

Properties of group I allergens from grass pollen and their relation to cathepsin B, a member of the C1 family of cysteine proteinases

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Expansins are a family of proteins that catalyze pH-dependent long-term extension of isolated plant cell walls. They are divided into two groups, α and β , the latter consisting of the grass group I pollen allergens and their vegetative homologs. Expansins are suggested to mediate plant cell growth by interfering with either structural proteins or the polysaccharide network in the cell wall.

Our group reported papain-like properties of β -expansin of Timothy grass (*Phleum pratense*) pollen, Phl p 1, and suggested that cleavage of cell wall structural proteins may be the underlying mechanism of expansin-mediated wall extension. Here, we report additional data showing that β -expansins resemble ancient and modern cathepsin B, which is a member of the papain (C1) family of cysteine proteinases. Using the *Pichia pastoris* expression system, we show that cleavage of inhibitory prosequences from the recombinant allergen is facilitated by its N-glycosylation and that the truncated, activated allergen shows proteolytic

activity, resulting in very low stability of the protein. We also show that deglycosylated, full-length allergen is not activated efficiently and therefore is relatively stable. Motif and homology search tools detected significant similarity between β -expansins and cathepsins of modern animals as well as the archaean *Giardia lamblia*, confirming the presence of inhibitory prosequences, active site and other functional amino-acid residues, as well as a conserved location of these features within these molecules. Lastly, we demonstrate by site-directed mutagenesis that the conserved His104 residue is involved in the catalytic activity of β -expansins. These results indicate a common origin of cathepsin B and β -expansins, especially if taken together with their previously known biochemical properties.

Keywords: cathepsin B; cell wall; expansin; group I allergen; proteinase.

Pollen triggers allergic reactions such as hayfever and seasonal asthma, which affect up to 25% of adults in industrialized countries. Of the diverse allergens of grass pollen, group I allergens are the major components [1] to which most patients possess specific IgE antibodies. They are glycoproteins of about 30 kDa with a carbohydrate content of 5% and are exclusively expressed in pollen of all grasses [2,3]. Grass group I allergens constitute the β -expansin subfamily of expansins [4]. Besides functioning as mediators of acid-induced cell wall loosening in plants, expansins are also essential for fruit ripening [5–8], fertilization [9] and differentiation [10,11]. However, the mechanism by which they mediate plant cell wall growth is highly controversial. Three main hypotheses have been put forward to explain their wall-loosening properties.

Several reports have suggested that expansins may interfere with hydrogen bonds between cellulose and hemicellulose microfibrils by a unique and novel mechanism,

reducing the rigidity of the cell wall [12]. This was supported by experiments showing that α -expansins associate with hemicellulose-coated cellulose microfibrils *in vitro* [13]. Expansins were therefore suggested to possess a C-terminal cellulose-binding domain (CBD) resembling bacterial CBDs, based on the spacing between highly conserved Trp (W) residues. They were also reported to be able to induce loosening of cellulosic paper [14]. On the basis of these findings, expansins were suggested to bind cellulose fibrils with their C-terminal CBDs, allowing interference with hydrogen bonds between wall polysaccharides via their N-terminal domain. The resulting weakening of the polysaccharide network was suggested to subsequently allow turgor-driven extension (relaxation) of the structure.

Another model indicates possible hydrolysis of polysaccharides, based on a $\approx 30\%$ sequence similarity within a restricted region between expansins and a small (F45) family of fungal endoglucanases. However, hydrolytic activity (exo and endo type) of expansins on polysaccharides has never been detected, and F45 hydrolases fail to stimulate plant cell wall extension [15,16]. Transglycosidase activity, another proposed mechanism, has also not been established. A summary of these models was recently published [17].

The third hypothesis proposed that expansins possess C1 (papain) proteinase family-related proteolytic activity, mediating plant cell wall loosening by cleavage of structural wall proteins, namely the extensins (hydroxyproline-rich glycoproteins) and associated proteins [18]. This concept requires a fundamental revision of the model of plant cell wall organization and growth. In accordance with this hypothesis, several potent allergens have been identified as

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Abbreviations: Phl p 1, grass group I allergen derived from *Phleum pratense*; Hol 1 1, grass group I allergen derived from *Holcus lanatus*; CBD, cellulose-binding domain.

Enzymes: cathepsin B (EC 3.4.22.1); papain (EC 3.4.22.2); bromelain (EC 3.4.22.32).

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proteinases, and their function suggested to contribute to their allergenicity. Thus, a proteinase function of group I allergens could explain the high prevalence of allergic individuals sensitized to these molecules. This model of expansins acting as proteinases was based on the finding that the recombinant β -expansin/allergen of Timothy grass, *P. pratense*, expressed in the yeast *Pichia pastoris*, catalyzed the degradation of a synthetic substrate containing a papain-cleavage site, as well as other proteins. Moreover, a protein with strong proteolytic activity was coeluted with the recombinant allergen after affinity purification using the mAb IG12 [18]. The natural allergen Phl p 1 was also found to be capable of degrading a synthetic substrate at a papain-cleavage site after incubation under acidic and reducing conditions, which are known to activate C1 proteinases. At that time, limited sequence similarity to motifs surrounding the active-site residues of papain was also established. However, the proposed putative proteinase identity of expansins seemed to be at odds with reports in the literature, e.g. that expansins loosened cellulosic paper [14] and that proteinases did not mediate plant cell wall extension *in vitro* [19,20].

EXPERIMENTAL PROCEDURES

Site-directed mutagenesis and subcloning

Phl p 1 cDNA (GeneBank/EMBL accession number Z27090) was ligated in pBluescript (Stratagene, La Jolla, CA, USA). Elimination of the putative N-glycosylation site NIT to QIT in position 9 of the mature protein product was performed by PCR with modified sense primer Phl p 1 Q (5'-ATCCCCAAGGTCCCCCGGCCCGCAGATC ACG-3'). Here, AAC coding for Asn (N) in the wild-type sequence Phl p 1 N (5'-ATCCCCAAGGTCCCCCGGCCCGAACATC ACG-3') was changed into CAG coding for Gln (Q). PCR in combination with antisense primer Phl p 1 rev (5'-TGGTGATCTTCTCGAGTCAAAATTG AACTT-3'), containing a *Xho*I site, was performed using *Pfu* polymerase (Stratagene) under the following conditions: Hot start for 5 min at 95 °C; followed by 20 cycles consisting of 95 °C for 30 s, 70 °C for 1 min and 72 °C for 2 min; and terminated by an extension step for 5 min at 72 °C. The reaction mixture consisted of 10 ng template DNA, 0.5 mM dNTPs and 1 μ M each primer in a total volume of 20 μ L. The PCR products were purified using the PCR Purification Kit (Qiagen, Hilden, Germany), subcloned into *Eco*RV-digested pBluescript, and sequenced. Inserts coding for rPhl p 1 N and Q were then separated using *Eco*RV and *Xho*I; the latter restriction site was then blunted with *Pfu* polymerase. This was followed by ligation into *Sna*BI-digested vector pPIC9 (*P. pastoris* Expression Kit; Invitrogen, San Diego, CA, USA), directly after the α -mating factor leader sequence, which mediates export of rPhl p 1 into the medium. Correct orientation of the constructs was confirmed by restriction analysis with subsequent sequencing and resulted in pPIC9 Phl p 1 N and Q, which were used for transformation after linearization with *Bgl*II. Mutagenesis of His104 to Val was performed by PCR using the primer phlp1s (5'-ACCGGGAGGAGGAATCCCCAAGGTCCCCCGG-3') with phlp1-Hs (5'-TACGTACGCGCGATGGGCTCC TCG-3'), and phlp1as2 (5'-AGAATTCTCAGTCCTT

GGCCTCGCCCTTG-3') with phlp1-Hs (5'-TACGTAT TCGACCTCTCCGGCATCGC-3'). The wild-type control was produced by using the primers phl p1s with phlp1as2. PCR products were obtained as described above, TA-cloned (pGEM, Promega, Madison, WI, USA), and sequenced. To construct the mutated form, fragments were released using the restriction enzymes *Sma*I and *Sna*BI or *Eco*RI and *Sna*BI, respectively, and both ligated in pBS, which had previously been linearized using the restriction enzymes *Eco*RI and *Sma*I. After transfection, positive clones were sequenced. All restriction enzymes were obtained from New England Biolabs, Beverly, MA, USA.

Pichia-transformation, identification of transformants, and expression

Transformation of *P. pastoris* strains GS115 and PEP4- (thus proteinase A)-deficient SMD1168 (Invitrogen) was performed by electroporation (Gene Pulser, Bio-Rad, Hercules, CA, USA) at 1.5 kV, 200 Ω and 25 μ F with 5 μ g linearized pPIC9 Phl p 1 per transformation, using 1-mm cuvettes (Bio-Rad). Transformants were identified by Mut^s phenotype (*methanol utilization slow*) and PCR with Phl p 1-specific primers. Cells were grown in BMDY (buffered minimal glucose + yeast extract; 2% bactotryptone, 1% yeast extract, 1.3% yeast nitrogen base with ammonium sulfate, 1% glucose, 0.00004% biotin in 0.1 M potassium phosphate buffer, pH 6.0) for 2 days, transferred in BMGY [buffered minimal glycerol (1%) + yeast extract] for 1 day and subsequently induced in BMMY Mod [buffered minimal methanol (0.5%) + yeast extract; 10 g L⁻¹ milk powder, 1 g L⁻¹ cysteine, 0.5% glycerol, in 0.1 M potassium phosphate buffer, pH 5.0] at a cell density of 10 D₆₀₀ units for 1 day, all at 30 °C and shaking at 150 r.p.m. in baffled flasks. Expression and export of rPhl p 1 was confirmed by dot-blotting of culture supernatant on to nitrocellulose membranes and detection with Phl p 1-specific mAbs IG12 [2], Bo14 and HB7. Cells were centrifuged at 1500 g for 10 min. The supernatant was collected, concentrated 20 times using Amicon concentrators (10-kDa membrane filters; Amicon, Beverly, MA, USA), and stored at -20 °C. The supernatant was washed twice with 0.1 M potassium phosphate buffer at pH 5.0.

Baculovirus expression of rPhl p 1 and rPhl p 1*His104

Sequences coding for rPhl p 1 as well as rPhl p 1*His were released from pBS or pGEM and ligated into the expression vector pAcSecG2T (Pharmingen, San Diego, CA, USA) using the restriction endonuclease sites *Sma*I and *Eco*RI. Recombinant virus was produced and amplified according to the manufacturer's instructions (Pharmingen). Briefly, recombinant virus was produced by cotransfection of Sf9 cells with linearized BaculoGold virus and pAcSecG2T-Phlp1 or pAcSecG2T-Phlp1*His. AcNPV wild-type virus was used as a control. Pure virus clones were isolated by plaque purification. Three clones each were tested for levels of expression, which was confirmed to be identical among a given construct. Virus was amplified until a titer of 10⁹ mL⁻¹ was achieved. An infectious dose of 10 virus particles per cell (multiplicity of infection = 10) was used for infection of cells (1 $\times$ 10⁷ Sf9 cells in a 10-cm dish). Expressing cells were either lysed 3 days after infection directly in SDS buffer, or

recombinant GST fusion protein was purified from the medium using glutathione/agarose (Sigma, St Louis, MO, USA), eluted in 50 mM acetic acid/sodium acetate buffer, pH 6.0, containing 5 mM GSH (Sigma), and processed as described below. Recombinant protein was detected after Western blotting using a monoclonal antibody to GST (Pharmingen).

SDS/PAGE and Western-blot analysis

Proteins were separated by discontinuous SDS/PAGE (T = 15%, C = 4%) and transferred to nitrocellulose membrane by semidry blotting [21]. Immunostaining was performed with mAbs IG12, Bo14, and HB7, binding to the peptide epitopes K(48)PPFS(52) (unpublished result), a C-terminal peptide and an N-terminal peptide, respectively (A. Petersen, personal communication). Subsequently alkaline phosphatase-conjugated goat anti-(mouse IgG and IgM) Ig (Dianova, Hamburg, Germany) was added before development (Nitro Blue tetrazolium/5-bromo-4-chloroindol-2-yl phosphate). Polyacrylamide gels were stained with Coomassie Brilliant Blue R250 [21]. For dot-blots, probes were applied directly to nitrocellulose and developed.

Zymograms

Zymograms were run as for SDS/PAGE, with 1% evaporated milk powder copolymerized in the resolving gel [22]. After electrophoresis, the gels were incubated in buffer containing 0.1 M glycine, 10 mM Ca^{2+} , 5 mM dithiothreitol and 10 mM cysteine, pH 3.6, for 16 h, followed by staining with Coomassie Blue. Protein probes (rPhl p 1 Q and rPhl p 1 N) were incubated in SDS sample buffer under nonreducing conditions at 65 °C for 10 min, before being loaded on to the Zymogel.

Preparative isoelectric purification of allergen

Concentrated *P. pastoris* expression supernatant was centrifuged at 3500 g for 30 min, filtered through a 0.2-µm filter, and dialyzed overnight against double-distilled water at 4 °C. A preparative Rotaphor cell (Bio-Rad) was assembled according to the manufacturer's instructions, and precooled to 4 °C. Ampholyte (pH 2–11; Serva, Heidelberg, Germany) was added to 60 mL dialyzed expression supernatant to a 2% final concentration, and separation was achieved at 12 W constant power for 5 h at 4 °C. The fractions were collected, and the respective pH values determined; the fractions were stored at –20 °C until analyzed.

Deglycosylation of Phl p 1 N with N-glycosidase A

Deglycosylation of proteins in the Phl p 1 N expression supernatant was achieved using N-glycosidase A (Boehringer-Mannheim, Mannheim, Germany). Twenty microliters deglycosylation buffer (100 mM citrate/sodium dihydrogen-phosphate buffer, pH 5.0, 1 mM dithiothreitol) and 0.3 mU N-glycosidase A were added to 10 µL expression supernatant (25 µg total protein) and incubated at 37 °C overnight. Buffer alone served as the negative control. Deglycosylated and control supernatant was subsequently analyzed in zymograms.

Alignments and computer analysis

Sequence data were analyzed with PCGENE software (Intelligenetics, Geel, Belgium). Alignments to conserved sequences of cysteine proteases and among Phl p 1 and Hol 1 1 were performed using WU-BLASTp2 (PAM270 matrix), modified manually and displayed using CLUSTALW and SEQVU software. Motifs were analyzed by the IMPALA BLOCKS search tool using the BLOSUM62 matrix. The percentage of identical amino acids between each pair of proteins was calculated by setting the number of comparable (e.g. within the same position) amino acids at 100%.

RESULTS

Expansins show significant sequence similarities to cysteine proteinases, especially cathepsin B

Analysis of the amino-acid sequence of Phl p 1 for conserved, functional motifs using the IMPALA BLOCKS search tool resulted in the following order of hits: (1) major pollen allergen Lol p 1 signature (e^{-103}); (2) expansin signature ($1e^{-13}$); (3) allergen pollen CIM1/Hol 1 1 signature (0.009); (4) eukaryotic thiol (cysteine) proteases active-site signature IPB000169 (1.6). Moreover, the WU-BLASTp2 program, employing the PAM270 matrix which allows detection of distantly related proteins, computed similarities between expansins and cathepsin (Q10834, $1.9e^{-7}$) as well as other cysteine proteinases (Q40261, $4.2e^{-6}$). Additional cathepsin B-like cysteine proteinases as well as cysteine proteinases from *Giardia lamblia* could also be detected [23]. From these findings, an alignment of Phl p 1 with *Gallus gallus* and *G. lamblia* cysteine proteinases was generated (Fig. 1). The identity between Phl p 1 and CP2 of *Giardia* within comparable regions was 21%, and the combined identity and similarity amounted to 34%. The identity between Hol 1 1 and CP2 was $\approx 22\%$, between Phl p 1 and CP1 as well as CP3 19%, and between Phl p 1 and CatB $\approx 15\%$. Moreover, the putative active-site amino acid Cys72 of Phl p 1 and Hol 1 1 is very similarly positioned if compared with the *Giardia* proteinases 1 (residue 71), 2 (residue 67) or 3 (residue 66). The catalytically essential Trp residues are also similarly located within the C-terminal region. Another striking feature is the well-conserved relative location of Cys41, 57, 69, 72, 83 and 139 when compared with the proteinases of *G. lamblia* and *G. gallus*. All Cys and Trp residues are absolutely conserved in α -expansins and β -expansins as well as in the C1 cysteine proteinases. Other highly conserved amino acids of cathepsin B-like proteinases are also well conserved in most β -expansins, notably the Pro2 residues and the Glu216 residue. However, the amino acids His158 and Asn193 (C1 numbering; cathepsin B: His260 and Asn280) of the catalytic triad are not present in a comparable position in C1 proteinases and group I allergens. Subsequently, functional tests on recombinant (r) Phl p 1 were refined to further explore the biochemical function of group I allergens.

Expression of glycosylated and nonglycosylated rPhl p 1 reveals differing stability of these allergens

The expression of rPhl p 1 in the yeast *P. pastoris* was attempted to obtain a post-translationally modified allergen in a natural conformation. In addition to the wild-type

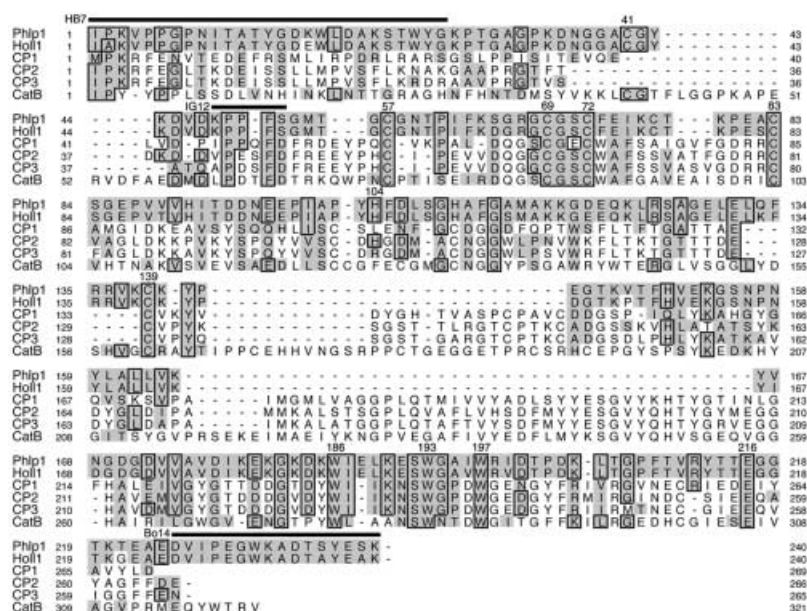


Fig. 1. Alignment of Phl p 1 (GenBank Z27090), Hol 1 (Z68893), *G. lamblia* cathepsins 1–3 (U83275, U83276 and U83277, respectively) and *G. gallus* cathepsin B (U18083). Areas of identity are boxed, and areas of similarity are shaded. Binding epitopes for mAb HB7 (N-terminal), IG12 (central) and Bo14 (C-terminal) are marked. Important conserved cysteine residues and other essential amino acids are numbered. Areas of high similarity exist around Cys69 and Cys72 and around Trp186, Trp193 and Trp197. The positions of Cys41, 57, 69 and 72 are especially conserved between β -expansins and cathepsin B. The His104 residue, which is absolutely conserved in all expansins, is also present in CP2 of *G. lamblia*.

sequence rPhl p 1 N, which contains an N-glycosylation site in position 9, another recombinant allergen rPhl p 1 Q lacking this site was produced by site-directed mutagenesis, the N-glycosylation site NIT being changed to QIT in the mutant protein. This allowed absolute discrimination of the biochemical characteristics between glycosylated and non-glycosylated allergens compared with other methods, such as enzymatic deglycosylation or expression in the presence of tunicamycin. Both proteins were produced by proteinase A-deficient *P. pastoris* SMD1168 cells and secreted into the medium. The identity of the proteins was confirmed by Western blotting, using grass group I-specific monoclonal antibodies or sera from patients. Figure 2A shows a Western blot of rPhl p 1 N, rPhl p 1 Q and the albumin-expressing control as detected with mAb IG12. The expressions were performed in a protein-enriched medium for a limited time (< 24 h). The hyperglycosylated ($\approx 15\%$ carbohydrate content) rPhl p 1 N has a size of about 40 kDa, whereas the nonglycosylated form, Phl p 1 Q, has a size of about 33 kDa. The identity of the respective N-termini was determined by N-terminal sequencing, resulting in the sequence Y-I-P-K-V, confirming the correct processing of the yeast (α -mating factor) signal sequence for protein export. The additional tyrosine resulted from the cloning site.

Induction of expression of rPhl p 1 in protein-free medium, even for a short time (< 24 h), consistently led to heavy degradation and a low yield of the recombinant proteins, which was not seen in albumin-expressing controls. In particular, rPhl p 1 N displayed very low stability (data not shown). By using a modified, protein-enriched medium and a short expression time (< 24 h) expression of nondegraded allergen could be achieved (Fig. 2A). However, expression over prolonged periods of time, even in protein-rich medium, did not allow expression of intact allergens. Elimination of the protecting proteins during purification also led to degradation of the allergens, predominantly at low pH. As rPhl p 1 N was consistently much less stable than rPhl p 1 Q during expression and purification, and rPhl p 1 N-containing supernatant

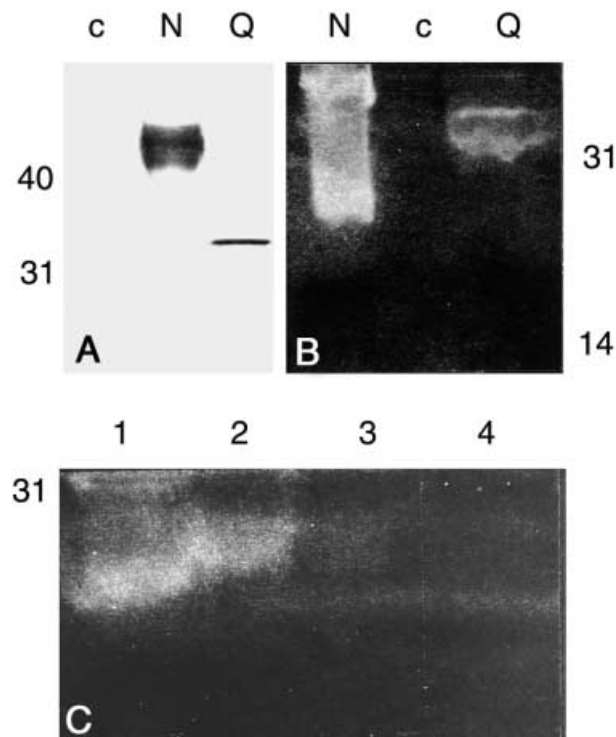


Fig. 2. Comparison of the expression and enzymatic activity of recombinant Phl p 1 N and Q. Sizes in kDa are indicated. (A) *P. pastoris* expression of Phl p 1 N (N) and Phl p 1 Q (Q), as well as albumin (c) as detected by Western blotting and IG12 binding. IG12 specifically detected the recombinant allergens, but not any yeast proteins. (B) Activity of Phl p 1 N (N) or Phl p 1 Q (Q) after prolonged expression in zymograms. The glycosylated allergen Phl p 1 N shows a much more pronounced proteolytic activity than the nonglycosylated allergen. (C) Effect of enzymatic deglycosylation on rPhl p 1 N activity in zymograms. Expression supernatant was applied in lane 1. Expression supernatant in deglycosylation buffer is applied in lane 2. Addition of N-glycosidase A leads to abolished proteolytic activity of the allergen in lane 3. Lane 4, albumin-expressing control.

showed much more pronounced proteolytic activity in zymograms (Fig. 2B), we investigated whether enzymatic deglycosylation of protein in rPhl p 1 N-containing supernatant after brief expression would lead to decreased (rPhl p 1 Q-like) enzymatic activity. As shown in Fig. 2C, deglycosylation of the allergen by N-glycosidase A indeed resulted in reduced proteolytic activity in zymograms compared with rPhl p 1 in buffer without N-glycosidase A. This result led us to investigate the behavior of full-length glycosylated vs. nonglycosylated recombinant allergens at various pH values by preparative isoelectric focusing.

Protein-rich BMMY Mod expressions of intact rPhl p 1 N and rPhl p 1 Q (as judged by Western blotting, Fig. 2A) were subjected to isoelectric focusing, concentrating the allergen according to the isoelectric point (pI) of the molecule. A pH gradient from 2 to 11 was established for the characterization of Phl p 1. After completion of the run, the individual fractions were collected and their pH values determined. Proteins in the respective fractions were subsequently analyzed by SDS/PAGE followed by Western blotting, using mAb IG12 and Bo14 for detection of the allergen. As can be seen in Fig. 3B,D, rPhl p 1 Q showed the appropriate size of 33 kDa and was detected by both antibodies close to the theoretical pI of about 8.0. No degradation products of the allergen could be detected by either antibody. However, the expression supernatant containing rPhl p 1 N showed strong degradation of the full-length allergen and accumulation of a truncated ≈ 15 -kDa fragment at about pH 4.5 (Fig. 3A). This sharp band lacked the N-terminal peptide, as the N-glycosylated Asn residue was located in position 9 of the molecule, and glycosylation would have resulted in a fuzzy band (Fig. 1A). It also lacked the C-terminal peptide normally detected by mAb Bo14. It is notable that degradation of the glycosylated form yielded only a limited number of defined fragments but no smear. Thus, a specific endoproteinase, and not a nonspecific digestive enzyme or exoproteinase, probably produced the observed fragments of rPhl p 1 N.

The detection of the respective antibody-binding sites (HB7: N-terminal; IG12: central; Bo14: C-terminal, Fig. 1) on dot-blots of various allergen expressions in various systems confirms that N-terminal and C-terminal peptides were cleaved off the active allergen Phl p 1 N (Fig. 4). The allergens were expressed in *Pichia* over a prolonged period of time (5 days) in protein-rich medium, concentrated, washed over 10-kDa membranes to eliminate short peptides and tested in zymograms (Fig. 2B). Also, a natural allergen isolated from pollen, *Escherichia coli*-expressed allergen as well as *Pichia*-expressed rPhl p 1 Q were tested in zymograms and turned out to be inactive or weakly active in the case of rPhl p 1 Q (Fig. 2B). They all possessed binding sites for antibodies HB7, IG12 and Bo14. However, the proteolytically active Phl p 1 N was not bound by the mAbs HB7 and Bo14, indicating truncations on both sides of the allergen. Supernatant of albumin-expressing *P. pastoris* showed no cross-reactivity with any allergen-specific monoclonal antibody. It was further tested whether the truncated IG12-binding forms still possessed proteolytic activity after affinity purification. IG12 affinity purification of supernatant containing the truncated, active allergen as shown in Fig. 4 was thus performed. This led to strong proteolytic activity of the eluate, whereas supernatants of albumin-expressing *Pichia* cells did not show any proteolytic

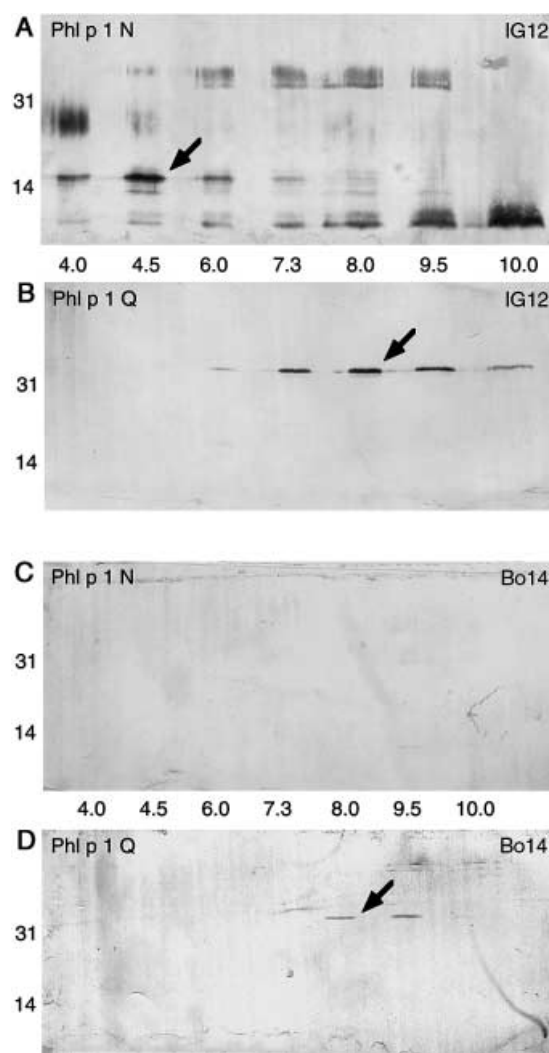


Fig. 3. Isoelectric focusing of rPhl p 1 N and rPhl p 1 Q, as detected by mAb IG12. The pH values of the respective fractions and the size of the protein markers used are indicated. (A) The full-length allergen Phl p 1 N mostly disintegrates. Notably, a ≈ 15 -kDa fragment can be seen in the pH 4.5 fraction (arrow). Further degradation products can be seen in the more basic fractions. (B) The allergen Phl p 1 Q can be detected at about pH 8.0, which is the pI computed for Phl p 1 and does not show any degradation products (arrow). (C) and (D) Isoelectric focusing of rPhl p 1 N and rPhl p 1 Q, as detected with mAb Bo14. As can be seen, only Phl p 1 Q was detected with this antibody, indicating an intact allergen (arrow). None of the Phl p 1 N fragments could be detected with mAb Bo14, demonstrating lack of a C-terminal peptide.

activity [18]. Even preincubation of the supernatant containing truncated rPhl p 1 N with 0.5% SDS, which strongly interferes with protein–protein interactions and thus further reduces the possibility of coelution of another proteinase, allowed IG12 affinity purification of a proteolytically active allergen (data not shown).

Site-directed mutagenesis was then conducted in order to identify the catalytic His residue of the C1 catalytic triad. Analysis of hydrophobicity plots of the Phl p 1 amino-acid sequence (data not shown) indicated that His104, which is the only histidine residue conserved in all α and β -expansins,

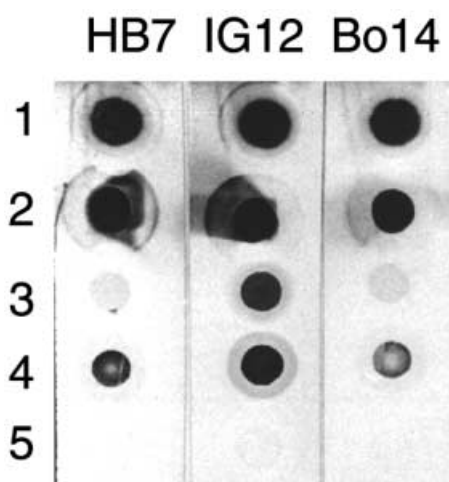


Fig. 4. Dot-blot of various inactive and active preparations of Phl p 1, as detected with mAbs HB7, IG12 and Bo14. Concentration and washing using 10-kDa Amicon filters resulted in the removal of small fragments. All mAbs detect the inactive allergen nPhl p 1 from pollen (1), inactive, *E. coli*-expressed recombinant Phl p 1 (2), and inactive *Pichia*-expressed rPhl p 1 Q (4). None of the antibodies detect proteins in the albumin-expressing *Pichia* supernatant (5). However, the active allergen Phl p 1 N (3) is only detected by IG12, demonstrating cleavage of the N-terminal and C-terminal propeptides (Fig. 1).

is located within a hydrophobic pocket of the enzyme. Therefore, His104 was replaced by a valine residue. As shown in Fig. 6, the mutated rPhl p 1*His is expressed and secreted into the supernatant as a stable molecule, unlike the natural allergen rPhl p 1, which is expressed at a low level. Analysis of the expressing Sf9 cells 3 days after infection confirmed the finding of very low expression of rPhl p 1, whereas the mutant rPhl p 1*His was stably expressed at a high level (data not shown).

DISCUSSION

Computer analysis of β -expansins reveals significant similarity to cathepsins, which are members of the C1 family of cysteine proteinases

WU-BLAST homology searches for β -expansins (PAM250-270) led to detection of significant similarity to a variety of cysteine proteinases, which could be confirmed by the IMPALA BLOCKS homology search tool. On the basis of these results, a full-length alignment of two β -expansins with cathepsin B of *G. gallus* and the cysteine proteinases from *G. lamblia* was generated [23]. As shown in Fig. 1, the alignment yields a moderate similarity between these enzymes. The proteinase CP2 of *Giardia* possesses $\approx 22\%$ identity with the β -expansin Hol I 1 (21% with Phl p 1), a high value when compared with the identity among α and β -expansins, which is $\approx 25\%$. Furthermore, high similarity is detected between regions surrounding the active sites of the proteinases and their expansin counterparts [4], and their comparable location enhances the significance of the sequence similarities. Other functional amino acids in cathepsins are also conserved in most or all expansins.

First, the distribution of cysteine residues is almost identical between cathepsins and expansins. The pro-

sequences of modern cathepsin B proteinases share a critical (inhibitory) cysteine residue in position 41 with all expansins [4,24,25]. Cysteine residues Cys57, 69, 72, 83 and 139 are also similarly located. Secondly, proline residues in position 2 stabilize the N-termini of cysteine proteinases [26] and can also be found in most expansins. Lastly, functionally relevant Gly70, Gly113 [27], Ser192 [28] and Glu216 [29] residues of cathepsin B are also highly conserved in expansins. Taken together, the presence of several conserved motifs and functional amino acids as well as their similar location in expansins and C1 proteinases is not likely to have occurred by chance.

The lack of the essential His260 (cathepsin B) can be explained by an expansin-specific protein folding. The tertiary structure of C1 family proteinase members is generally very diverse [30]. His104 is present in all α and β -expansins and thus was assumed to have functionally replaced the His260 found in cathepsin B. Asn280 (cathepsin B) is lacking in all expansins but is also absent from the C1 proteinase bromelain [26] and is not considered essential for catalysis in papain [31].

β -Expansins possess closest similarity to cell wall-degrading cathepsin B

Interestingly, CP2 of *G. lamblia* is a hatching (exocystation) enzyme, thus showing a functional resemblance to the cell wall-degrading expansins. The *Giardia* cyst wall consists of a carbohydrate/peptide complex [32] which is resistant to cleavage with chymotrypsin, trypsin, papain, or pronase. Protozoan parasites of the genus *Giardia* are one of the earliest lineages of eukaryotic cells, and the *Giardia* protease is the earliest known branch of the cathepsin B family [23]. Its phylogeny confirms that the cathepsin B lineage evolved in archezoa, before the divergence of plant and animal kingdoms and underscores the diversity of cellular functions that this enzyme family facilitates.

The sequence and functional similarities led us to speculate that plant cell wall-extending expansins and the cyst wall-degrading *Giardia* proteinases may stem from a common ancestor. We believe that expansins act like the cell wall-digesting proteinases of *Chlamydomonas*, which fragment proline-rich and hydroxyproline-rich structural proteins of the cell wall [33] also known to be present in all higher plants.

The glycosylated rPhl p 1 is very unstable during expression and purification, indicating a role for N-glycosylation in enzyme activation

The glycosylated rPhl p 1 β -expansins were very unstable during expression in the methylotrophic yeast *P. pastoris*. The mutated, nonglycosylated allergen Phl p 1 Q was found to be more stable. This is in accordance with the finding that N-glycosylation of prepropapain is necessary for production of active papain in Sf9 cells [34]. The difference in rPhl p 1 stability showed very clearly after purification according to the isoelectric point. Phl p 1 Q was not degraded, and focused at $\approx$ pH 8.0. mAb IG12, which detects a central Phl p 1 peptide, as well as mAb Bo14, which detects a peptide at the C-terminus, bound to this expression product (Fig. 3B,D). In contrast, Phl p 1 N was mostly degraded. A fragment of ≈ 15 kDa focused at

≈ pH 4.5 and was bound by mAb IG12, but not mAb Bo14 (Fig. 3A,C), indicating truncation of the C-terminus. The different stability of glycosylated and nonglycosylated rPhl p 1 rules out the presence of a contaminating *Pichia* proteinase, as this would have led to equal degradation of the two allergens.

Most interestingly, the 15-kDa fragment of Phl p 1 N accumulated at ≈ pH 4.5 after isoelectric focusing, which is the approximate pH of the growing wall region *in vivo* and the optimum pH for expansin activity (Fig. 3A). Thus, active expansins, which are highly soluble [4], could migrate to and concentrate within the acidic (= growing) areas of the cell wall *in vivo*. This enables expansin activation, accumulation and catalysis under identical pH conditions and explains how expansins may mediate acid growth of plant cell walls. Theoretical pI calculations show that N-terminal and C-terminal truncation of the allergen leads to a pI shift to ≈ 5.0 (data not shown), confirming the experimental findings.

Removal of putative prosequences is essential for C1 proteinase activation

Various active and inactive allergens were analyzed by dot-blotting after removal of small peptides by filtering. Natural Phl p 1 pollen allergen as well as *E. coli*-expressed rPhl p 1, both of which do not possess proteolytic activity, were bound by mAbs HB7, IG12 and Bo14. The *Pichia*-expressed, active allergen rPhl p 1 N, however, was only bound by IG12. In contrast, *Pichia* expression of mostly inactive rPhl p 1 shows binding of all three antibodies. The active rPhl p 1 was tested in zymograms and by affinity purification, followed by elution of high proteolytic activity [18]. This indicates that N-terminal and C-terminal truncation of rPhl p 1 is a prerequisite for its proteolytic activity and allows further conclusions from the alignment shown in Fig. 1.

C1-proteases possess N-terminal and C-terminal inhibiting prosequences, the cleavage of which results in enzyme activation [26,35,36]. Lys61 is the N-terminal amino acid of active cathepsin B, followed by a highly conserved, stabilizing Pro residue, which is present in all β -expansins. The finding that enzymatically active β -expansins lack N-terminal peptides was also reported for CIM1 [37]. Proteolytic fragmentation of the β -expansin at the end of the growth phase was also shown, possibly protecting the growing wall from rupturing. Concurrent with this, exogenous application of large amounts of expansin described in another report [38] caused bursting of root hairs, underlining the importance of effective down-regulation of expansin activity.

Mutagenesis of His104 in the highly conserved HFD motif stabilizes the recombinant allergen

Expression of native and mutated rPhl p 1 in the baculovirus expression system was conducted to identify the His residue involved in the proteolytic activity of β -expansins. His104 was identified as part of the catalytic triad, because the mutated protein rPhl p 1*His was expressed stably at much higher levels than the nonmutated allergen rPhl p 1. We herewith have confirmed that autodegradation is the likely cause of the observed low expression levels and instability of recombinant allergen in *Pichia* and Sf9 cells.

This was confirmed with three independent virus clones in expression supernatant as well as in lysed cells. This finding is important in two ways. First, mutation of His104 now allows high level production of stable recombinant grass group I allergens in eukaryotic systems, which may prove useful for diagnostic methods or even future therapeutic protocols. It also demonstrates that β -expansins are a novel group of proteinases with a unique catalytic triad, in which His104 replaces the cathepsin B-typical His260 residue. Notably, this finding implies a predominant role for the putative target proteins, the extensins, in the growing plant cell wall.

All models that propose that expansins work as polysaccharide-modifying enzymes are not in agreement with their biochemical properties

A recent publication [20] claimed that β -expansins lack proteinase activity. However, highly purified β -expansins were (auto)degraded almost completely after a C1 activation step, which was not noted. Also, the claim that proteinases have no effect on wall extension contradicts another report by this group [39]. Most importantly, in one particular assay conducted under unfavorable, nonreducing conditions, up to 25% reduction in wall extension was still observed when the cysteine proteinase inhibitor *N*-ethylmaleimide was employed, indicating involvement of a free cysteine residue in catalysis. Unfortunately, no dose dependence of this reaction (under reducing conditions) was investigated to clarify this result.

A ≈ 30% similarity between expansins and family 45 glycosyl hydrolases was also reported. However, a hydrolase function for expansins could not be established, and family 45 hydrolases fail to catalyze expansin-like wall extension [17]. A similarity of the C-terminal region of expansins to CBDs of bacterial cellulases was further suggested on the basis of conserved spacing of Trp residues [40]. As shown in Fig. 5A, Trp spacing is much more similar to that in cysteine proteinases than to any bacterial (or fungal) CBD. Figure 5B shows that the Trp residues of the Phl p 2 allergen, which is homologous to Phl p 1, do not form a flat surface that would allow binding of cellulose.

The suggested functional mode of expansins as turgor-dependent hydrogen-bond-weakening agents (for a review, see [17]) is also not supported by the known experimental data. Weakening of hydrogen bonds should be energy dependent, but no energy or cofactors were found to be required for expansin action. Also, chemical substances that interfere with hydrogen bonding do not mediate cell wall extension. Addition of 8 M urea or other chaotropic reagents does not have any effect reminiscent of expansin activity [19]; instead, the observed shrinkage of the walls points towards a predominant role for structural proteins in mediating wall rigidity. Also, expansins cause softening of fruit [6,9], but as ripening fruit does not grow, turgor-driven wall relaxation does not seem to occur.

Proteinase function of expansins is consistent with their biochemical data

In contrast with the above models, a proteinase identity of expansins is in very good agreement with the published

A

I.
CxxxxWxxxxNxxxWxxxxxxWxxxxxxWN

II.
xxxxxxCxxxxxxxxCxxxxCxxxxxxxxCxx

III.
xxxxxWlxKNSWxxxWxxxxxxLxxxxx

IV.
xxxxWlxxKxSWxxxWxxxxxxLxxxxx

B

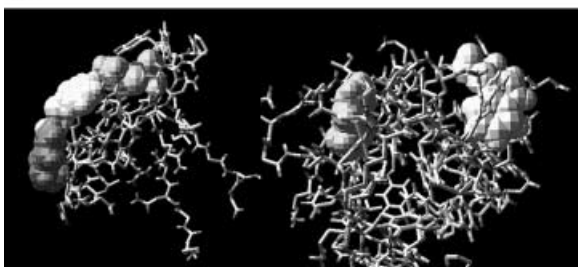


Fig. 5. Three-dimensional and Prosite motifs of CBDs, proteinases and expansins. (A) The consensus pattern of bacterial CBD (I, Prosite PS00561) and CBDs of fungi (II, Prosite PS00562) are shown. Pattern III denotes the consensus of the Trp-rich region in cysteine proteinases as identified by IMPALA BLOCKS (PS00640), and pattern IV shows the corresponding expansin region. The similarity of expansins to CBD of bacteria and fungi is low in this region, but identity with C1 proteinases is high. (B) Three-dimensional structure of CBD of *Pseudomonas* xylanase A (PDB:1E8R, left) and the allergen Phl p 2 (PDB:1WHP, right). Phl p 2 shares $\approx 50\%$ identical amino acids with Phl p 1 in the putative CBD. Trp residues are shown as solid molecules in the stick structures. The Trp residues of the bacterial CBD form a flat surface which is essential for cellulose binding, but the corresponding region of the allergen Phl p 2 does not show this feature and does not bind cellulose.

experimental data. First, C1 proteinases and expansins are proteins of ≈ 25 – 30 kDa and are exported to the cell wall [26,40] as inactive proforms. The pH optimum for cathepsin B and expansins is ≈ 4.5 , and both enzymes are irreversibly inactivated at $\text{pH} > 7.0$ [19,41]. Expansins are activated by reducing agents such as dithiothreitol and NaCN, which are activators of thiol proteinases. Expansins are also inhibited by Cu^{2+} , Hg^{2+} , Al^{3+} and *N*-ethylmaleimide, all potent inhibitors of cysteine proteinases [18,19]. Moreover, deuterated water (D_2O) was shown to reduce expansin activity [14]. The stronger hydrogen bonds of deuterated water are known to inhibit the formation of the tetrahedral intermediate step and thus the reaction speed of C1 proteinases. Also, expansins mediate fruit softening without growth, which is in accordance with a proteolytic mechanism. No energy and cofactors are required for activity, which is also true for C1 proteinases. The observation that expansins catalytically mediate wall growth

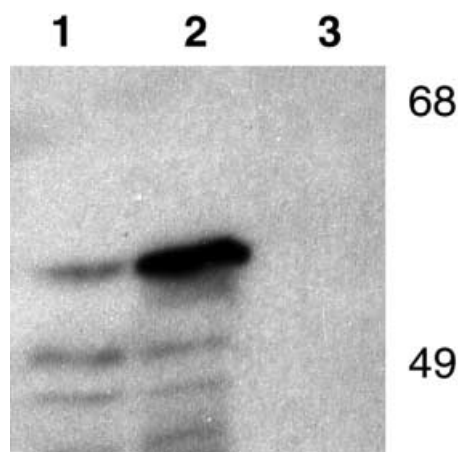


Fig. 6. Mutagenesis of His104 allows stable expression of rPhl p 1 in the medium of Sf9 cells. The natural allergen is expressed at a low rate (lane 1), whereas the mutated form rPhl p 1*His is strongly expressed (lane 2). Lane 3, AcNPV wild-type control. The molecular size is indicated.

(at an expansin to wall ratio of up to 1 : 12 500 [42]) is also in good agreement with a putative proteolytic function, as is the report that expansins may be present in digestive juices of snails [39], indicating that stable polymers need to be hydrolyzed.

The results presented here prove that expansins are proteinases that arose from the wall-digesting cysteine proteinase family of *Giardia*. Expansins form a novel group of proteinases, indicating early evolutionary divergence, but still possess numerous key features of modern and ancient cathepsin B. The major difference is the involvement of the unique His104 active-site residue in proteolysis.

We suggest a model in which expansins are activated by pH reduction or other proteinases located in the cell wall analogous to *Chlamydomonas* hydroxyproline-rich glyco-protein-degrading proteinases [33,43–45]. As cathepsin B can form a noncovalent complex between the mature enzyme and its precleaved prosequence, very rapid expansin activation upon pH reduction may occur [46]. The processed active β -expansin is suggested to concentrate within the acidic growing area of the wall because of its isoelectric properties. Subsequently, expansin degrades structural wall proteins, leading to slipping of the polysaccharide structures and thus slow controlled extension. Pectinases and cellulases synergistically enhance wall extension *in vivo*. These two independently regulated mechanisms, acting on structural proteins and the polysaccharide network, greatly enhance the fine tuning and safety of the growth process. Because of its low stability, expansin degrades rapidly, preventing rupture of the wall. As C1 proteinases are also capable of cleaving ester bonds, expansins may also act on suberin-type structural molecules in the primary wall. Moreover, the proteolytic function of group I allergens may determine their allergenicity.

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