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CHARACTERIZATION OF BnD22, A DROUGHT-INDUCED PROTEIN EXPRESSED IN *BRASSICA NAPUS* LEAVES

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Key Word Index—*Brassica napus*; Brassicaceae; rape; anti-proteolytic activity; circular dichroism; drought-induced protein; mass spectrometry; post-translational modification.

Abstract—BnD22, a protein shown to accumulate in *Brassica napus* leaves adapted to progressive drought stress and to salinity, was previously found to be related to the Künitz protease inhibitor family. It was purified to homogeneity by high performance liquid chromatography and characterized by circular dichroism and mass spectrometry. The protein was shown to undergo a post-translational C-terminal cleavage of 22 ± 1 amino acid residues proving that mature BnD22 is indeed a 19 kD holoprotein without side chain post-translational modification. Complementary to the sequence homology, BnD22 was found to be homologous to protease inhibitors with regards to its secondary structure and size. A negative correlation between the endogenous proteolytic activity in leaves adapted to progressive drought and the presence of BnD22 was established. The antiproteolytic activity of the purified mature BnD22 was assayed both on endogenous leaf proteases and purified serine proteases. A low level of antiproteolytic activity was observed, with a limited range of specificity. BnD22, nevertheless, might be involved in the decrease of the protease activity in the drought-adapted leaves, thus contributing to delay the leaf senescence.

INTRODUCTION

Various strategies of drought adaptation of plants have been described [1], but the molecular mechanisms involved are still poorly understood. Genes have been isolated which are expressed in vegetative tissues in response to water stress caused by different environmental conditions, such as desiccation, salinity, or cold [2, 3]. Some of these genes were also shown to be induced by exogenous treatment with the phytohormone abscisic acid whose endogenous level increases rapidly upon water stress [4]. The molecular responses to water stress were shown, by two-dimensional gel electrophoresis or by differential screening, to include the expression of many genes, the functions of which were only assigned by sequence homology if any, but not by protein isolation and use of functional assays [5].

Like other Cruciferae species, rapeseed (*Brassica napus* L. var. *oleifera*) displays an original drought adaptive strategy. When subjected to a progressive drought stress that aims to mimic the physiological conditions experienced by plants in the field during natural periods of drought, a dramatic alteration of its root system occurs, characterized by the emergence of numerous roots that remain short, hairless and often tuberized [6]. A specific behaviour is also observed in the aerial part of the plant: the first leaves, developed before the water stress, wilt progressively whereas the leaves that emerge after the onset of water deficit remain turgid, harden and display a bluish colouration due to epidermal wax bloom [7, 8]. Upon rehydration, these adapted leaves resume growth and lose their bluish tint.

Two-dimensional gel electrophoresis has indeed revealed several changes in *Brassica napus* leaf protein patterns during this adaptive response [8]. Of particular interest was the accumulation of a protein, with a M_r of 22 kD and an isoelectric point of 5.1. This protein, called BnD22 (*Brassica napus* drought-induced 22 kD protein), was not detected in well-watered plants, accounted for more than 1% of the total protein content in adapted leaves, and disappeared gradually after rehydration. Its amino acid *N*-terminal sequence proved BnD22 to be a novel protein and allowed the cloning and sequencing of the cDNA [9]. The deduced amino acid sequence indicates that BnD22 is homologous to the Künitz family of protease inhibitors, which are normally found in leg-

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uminous seeds or tubers. Actually BnD22 is not expressed in seeds and is only the second example of a protein expressed specifically in vegetative tissues in response to water stress. This protein should also be the first case of a member of the Künitz family to be involved in such a process. A similar protein, 80% homologous in sequence to BnD22, was also found in salt and water-stressed *Raphanus sativus* leaves [10].

Unlike the BnD22 transcript [9], the protein was detected neither in leaves of control plants nor in the D1 leaf* of drought-stressed plants. BnD22 accumulated abundantly in the youngest D3 leaf and to a lesser extent in D2 leaf. We have, therefore, undertaken to correlate the presence of BnD22, a putative protease inhibitor, with endogenous proteolytic activities determined in the different leaves. Because this protein was clearly separated from the other leaf proteins on 2D gels [8], the purification of BnD22 in order to get information on its structure and post-translational modifications has been carried out. This was a prerequisite to test the anti-proteolytic activity of the purified mature BnD22, which was assayed both on the endogenous leaf proteases and on purified serine proteases.

RESULTS AND DISCUSSION

Proteolytic activity in leaves submitted to progressive drought

We determined and compared the leaf proteolytic activity of crude extracts from the W1, W2, and W3 leaves of control plants and of the corresponding D1, D2 and D3 leaves of drought-stressed plants. The presence of BnD22 was also observed in D2 and D3 leaves (not shown). Figure 1 shows the proteolytic activities observed in 24-day-old drought-stressed and in well-watered leaves, as a function of pH. Two pH regions were observed as maxima, a broad one centred at pH 5, and a narrower one at pH 8, indicating that several proteases are involved in the total leaf proteolytic activity. Both proteolytic activities at pH 5 and 8 were clearly dependent on the leaf age, the oldest leaves exhibiting the greatest proteolytic activity as a result of increasing leaf senescence [11]. The proteolytic activities were drastically enhanced by the drought stress in the oldest leaf (a 83% increase at pH 5 and 43% at pH 8) and to a lesser extent in D2, but were reduced in the youngest D3 leaf, which exhibited a proteolytic activity 33% lower than W3. The progressive drought induced a significant stimulation of the proteolytic activity in the D1 leaf where BnD22 was not detected. In the D2 leaf where BnD22 was present in lower amounts, a lesser stimulation was observed, and in the D3 leaf characterized by large accumulation of BnD22, the proteolytic activity was significantly reduced. A negative correlation, suggesting an antiproteolytic role for BnD22, was therefore clearly found between the leaf proteolytic activity and the occurrence of this protein.

Purification of BnD22

The purification of BnD22 was carried out from the D3 leaf of drought-stressed plants using three chromatography steps monitored by SDS–PAGE. Firstly, a gel filtration, run in 25 mM NH_4OAc pH 6.0 buffer using Sepharcryl-300, led to a single fraction containing proteins with M_r compatible with BnD22. This fraction was largely contaminated with Rubisco and other proteins. Then, one of two procedures was used. The first, based on reverse phase HPLC, allowed a high purity of BnD22. A first reverse phase HPLC, run in 25 mM NH_4OAc pH 7.2 buffer, gave a single peak banding at 34% acetonitrile which was shown by silver stained SDS-PAGE to be slightly contaminated with a 28 kD protein (Fig. 2, lane 2). A following reverse phase HPLC, run in 0.1% TFA instead of NH_4OAc, removed this only contaminant (Fig. 2, lane 3). This first procedure allowed us to obtain 0.5 mg of purified BnD22 per g of leaf dry matter. The second was used to isolate undenatured proteins in order to test their biological activity. The RPLC was replaced by a single ion-exchange chromatography run on DEAE-Sephacell which produced a peak eluting at 0.14 M NaCl containing BnD22. However, the protein obtained was less pure than that resulting from HPLC. It showed contamination with a 17 kD protein (Fig. 2, lane 4) and with chlorophyll pigments that could not be separated without organic solvents. Anti-proteolytic activities and conformation of BnD22 were nevertheless identical whatever the purification procedure used (not shown).

Proteins obtained with either ion-exchange chromatography or NH_4OAc reverse phase HPLC were sequenced up to residue 20, while the pure BnD22 protein resulting from the second reverse phase HPLC was sequenced up to residue 45. None of these sequences exhibited any difference when compared to the mature BnD22 cDNA-deduced sequence [8, 9], definitely demonstrating that the purified protein was indeed BnD22. The proteins obtained by reverse phase HPLC exhibited a unique sequence without any distinguishable contaminant.

Post-translational modifications of BnD22

Figure 3 shows the mass spectrum of BnD22 obtained by electrospray mass spectrometry. Whereas a mass of 21 515 D was expected, we observed three major peaks at molecular masses of $18\,981 \pm 3$ D, $19\,054 \pm 1$ D and $19\,124 \pm 4$ D. No other peak revealed any contaminating protein. The mean difference (2460 D) with the expected mass corresponds to about 22 amino acids. Since the *N*-terminal end of BnD22 was sequenced and shown to be identical to the expected

* Leaves are numbered according to their development order. D is used for leaves of water-stressed plants, W for normally watered control plants.

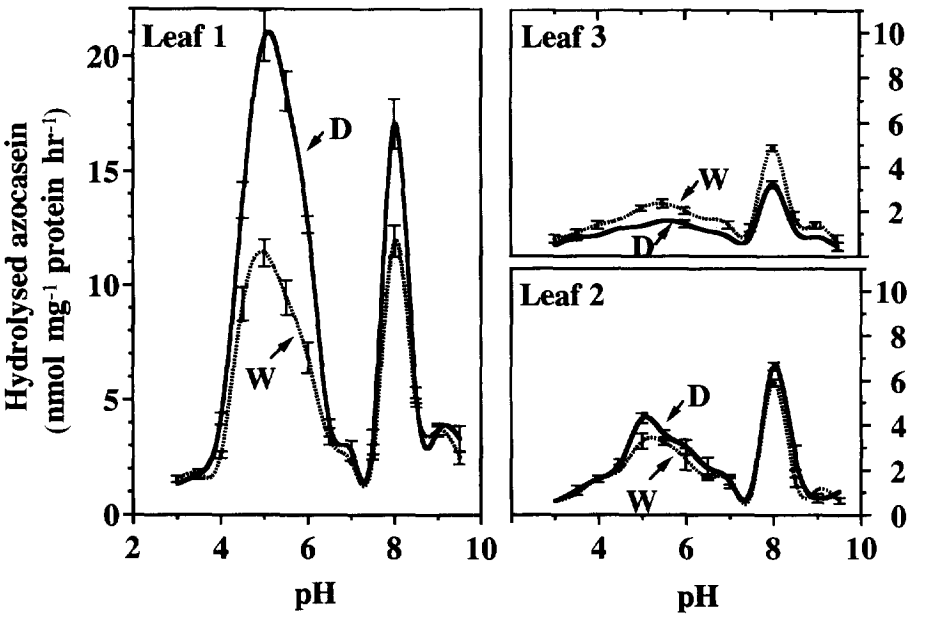

Fig. 1. Changes in proteolytic activity as a function of pH in the different leaves of 24-day old drought-stressed plants (D, solid line) and well-watered plants (W, dashed line). Each point is the mean value of six assays ± S.D. A similar scale is used for all plots.

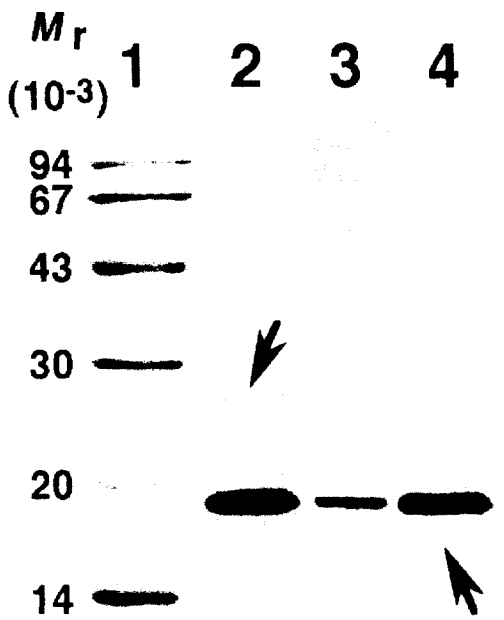

Fig. 2. SDS-PAGE of purified BnD22 fractions. Lane 1—Pharmacia LMW standards; lane 2—peak resulting from the first RPLC in NH_4OAc (silver stained); lane 3—peak resulting from the second RPLC in TFA (silver stained); lane 4—peak resulting from the alternative ion exchange chromatography (Coomassie Blue stained). Arrows indicate contaminants.

sequence [8], and also since the excision of an internal peptide was dismissed by SDS–PAGE under reducing conditions, the difference with the expected molecule was therefore looked for at the C-terminus. The masses calculated from the cDNA-deduced sequence of the protein with its 19-residue N-terminal signal peptide removed and ending at Asp176, Ala177 and Ala178 are, respectively, 18984 D, 19055 D and 19126 D. These values are in perfect agreement with the measured ones. Considering that only one N-terminus was observed and found to be strictly identical to that deduced from the cDNA sequence, this implies without ambiguity that BnD22 undergoes a stepwise C-terminal processing, excluding both the excision of an internal peptide and side chain modifications such a glycosylation.

This C-terminal processing could result from an artifactual proteolysis in the course of purification. To test this hypothesis, we compared the size of the reverse phase HPLC purified BnD22 to that of the protein in the crude extract of the D3 leaf after western-blotting and immunodetection. No difference in migration was observed, as shown in Fig. 4, that also confirmed the absence of BnD22 in the W3 leaf (watered control plant). A rapid extraction in the presence of several protein inhibitors (EDTA, PMSF, benzamidine, aprotinin, pepstatin and leupeptin), immediately followed by an NH_4OAc reverse phase HPLC, was also performed (not shown). The 34% CH_3CN peak was analysed by SDS–PAGE and ES-MS mass spectrometry. No difference in size was observed with regard to the BnD22 obtained with the standard procedure, strongly suggesting that the C-terminal processing naturally occurs *in planta*.

Therefore, the actual molecular mass of mature BnD22, which comprises 177 ± 1 amino acids, is 19054 ± 70 D. This size is in better agreement with known Künitz protease inhibitors, such as the soybean chymotrypsin inhibitor b with 181 residues [12] or the winged bean trypsin inhibitor 3 [13] with

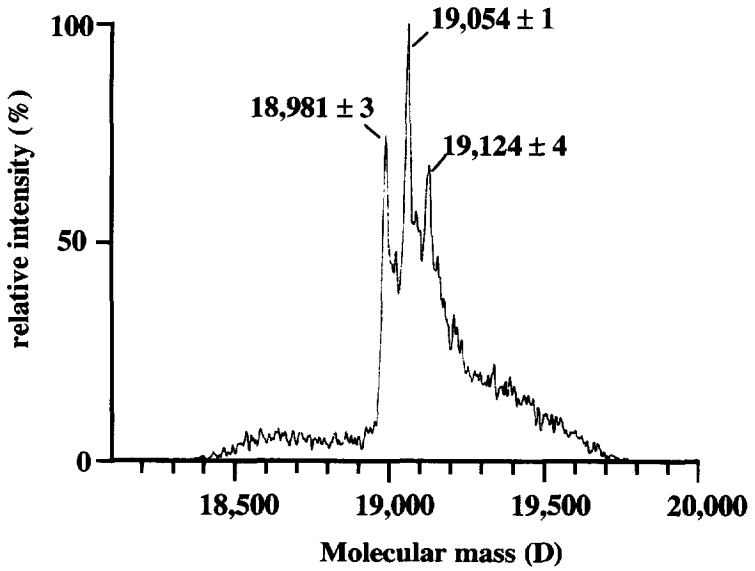

Fig. 3. Reconstituted mass spectrum of BnD22 purified using RPLC chromatography.

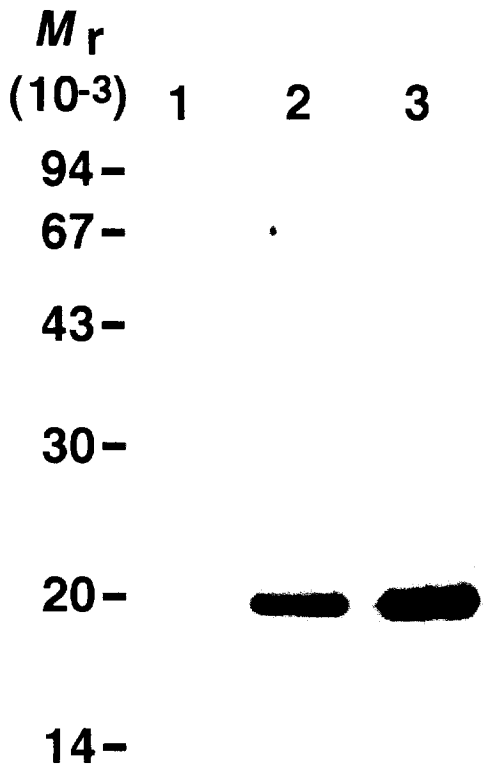

Fig. 4. Comparison of the size of purified BnD22 to that in D3 leaf crude extract. Western blots of well-watered W3 leaf extract (control, lane 1), drought-stressed D3 leaf extract (lane 2) and purified BnD22 (lane 3).

183 amino acids. Taking into account the mature BnD22 size, the sequence homology with these inhibitors is calculated to be 26% instead of 23% for the soybean trypsin inhibitor b and 20% instead of 18% for the winged bean chymotrypsin inhibitor 3. It is also noteworthy that the C-terminal removed sequence of BnD22 is almost strictly identical to the C-terminus of P22, a radish water and salt stress induced protein, whose cDNA-deduced sequence is 80% homologous to BnD22 [10]. The removal of this conserved C-terminal end may, therefore, have a biological meaning. One could postulate that this region of the molecule might play a role in the targeting towards a particular cellular compartment, as has been found for barley lectin [14]. It has also been suggested that the 20 residues which differentiate osmotin from thaumatin at the C-terminal end would address differently these proteins in the cell [15]. So BnD22 could be targeted to a cell compartment different from standard Künitz protease inhibitors.

Secondary structure of BnD22

Figure 5 illustrates the CD spectrum of BnD22 compared with that of the soybean trypsin inhibitor in 10 mM K-Pi buffer pH 7.4. Similar results were obtained

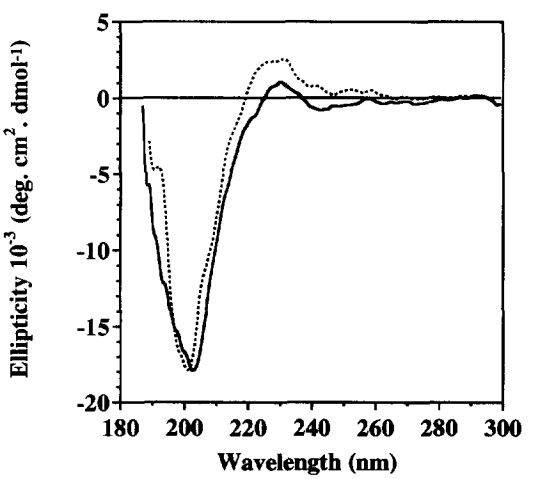

Fig. 5. Far UV circular dichroism spectra of BnD22 (solid line) and of soybean trypsin inhibitor (dashed line).

with BnD22 purified either by reverse phase HPLC or by ion-exchange chromatography, revealing that the use of organic solvents did not induce any conformational change and that the interaction with pigments was without effect on its folding. The soybean trypsin inhibitor CD spectrum we obtained was identical to the previously reported one [16]. The BnD22 and soybean trypsin inhibitor spectra are very close to each other and characterized by a main negative peak banding around 200 nm and a weak positive band at 230 nm. They do not exhibit the typical extrema of known periodic secondary structures. Nevertheless computation of the relative amounts of these structures shows that β-sheet accounts for 45 ± 34 and $21 \pm 72\%$, β-turns for 11 ± 11 and $19 \pm 24\%$ and aperiodic structure represents 44 ± 6 and $60 \pm 12\%$ of the molecules of BnD22 and soybean trypsin inhibitor, respectively, while α-helix is apparently absent in both proteins. The secondary structure prediction indicated in BnD22 is 25% β-sheet, 72% aperiodic and 3% α-helix. It is worth noticing that the BnD22 CD spectrum and secondary structure are very similar to those of plant Künitz protease inhibitors, such as soybean trypsin inhibitor [16, 17] and alfalfa protease inhibitor I [18]. It has been observed by X-ray crystallography that *Erythrina caffra* trypsin inhibitor, another member of the Künitz family, exhibits large amounts of distorted β-sheets [19], which explains why protease inhibitor CD spectra are atypical (note the large standard deviation of the measured structure amounts [16]). In addition to the sequence and the size, the secondary structure and the CD spectrum of BnD22 support the homology of this protein with members of the Künitz protease inhibitor family.

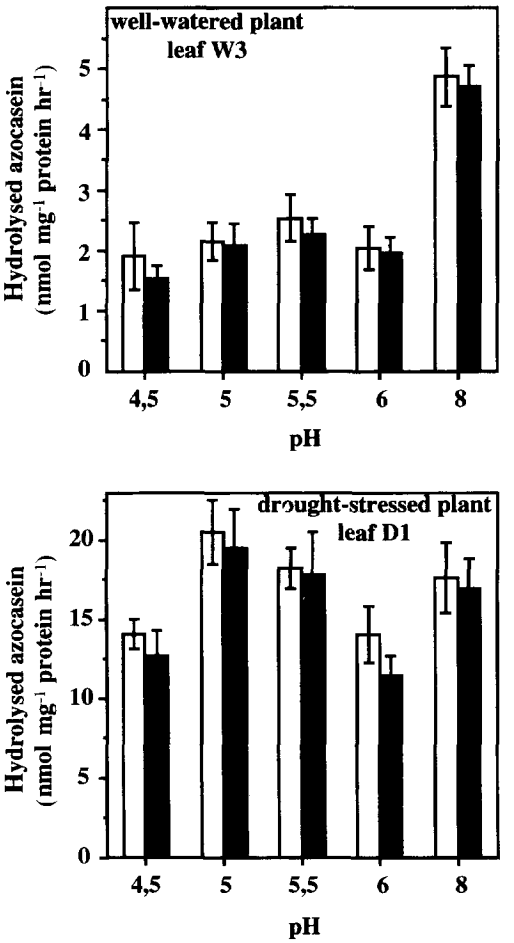

Fig. 6. Proteolytic activity at various pH of the youngest leaf of well-watered plant (W3) and of the oldest leaf of drought-stressed plant (D1) in the presence (■) or in the absence (□) of 15 μM BnD22. Crude leaf extracts were incubated with or without BnD22 at 4 for 120 min. Each point is the mean value of six assays $\pm$ S.D.

Antiproteolytic activity of purified BnD22

Once purified and more precisely characterized as a putative member of the Künitz protease inhibitor family, BnD22 was tested for its ability to inhibit *in vitro* the proteases present in *Brassica napus* leaves and some heterologous standard proteases. We used BnD22 purified by either of the two procedures, since the results indicated no difference in activity, in agreement with the CD conformation determination.

Tests on leaf proteases were performed at various pH values in the presence of 15 μM BnD22 using various incubation times between enzymes and BnD22 (from 3 to 90 min). BnD22 was assayed against the endogenous proteases of the W1, W2, and W3 leaves of control plants and of the corresponding leaves (D1, D2, D3) of drought-stressed plants. Figure 6 illustrates results obtained with the oldest D1 leaf, in which a high proteolytic activity stimulation was observed under water stress and with W3-leaf of the control plant. Data for this leaf are presented because it is homologous to the leaf D3 in which BnD22 is expressed together with a significant inhibition of proteolytic activity. Whatever the BnD22-enzyme incubation conditions, and independently of the pH of the assay, only a slight decrease of the endogenous proteolytic activity was systematically observed for the various leaves: for instance a 14% decrease of the proteolytic activity in leaf D1 was noted at pH 6.0. Nevertheless, due to the range of values obtained on replicates, the differences may not be significant. The endogenous proteolytic activity in leaf extracts was also measured with D3 leaf crude extract instead of purified BnD22 (not shown). Since no significant inhibition was observed, the lack of activity of the purified BnD22 is shown to be independent of the purification process.

Tests on purified serine proteases from different origins (trypsin, chymotrypsin, subtilisin, proteinase K) were performed at their optimal pH with various BnD22 concentrations and various incubation times (from 3 to 90 min) between the enzyme and BnD22 at 4, 10, 20, 30 and 37°. Figure 7 shows the proteolytic activity of chymotrypsin and trypsin as a function of increasing concentrations of inhibitors, i.e. purified BnD22 or aprotinin used as a control. Chymotrypsin

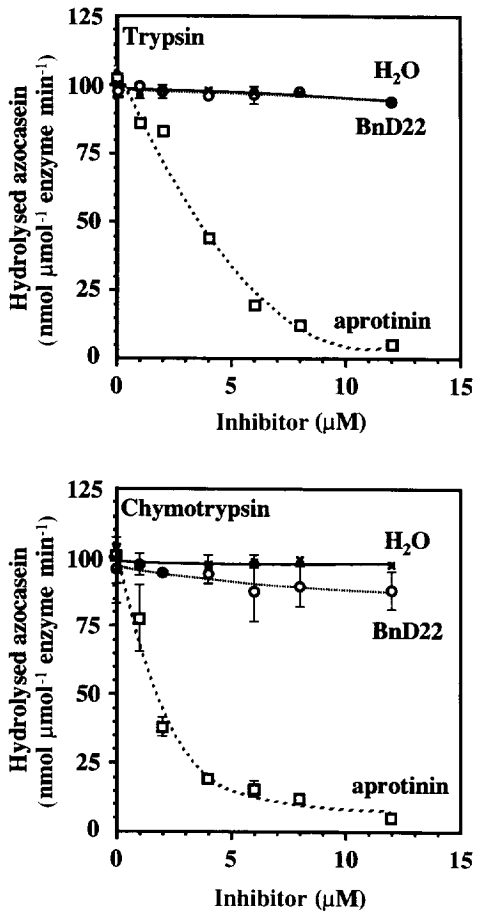

Fig. 7. Evolution of proteolytic activity at pH 8.0 of trypsin and chymotrypsin as a function of increasing concentration of aprotinin (--□--) or BnD22 (---○--). H_2O was used as control (—×—). Proteases were incubated with inhibitor at 37 during 90 min. Each point is the mean value of six assays ± S.D.

was progressively inhibited by BnD22, but to an extent considerably reduced when compared with aprotinin (about 10% of the maximal aprotinin inhibition at 12 µM BnD22). Taking into account the number of replicates and the error bar, the measured inhibition was considered as significant ($P = 0.05$). In the case of trypsin, the proteolytic activity was identical in the presence or absence of BnD22; results also obtained with subtilisin and proteinase K (not shown). Such a differential response between chymotrypsin and the other serine proteases could result from a difference in affinity between inhibitors and proteases [20]. For instance, three trypsin inhibitors, the soybean trypsin inhibitor, the Lima bean inhibitor and the egg white inhibitor, inhibit trypsin and chymotrypsin from 12 animal species with large differences. The soybean trypsin inhibitor is not able to inhibit ovine chymotrypsin, but strongly inhibits porcine chymotrypsin [21].

The lack of trypsin inhibition by BnD22 is in agreement with the absence of the expected active site residues of the Künitz protease inhibitors (Arg or Lys). Residues such as Phe, Tyr, Leu and Ser are known to be found in the P1 active site of chymotrypsin inhibitors [13, 22]. Such residues are effectively found in BnD22 in a homologous region, in agreement with the specificity of its activity, but the high variability of the active site sequences of protease inhibitors [22, 23] does not allow any reliable prediction of the residues of the BnD22 active site in the absence of knowledge of its 3D structure.

CONCLUSION

Proteases are known to occur in all examined plant species and to be involved in many stages of plant life such as development, senescence or defence against pathogens [24]. In plants, the activity of such enzymes is controlled by more or less specific inhibitors [25]. We observed that, when submitted to progressive drought stress, *Brassica napus* responds by increasing the level of the leaf proteolytic activity, in a process akin to precocious senescence, except in the leaves that develop a resistance against drought [7], i.e. those which accumulate BnD22 [8]. A clear correlation was observed between the presence of BnD22 in the resistant leaves and a lower endogenous leaf proteolytic activity. BnD22 was thoroughly purified by RPLC without protein or pigment contamination, as revealed by SDS–PAGE, ES-MS and sequencing. This purified protein was not shown to induce a significant inhibition of the endogenous leaf proteases. It was, nevertheless, able to slightly inhibit chymotrypsin, but no other serine proteases tested. BnD22 could, therefore, be involved in the decrease of protease activity, thus contributing to a delay in the senescence process. Since several proteolytic activities were observed with different optimal pHs and also because BnD22 exhibits a certain specificity, other factors are probably necessary to explain the *in vivo* decrease of the leaf proteolytic activity.

The BnD22 protease inhibitor activity was observed to be very low with a limited range of specificity. The absence of activity of BnD22 against endogenous proteases can be linked to previously reported results showing that inhibitors active *in vitro* against purified proteases were ineffective against endogenous proteases [26]. Besides, the standard proteases tested were in limited number and, furthermore, a specific leaf protease might be more strongly inhibited by BnD22. This protein could act against some proteases confined to a precise location in the cell. The subcellular location of BnD22 might then be responsible for its physiological role. When BnD22 was tested in a system where all cell compartments were disrupted, a specific process controlled by BnD22 might therefore have been hidden. Such a location might arise not only from the N-terminal signal peptide excision, but also from the removal of the C-terminal end that would play a role in the targeting towards a specific cellular compartment. The likely biological importance of the C-terminal end removal is supported by

the observation that P22, another water and salt stress induced protein, exhibits a quite homologous C-terminal end [10].

With this C-terminal end removed, the mature BnD22 was found to be closely related to Künitz inhibitors in size and sequence. This homology was also supported by the secondary structure determination. The structural data reinforce the aptitude of BnD22 to play the role of a protease inhibitor. We conclude that some unknown specific proteases would, therefore, be inhibited by BnD22.

EXPERIMENTAL

Rapeseed culture under progressive water deficit. Rapeseed *Brassica napus* L. var. *oleifera* Metz. cv. Darmor were germinated on moistened filter paper in the dark for 4 days. The seedlings were transferred (day 0) to individual plastic pots filled with 1.5 kg sandy soil, watered to field capacity, i.e. 5.6% dry wt humidity at a soil Ψ of -0.01 MPa [27]. The soil surface was covered with a sheet of Parafilm to prevent evaporation; subsequent H_2O loss thus occurred through plant transpiration only. Plants were grown in a microphytotron at 22 , 50% relative atmospheric humidity and 200 μE m^{-2} s^{-1} PAR during a 16 hr photoperiod. Control plants were watered daily to maintain the soil humidity at field capacity. Progressive drought stress was imposed by withholding H_2O from day 0. Each experiment was carried out during the vegetative phase of growth at the rosette stage on six plants grown simultaneously. Leaves were collected between days 20 and 30. At day 20, leaves 1, 2 and 3 were, respectively, 16, 15 and 9 days old.

Proteolytic activity of leaf extracts. Lyophilized leaves (40 mg) were ground at 4 in 4 ml of 50 mM Tris–HCl pH 7.5, 1 mM DTE with 40 mg insoluble PVP (extracting buffer) in a Potter homogenizer. The sample was centrifuged at 15000 *g* for 10 min at 4 and the supernatant collected. $(NH_4)_2SO_4$ was added to reach 3.65 M. After a 30 min stirring at 4 the suspension was pelleted at 15000 *g* for 20 min at 4 . The pellet was resuspended in 2.5 ml of extracting buffer, then desalted using a Sehedex G-25 column to obtain the purified leaf extract. Leaf extract proteolytic acitivty was colorimetrically determinated with azocasein as substrate. Purified leaf extract (200 μl) was added with 400 μl of 0.4% (w/v) azocasein in H_2O and 400 μl of test buffer. Depending on pH, the test buffers used were: 50 mM Gly–HCl (pH 3), 50 mM Na citrate–HCl (pH 3.5), 50 mM NaOAc (pH 4, 4.5 and 5), 50 mM MES–NaOH (pH 5.5, 6 and 6.5), 50 mM Tris–HCl (pH 7, 7.5 and 8) and 50 mM Bicine–HCl (pH 8.5, 9 and 9.5), every buffer contained 10 mM $CaCl_2$ and 1 mM DTE or 0.1% 2-mercaptoethanol. The mixture was incubated at 37 for 3 hr. The reaction was stopped by a 200 μl addition of 50% (w/v) TCA, then maintained for 30 min at 4 before centrifugation at 15000 *g* for 10 min. The supernatant was collected for *A* 336 nm determination. The proteolytic acitivty is expressed by hydrolysed azocasein (nmol mg^{-1} protein hr^{-1}). Soluble proteins were determined according to ref. [28]. Each assay was repeated six times on six different extracts from six different plants.

Determination of BnD22 anti-proteolytic acitivty. Purified BnD22 was incubated with leaf extracts, obtained as above, for 3–120 min at 4 and at the pH of the assay mixture, before proteolytic activity measurement. Determinations were run, as already described, at pH 4.5, 5, 5.5, 6 and 8. The inhibition capability of BnD22 was also assayed on the following purified proteases: Trypsin (EC 3.4.21.4, bovine pancreas), α-chymotrypsin (EC 3.4.21.1, bovine pancreas), subtilisin (EC 3.4.21.14) and proteinase K (EC 3.4.21.14). Aprotinin, an inhibitor of all the serine proteases mentioned above was used as a control at various concns. Incubation of enzymes (1 μM) with various amounts of either BnD22 or aprotinin (15 μM) was performed in 600 μl containing 10 mM $CaCl_2$ in 50 mM Tris–HCl pH 8 for 3–90 min at 4, 10, 20, 30 and 37 . Further addition in the incubation mixt. of 400 μl of 0.4% (w/v) azocasein in H_2O started the reaction, and was allowed to develop for 60 min at 37 . The assay was performed and repeated as above.

BnD22 extraction and purification. Lyophilized 30-day-old D3 leaves (400 mg) were ground at 4 in 4 ml of 25 mM NH_4OAc pH 6, 1 mM DTE with 200 mg insoluble PVP in a Potter homogenizer with or without a cocktail of protease inhibitor composed of 1 mM EDTA, 0.5 mM PMSF, 0.5 mM benzamidine, 5 μg ml^{-1} aprotinin, 5 μg ml^{-1} pepstatin and 5 μg ml^{-1} leupeptin. The sample was centrifuged at 15000 *g* for 20 min at 4 and the supernatant collected.

Gel filtration was first performed with a Sephacryl-300 (Pharmacia) 2.4×50 cm column in 25 mM NH_4OAc buffer pH 6, 1 mM EDTA and 1 mM DTE at a flow rate of 1.8 ml min^{-1} and monitored by UV absorbance. BnD22 containing fractions were identified by SDS–PAGE and lyophilized before submission to either reverse phase HPLC or ion-exchange chromatography.

Reverse phase HPLC was run on a Spectra-Physics device using an Aquapore Octyl-RP300 (Brownlee Labs) cartridge (4.6×30 mm) equilibrated with 25 mM NH_4OAc buffer pH 7.2 with 5% CH_3CN. Elution was performed with a linear 5–50% CH_3CN gradient longing for 65 min at a flow rate of 0.5 ml min^{-1}. BnD22 containing frs were submitted to a second reverse phase HPLC run in H_2O–TFA medium using the same column eluted with a linear 0–60% CH_3CN gradient for 60 min at a flow rate of 0.5 ml min^{-1}.

In the alternate procedure, ion-exchange chromatography was performed with a DEAE-Sephacell (Pharmacia) 16×1.2 cm column equilibrated with 20 mM Tris–HCl buffer pH 8.1, 20 mM $CaCl_2$. Elution was run with a 0–0.3 M NaCl gradient at a flow rate of 0.12 ml min^{-1}. BnD22 containing frs were desalted by gel filtration on Pharmacia PD10 column before analysis.

SDS polyacrylamide gel electrophoresis and immunodetection. SDS–PAGE was performed as described in ref. [29] with modification with 12.5% polyacrylamide gels, using a Biorad mini-Protean II system. Proteins were stained either with Coomassie Blue in 95% EtOH or with $AgNO_3$ using a Bio-Rad kit. LMW standard electrophoresis kit (Pharmacia) was used for M_r determination. For Western immunoblot analysis, the polypeptides were transferred from the SDS–PAGE gel to a nitrocellulose membrane (Schleicher and Schuell, BA85 0.45 μm) using a Biorad Trans-blot cell. After transfer, antigenic proteins were detected using the Protoblot system (Promega, France) and an anti BnD22 serum obtained in rabbits from purified BnD22.

N-terminal sequencing and mass spectrometry. The *N*-terminal sequences of the purified proteins were determined using automated Edman degradation using an Applied Biosystems 475 sequencer equipped with an on-line phenyl thiohydantoin amino-acid analyser. Precise determination of the M_r BnD22 was performed on a Perkin Elmer API 100 mass spectrometer using an ion spray source. Twenty microlitres of a 5.8 pmol μl^{-1} BnD22 soln in 50% CH_3CN, 49.9% H_2O, 0.1% HCO_2H were infused into the source at a flow rate of 2 μl min^{-1}. On the spray needle 4800 V were applied. The quadrupole was calibrated with a Perkin Elmer polypropylene glycol soln. The calcd and experimental M_r are the average mass and not the monoisotopic mass.

Circular dichroism and secondary structure prediction. CD spectra were obtained and analysed as described previously [30]. BnD22 and soybean trypsin inhibitor concns were determined using UV spectroscopy employing molar ε of 27 055 and 15 470 M^{-1} cm^{-1}, respectively, at 278 nm, calcd from the amino acid content of the mature processed molecules [31]. Secondary structure prediction from the BnD22 cDNA-deduced amino acid sequence [9] was performed according to ref. [32].

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