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Antifungal activity of chitin-binding PR-4 type proteins from barley grain and stressed leaf

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Antifungal activity in vitro has been associated with barley leaf and grain proteins which are homologous with pathogenesis related proteins of type 4 (PR-4) from tobacco and tomato and with C terminal domains of potato *win* and *Hevea* hevein precursor proteins. One protein (pI ~9.3, M_r ~13.7 kDa) from barley grain and two very similar proteins from leaves infected with *Erysiphe graminis* were isolated by chitin affinity chromatography, but none of the proteins showed chitinase activity in vitro. The leaf proteins were increased several fold in response to either *Erysiphe* infection or $NiCl_2$ infiltration and accumulated extracellularly. The three barley proteins were found to inhibit growth of *Trichoderma harzianum* in microtiter plate assays using ~10 μg/ml concentrations and in lower concentrations in a synergistic way when mixed either with barley chitinase C (a PR-3 type protein) or with barley protein R (a PR-5 type protein). Structurally similar proteins were detected in wheat, rye and oats grain extracts.

Amino acid sequence; Antifungal; Hevein; Pathogenesis related; Win protein; *Hordeum vulgare*

1. INTRODUCTION

Infection with fungal or viral pathogens may induce a systemic synthesis of about 10 families of homologous pathogenesis-related proteins (PR proteins) in vegetative tissues of plants [1,2]. A simultaneously acquired resistance arresting or delaying further infection suggests a direct defensive action of the induced proteins against invading pests. The first direct proof of increased resistance due to expression of a chitinase gene in transgenic tobacco plants appeared recently [3]. Homologous, but not identical, PR-like proteins are constitutively expressed in many tissues. In cereal seeds these proteins are synthesized in the late stages of development together with or after the storage proteins [4,5]. The relatively high seed contents indicate protective roles of importance for seed storage.

The hypersensitive response of tobacco to TMV infection is well-characterized and the induced PR proteins have been classified in five groups [1]. The PR-2, PR-3 and PR-5 proteins are β-1,3-glucanases, chitinases and thaumatin-like proteins, respectively, which all possess antifungal activity. Specific functions have not been associated with PR-1 and PR-4 proteins. Recently, cDNA clones with open reading frames for tobacco and tomato PR-4 proteins were characterized [6,7]. Comparisons with partial amino acid sequences showed that the encoded acidic proteins are very similar to the C-terminal domains of prohevein and the putative wound-induced *win* proteins of potato, thus lacking the N-terminal hevein domain.

The present communication describes proteins of cereal grain and stressed barley leaf, which are basic counterparts of these acidic PR-4 proteins. Although the chitin-binding (CB) hevein domain is absent in their structure the barley proteins were isolated by chitin affinity chromatography. The proteins were characterized and found to possess antifungal activity towards *Trichoderma harzianum* presumably related to the affinity for chitin.

Abbreviations: CB, chitin binding; IWF, intercellular washing fluid; PDMS, plasma desorption mass spectrometry; PR, pathogenesis related; RP-HPLC, reversed-phase high-performance liquid chromatography.

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2. MATERIALS AND METHODS

2.1. *Plant material*

Seeds of barley (*Hordeum vulgare* L. cv Bomi mutant 1508) were used for purification of the grain protein. Barley line P02, a near-isogenic line of the cv Pallas possessing the resistance gene *Ml-a3*, was grown as described [8]. The powdery mildew (*Erysiphe graminis* f. sp. *hordei*) isolate C 15 was used for inoculation day 7 after sowing and primary leaf material was harvested at day 14 and stored at −20°C until used for purification. For elicitor infiltration pots with 7-days-old plants were inverted and leaves submerged in a degassed solution of 0.01% Tween-20 containing 2 mM sodium salicylate, pH 6.5 or 2 mM $NiCl_2$ for 5 min at 2,700 Pa whereafter the vacuum was released. At day 10 leaf material was harvested, intercellular washing fluid (IWF)

was isolated by vacuum infiltration before extraction of leaf cells as described [8].

2.2. *Isolation and characterization*

The grain ~14 kDa CB protein was purified by chitin affinity chromatography and cation exchange chromatography as described for isolation of the grain chitinases C and T [5]. Extraction and purification of ~14 kDa CB proteins from infected leaves by combination of chitin affinity and ion exchange chromatography has also been described [8]. Chitinase, lysozyme and chitosanase activities were assayed with tritiated chitin [5], *Micrococcus lysodeikticus* cell walls [9] and glycolchitosan [10] as substrates, respectively. M_r of the purified proteins was determined by plasma desorption mass spectrometry (PDMS) on a BIO-ION 20. Other methods used for protein characterization, including amino acid analyses, sequencing of separated tryptic peptides, RP-HPLC, SDS-PAGE and immunoblotting were described recently [5,8,11,12].

2.3. *Antifungal activity*

Inhibition of fungal growth was measured in 96-well microtiter plates at 540 nm [12]. The desalted antifungal proteins were preincubated with ~10^3 pregerminated spores of *Trichoderma harzianum* in 200 μl 25× diluted potato dextrose broth (Difco) for 2 h at 31°C. Then 50 μl 5× concentrated medium was added and the plates were read after 20–50 h at 31°C. The concentration (μg protein/ml final medium) resulting in 50% inhibition of growth as measured at an absorbance of about 0.6 in the control wells was termed I_{50}. To test for synergistic effects, two proteins were mixed in proportion to their I_{50}. The medium contained chloramphenicol (30 μg/ml) and chlorotetracycline (30 μg/ml) as antibacterial agents.

3. RESULTS

3.1. *Characterization*

During purification of chitinases from barley leaf [8] and grain [5] using chitin affinity columns minor components with M_r ~14 kDa were eluted with acetic acid, pH 3.2 together with the ~27–33 kDa chitinases. Subsequent cation exchange chromatography on Mono S resulted in isolation of three ~14 kDa proteins from the leaf extract [8]. Two of these CB proteins, CBP 4 and 5, were isolated in higher amounts for the present study. A similar separation of the CB grain proteins resulted in isolation of one ~14 kDa component, termed CBP N. Amino acid analyses and immunochemical tests (not shown) confirmed that CBP N was identical with a previously characterized basic protein N with pI ~9.3 [11]. Purity of the three CB protein preparations was confirmed by SDS-PAGE and RP-HPLC. In the RP-HPLC system CBP N and 5 had same retention time, but in cation exchange chromatography on Mono S CBP N eluted close to CBP 4 in the salt gradient (compare with [8]). The three CB proteins had very similar, but not identical, amino acid compositions [8,11], M_rs and immunochemical properties (Fig. 1A). After tryptic digestion of CBP N and CBP 5 selected peptides were sequenced. About 50% of the CBP N sequence was determined and comparisons showed ~80% amino acid identity with the tomato and tobacco PR-4 proteins and the C-terminal domains of prohevein and the putative *win* proteins (Fig. 2). The two short sequences of CBP

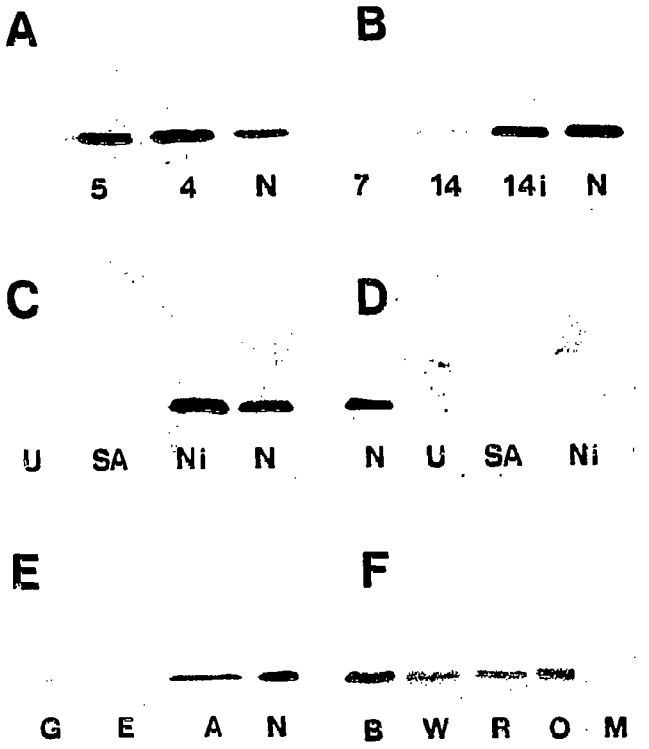

Fig. 1. Immunoblotting of PR-4-like barley leaf and grain proteins. Proteins were separated by SDS-PAGE and detected with antibodies towards CBP N after blotting on nitrocellulose. (Panel A) Purified chitin-binding proteins from barley leaf (4 and 5, ~50 ng) and grain (N, ~30 ng). The same preparation of CBP N was applied as reference in panels B–E. (Panel B) Effect of *Erysiphe graminis* infection on leaf. Extracts of 7- and 14-days-old leaves (7 and 14) are compared with 14-days leaf infected at day 7 (14i). About 2 μg protein was applied in each well. (Panels C and D) Effect of salicylic acid (SA) or $NiCl_2$ (Ni) treatment of leaf. IWF (panel C) and cell homogenate (panel D) were isolated at day 10 after treatment at day 7. Leaves from unstressed plants were included as controls (U). See section 2 for details. About 1 μg protein was applied in each well. (Panel E) Extract of dissected embryo (G), endosperm (E) and aleurone (A) tissue from immature barley grains harvested 25 days after anthesis. Extract corresponding to 12.5 μg protein was applied in each well. These amounts correspond to about 8, 1.6 and 8% (wet weight) of the embryo, endosperm and aleurone tissue, respectively, present in a grain at this stage. (Panel F) Immunochemically related proteins in cereals: barley (B), wheat (W), rye (R), oats (O) and maize (M). Flour of mature grain was extracted and about 15 μg was applied in each well.

5 were identical with sequences in CBP N and the PR-4 proteins (Fig. 2). The M_rs determined by PDMS were 13,718±10 Da, 13,605±14 Da and 13,699±14 Da for CBP N, 4 and 5, respectively. CBP N contains an insertion of two residues between residue 24 and 25 in comparison with tobacco and tomato PR-4 proteins [6,7] with calculated M_rs in the range 13,468–13,485 Da (122 or 123 residues). The three barley proteins had blocked N-terminals and contained five Cys residues as the homologous tobacco and tomato proteins. These data further confirm that the barley proteins are PR-4 type proteins without Cys-rich N-terminal hevein domains.

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          8                                  36
CBP N     ATYHYYRPAQNNWDLGAPAVSAYCATWDASK
PR-P2     R****L*N*QNI****RT   A*V*******D*
PR-4      RS***L*N*QNI****R*   A**F*******D*
Win       R****I*N*QNVG***N*   *****S****N*
Hev       L****L*NSQDHG***N*   *****S****N*

          43   47            80              94
CBP N     YGWTAF             IVDQCANGGLDLDWN
PR-P2     R******            R*****R*******V*
PR-4      K******            R*****S*******V*
Win2      K******            R*****S*******I*
Hev       K******            R*****S*******I*
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Fig. 2. Partial amino acid sequences of barley grain and leaf proteins. CBP N, barley grain chitin-binding protein N. Residues 7, 42 and 79 are most likely either Arg or Lys. The underlined sequences were also identified in barley leaf chitin-binding protein 5. Sequences included for comparison [7]: PR-P2, tomato PR-4 type protein P2; PR-4 tobacco PR-4 protein; Win, putative wound-induced potato protein; Hev, *Hevea brasiliensis* pro-hevein. Numbering refers to the mature PR proteins [7]. * indicate identity with the CBP N sequence. Note insertion of two residues in CBP N between residues 24 and 25.

CBP N did not exhibit chitinase, lysozyme or chitosanase activity. Neither did the protein enhance the chitinase activity or the (weak) lysozyme activity of chitinase C from barley [5].

3.2. *Induction and localization*

Immunoblotting experiments (Fig. 1B) showed that the low content of ~14 kDa CB proteins in healthy leaves of the susceptible line P-02 had increased at day 3 and at least 5-fold 7 days after inoculation with *Erysiphe graminis* (Fig. 1B). A similar increase in chitinase activity was observed [8]. Very low amounts of the CB proteins were found both in IWF (Fig. 1C) and in the subsequently prepared cell extracts (Fig. 1D) of unstressed as well as salicylate treated leaves. In contrast, nickel chloride infiltration strongly increased synthesis of the CB proteins which were only detected in the IWF and thus exported and accumulated outside the cell membrane (Fig. 1C). The tissue distribution of CBP N in developing Carlsberg II barley grain 25 days after anthesis was also estimated by immunoblotting (Fig. 1E). Only low concentrations were detected in the embryo and starchy endosperm and, apparently, the aleurone layer is the main site of expression. Other cereals of the tribe *Triticeae* (wheat, rye and oats) all contain immunochemically related proteins of same size, while cross-reacting proteins could not be detected in maize, sorghum or rice grain extracts with the CBP N antiserum (Fig. 1F).

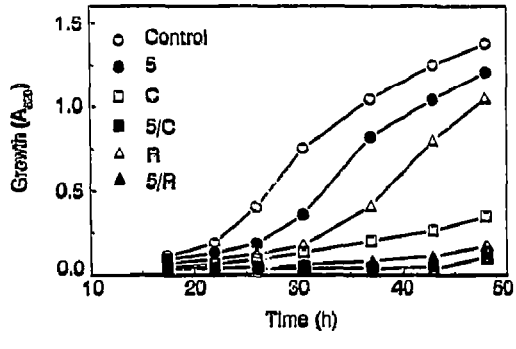

Fig. 3. Growth curves for *Trichoderma harzianum* in microtiter wells in the presence of antifungal barley proteins. 5, barley leaf CBP 5 (14 μg/ml); C, chitinase C (3 μg/ml); R, thaumatin-like protein R (7 μg/ml). X/X indicate mixtures of two proteins *in half concentrations*. A water control experiment is included. Very similar growth curves were obtained in identically performed experiments where CBP 5 was substituted with CBP 4 or CBP N.

3.3. *Antifungal activity*

Originally, the grain protein N was isolated together with several other basic proteins appearing very late during seed development [4,11]. Recently, five of these proteins: the chitinases C and T, the ribosome inactivating protein K and the thaumatin-like proteins R and S were found to retard fungal growth [5,12,13]. The three CB proteins were tested in vitro for antifungal activity in growth inhibition experiments with *Trichoderma harzianum*. Time course experiments (Fig. 3) illustrate that all three CB proteins can retard growth almost as efficiently as chitinase C and protein R [12], when added in a final concentration of ~10 μg/ml. Mixing half amounts of one of the CB proteins with half amounts of either chitinase C or protein R further delayed onset of growth and the experiments suggested synergistic inhibition in both combinations (Fig. 3). After incubation with substrate for more than 50 h no growth could be detected in 3 out of 10 wells contaning the CBP 5/chitinase C mixture and 6 of 10 wells with CBP 5/protein R. Synergism was clearly confirmed in dilution experiments where CBP N was mixed with chitinase C (Fig. 4A) or protein R (Fig. 4B) in proportion to the I_{50} values.

4. DISCUSSION

We have identified three new antifungal proteins, one from barley grain (CBP N) and two from primary leaves challenged with *Erysiphe graminis* (CBP 4 and 5). However, further sequencing is necessary to confirm that the proteins are not modifications of the same gene product. The three proteins are very similar in size, amino acid composition and sequence to PR-4 proteins from leaves of infected tobacco and tomato plants (Fig. 2). Like these acidic proteins the basic barley counterparts have blocked N-terminals. The tobacco and tomato PR-4 proteins appear to be synthesized as precursors with short N-terminal signal peptides involved in extracellular targeting [6,7] as found for other acidic PR proteins. Although basic, our experiments suggest that the barley PR-4-like proteins of stressed leaves also accumulate in

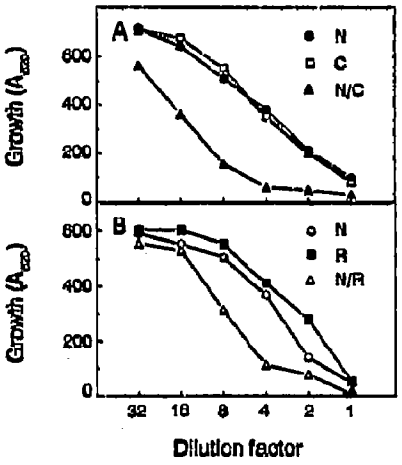

Fig. 4. Synergistic effect of barley antifungal proteins on *Trichoderma* growth in dilution experiments. (A) Grain CBP N and chitinase C. (B) CBP N and protein R. See Fig. 3. for explanation of symbols. Two proteins were mixed in ratios corresponding to the I_{50} values. The following highest concentrations (dilution factor 1) were used: N (28 μg/ml); C (6 μg/ml); R (12 μg/ml); N/C (14 μg N + 3 μg C/ml); N/R (14 μg N + 6 μg R/ml). Inhibition of *Trichoderma* growth was measured in microtiter wells in dilution assays as indicated.

the extracellular space (Fig. 1C). In contrast to the tobacco and tomato genes, the related wound induced genes of *Hevea brasiliensis* and potato encode protein precursors which contain a wheat germ lectin type domain, called hevein, between the N-terminal signal peptide and a C-terminal PR-4-type domain. In *Hevea* processing results in release of the cysteine-rich hevein domain of 43 amino acids, but the C-terminal peptide as well as the gene product(s) of the *win* genes in potato have not been identified. Hevein is present in high amounts in *Hevea* latex and possesses antifungal activity [14]. The present results suggest that also the C-terminal domain of prohevein and of the primary *win* gene product may be antifungal and have affinity for *N*-acetyl glucosamine polymers. This structural organization is similar to that of the plant class I chitinases which contain an N-terminal hevein-type domain in addition to the catalytic domain [5,15]. The barley PR-4 type proteins did not show chitin degrading activity in vitro, alone or in mixtures with chitinase. Neither did they show related (lysozyme or chitosanase) activities that could explain an affinity for (partly deacetylated) chitin. Yet, all three barley proteins inhibited *Trichoderma* growth alone and in a synergistic way both when mixed with chitinase C and, like chitinase C [12], in combination with the thaumatin-like protein R, which is assumed to have a membrane permeabilizing activity (Figs. 3 and 4). The leaf challenge experiments (Fig. 1B–D) suggest that CBP 4 and 5 are truly inducible PR-4 proteins. The grain protein CBP N appears to be deposited, together with the chitinases [5,13], in the outer aleurone layer (Fig. 1E) where combined action of the two proteins could retard fungal attacks efficiently. CBP N has previously been shown to be encoded by a gene on barley chromosome 3 (3 H) [16]. The total grain concentration in cultivated barleys is 0.2–0.4 mg/g grain [11] and CBP N can easily be isolated in sufficient amounts for detailed structural characterization and elucidation of the molecular basis for the chitin affinity as well as the antifungal activity of PR-4 type proteins.

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