Isolation and Characterization of a Thionin Proprotein-processing Enzyme from Barley*

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Stephan Plattner‡, Clemens Gruber‡, Johannes Stadlmann§, Stefan Widmann‡, Christian W. Gruber‡, Friedrich Altmann§, and Holger Bohlmann interpolation

From the Division of Plant Protection, Department of Crop Sciences, and Department of Chemistry, University of Natural Resources and Life Sciences, A-1190 Vienna and the Center for Physiology and Pharmacology, Medical University of Vienna, A-1090 Vienna, Austria

Background: Thionins are produced as preproproteins, implicating the existence of a protease responsible for proteolytic processing.

Results: The barley subtilase BAJ93208 was found to be a strong candidate for the barley leaf thionin-processing enzyme.

Conclusion: A candidate processing enzyme for barley thionin proproteins has been identified.

Significance: The three-dimensional structure of the antimicrobial peptide protects it against proteolysis during processing.

Thionins are plant-specific antimicrobial peptides that have been isolated from the endosperm and leaves of cereals, from the leaves of mistletoes, and from several other plant species. They are generally basic peptides with three or four disulfide bridges and a molecular mass of ~5 kDa. Thionins are produced as preproproteins consisting of a signal peptide, the thionin domain, and an acidic domain. Previously, only mature thionin peptides have been isolated from plants, and in addition to removal of the signal peptide, at least one cleavage processing step between the thionin and the acidic domain is necessary to release the mature thionin. In this work, we identified a thionin proprotein-processing enzyme (TPPE) from barley. Purification of the enzyme was guided by an assay that used a quenched fluorogenic peptide comprising the amino acid sequence of the enzyme was guided by an assay that used a quenched fluorogenic peptide comprising the amino acid sequence of the barley thionin domain (5 kDa). Thionins are produced as preproproteins containing an N-terminal signal peptide and a C-terminal propeptide, which is usually acidic. We isolated and sequenced the BTH6 thionin from barley to confirm the N and C terminus of the peptide in planta. Using an enzymatic assay, the recombinant TPPE was able to process the quenched fluorogenic peptide and to cleave the acidic domain at least at six sites releasing the mature thionin from the propeptide. Moreover, it was found that the intrinsic three-dimensional structure of the BTH6 thionin domain prevents cleavage of the mature BTH6 thionin by the TPPE.

Plants are continuously exposed to a large number of pests and pathogens and therefore have evolved different defense mechanisms to enhance their survival. Among those are a large variety of antimicrobial peptides, whose production can be constitutive or inducible. To date, many antimicrobial peptides have been isolated from different plant species and have been shown to possess antimicrobial activity in vitro (1, 2). The first antimicrobial peptide isolated and characterized was purothionin from the endosperm of wheat (3). Purothionin is actually a mixture of closely related isoforms (4), and similar peptides have subsequently been isolated from other cereals, mistletoes, and several other plant species (5, 6). Barley contains in addition to the endosperm-specific hordothionins a large group of so-called leaf thionins (7). Thionins contain on average 45 amino acids, are usually basic, and contain 6 or 8 cysteine residues that have been shown to form disulfide bridges. A variety of toxic and antimicrobial activities of different thionins have been found in vitro (6), and the overexpression of thionins in plants resulted in enhanced resistance against pathogens (8).

The first thionin was discovered by amino acid sequencing of the isolated peptides (5). In the meantime, DNA sequencing of thionin cDNAs and genes showed that thionins are produced as preproproteins (9) containing an N-terminal signal peptide for secretion and a C-terminal propeptide, which is usually acidic and has therefore been named acidic domain. A peptide corresponding to the acidic domain has never been isolated from plants. To date, no experimental information is available about possible functions of the acidic domain, but it is clearly not dispensable as shown by the high conservation of the cysteine residues. Even viscosides, which have several deletions in the acidic domain, have the same conserved pattern of cysteine residues (10). Possible functions of the acidic domain could be to target the mature thionin via the secretory pathway to its final destination (vacuoles, cell walls, and protein bodies), to neutralize the toxic basic thionins and thereby protect the cell against its own toxin, and as an intramolecular chaperone to assist in the folding of the thionin. The propeptide for a plant defensin is needed for protection of the plant against its own peptide and for subcellular targeting to the vacuole (11).

Subtilases represent a group of serine proteases with over 200 known proteins (12). Members of the subtilase superfamily have the same catalytic triad (Asp, His, and Ser) as subtilisin
Carlsberg and subtilisin BPN, two closely related serine proteases isolated from two strains of *Bacillus subtilis* in 1966 by Smith *et al.* (13). Subtilases can be classified into six families according to their catalytic domain structures, namely subtilisins, thermitases, proteinases K, lantibiotic peptidases, kexins, and pyrolysin (12). An alternative classification is available via the protease database MEROPS (14). In accordance with MEROPS, the subtilase family S8 is divided into the prokaryotic subgroup S8A and the eukaryotic subgroup S8B. All families mentioned above, except the kexin family, are represented in subgroup S8A, although the latter is found in S8B.

Subtilases are more abundant in plants than in mammals with 63 members present in rice and 56 in *Arabidopsis* (15, 16). All subtilases found in these two species belong to the pyrolysin subfamily. Some plant-derived enzymes have been studied in detail and are responsible for highly specific catalytic reactions in addition to nonselective general protein turnover.

Different *Arabidopsis* subtilases are involved in the processing of protein precursors, which indicated that the thionin proprotein-processing enzyme (TPPE) might also be a subtilase. Previously, Romero *et al.* (17) purified a protease from barley leaves that was able to process a thionin precursor protein *in vitro*. However, the authors claimed that they were only able to obtain a partial sequence (which was not published) of the protein, and no further studies have been reported since then. Here, we directly purified the TPPE from etiolated barley seedlings because these can be produced easily and contain large amounts of leaf-specific thionins (18). Because of massive expressed sequence tag and genomic sequencing efforts in the last years, it was likely that a partial sequence of the TPPE enzyme would lead to identification of the corresponding gene or cDNA sequence. We used a fluorescently labeled peptide that incorporated the flanking sequence between barley leaf-specific thionins and their acidic domains to identify the TPPE protein, and we were able to identify several peptides by mass spectrometry that corresponded to a barley proteinase for which a complete cDNA sequence was available. The barley TPPE was produced as a strep tagged protein in *E. coli* and used to study the processing of a recombinant leaf-thionin precursor *in vitro*.

**Experimental Procedures**

**Plant Growth**—Seeds of spring barley (*Hordeum vulgare*) variety Xanadu were soaked in water for 4 h at room temperature, transferred to vermiculite, and incubated at room temperature in light-tight plastic boxes. Etiolated seedlings were harvested after 5 days, frozen in liquid nitrogen, and stored at −80 °C until further usage.

**Protease Assay**—A fluorogenic substrate assay was used to measure TPPE activity. In a standard reaction, 10 μl of protease-containing sample was added to a solution containing a final concentration of 2 μM fluorogenic peptide (5-amino-2-nitrobenzoyl-SDYPKLLPK-7-methoxycoumarinyl-4-acetyl), 25 mM MES, pH 6.5, supplemented with 150 mM NaCl and 10 mM CaCl2. Kinetic assays were performed at 25 °C, and formation of methoxycoumarinyl-4-acetyl (MCA)-labeled cleavage product was followed at 330 nm excitation and 420 nm emission using black 96-well microtiter plates (Greiner) in a FLUOstar Omega spectrophotometer (BMG Labtech).

**Chromatographic Purification of Native TPPE from Barley**—All chromatographic purifications used to purify the native TPPE from barley were performed on an ÄKTA Purifier 10 System (GE Healthcare). The purification was done as described by Romero *et al.* (17) with some modifications. Five-day-old etiolated barley seedlings (500 g) were harvested, immediately frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle. Proteins were extracted in 1 liter of 20 mM sodium acetate buffer, pH 5.5. Insoluble material was removed by centrifugation at 7000 × g for 20 min at 4 °C. The extract was lyophilized, dissolved in 80 ml of 20 mM sodium acetate buffer, pH 5.5, centrifuged at 15,000 × g at 4 °C for 15 min, and filtered through a 0.22-μm membrane. The filtrate was applied to a self-packed, 80-cm Sephacryl S-200 HR gel filtration column (GE Healthcare). The column was equilibrated with 20 mM sodium acetate buffer, pH 5.5. A flow rate of 2 ml/min was used, and 5-ml fractions were collected. Fractions were analyzed for protease activity, and active fractions were pooled, centrifuged at 15,000 × g at 4 °C, and filtered through a 0.22-μm membrane. The solution was loaded onto a Highprep SP FF 16/10 cation exchange column (GE Healthcare) with a flow rate of 5 ml/min. A NaCl gradient (0 to 1 M) for 50 min was used to elute bound proteins. Protease activity was measured and found in the unbound fraction. This fraction was concentrated 10 times and dialyzed against 50 mM Tris/HCl, pH 8, using an ultrafiltration cell with a 3-kDa cutoff membrane and applied onto a Mono Q HR 16/10 anion exchange column (GE Healthcare) with a constant flow rate of 5 ml/min. The column was equilibrated with binding buffer (50 mM Tris/HCl, pH 8). Bound compounds were eluted using a linear NaCl gradient from 0 to 1 M NaCl for 50 min. 1-ml fractions were collected and analyzed for protease activity. Active fractions were concentrated five times, and 15-μl samples were separated on SDS-PAGE.

**Synthesis of cDNA**—RNA was isolated from etiolated barley leaves using the Qiagen RNA plant mini kit according to the protocol of the manufacturer. SuperScript® III reverse transcriptase (Invitrogen) was used to synthesize cDNA following the standard protocol with an oligo(dT) primer. The cDNA was amplified by PCR using specific primers (Table 1) incorporating restriction sites for subsequent cloning into the expression vector.

**Cloning, Expression, and Purification of BTH6 Thionin Proprotein**—To follow the destiny of the mature thionin and the acidic domain after cleavage, the BTH6 proprotein was produced with an N-terminal Myc tag and a C-terminal strep tag in *E. coli*. To overcome solubility problems during the expression in *E. coli*, the proprotein was expressed as a fusion protein with thioredoxin, which is known to enhance disulfide bond formation and solubility of its fusion partner during expression in the cytoplasm. After expression and purification, the fusion protein was cleaved from the proprotein by TEVSh protease (19), which
recognized a sequence between fusion protein and the double-tagged BTH6 proprotein.

The expression vector pETtrx_1a (20) was modified to introduce a sequence coding for a Myc tag. Vector-specific primers pETTRXfor2 and TRXmycDralBamH1rev2 were used in a PCR to amplify a part of the pETtrx_1a vector and to introduce the coding sequence for a Myc tag together with a multiple cloning site containing the restriction sites for DraI and BamHI. The amplicon was subsequently digested with XbaI and BamHI and ligated into the original pETtrx_1a vector digested with the same enzymes to produce pETtrxMyc_1a for expression of the BTH6 proprotein.

The BTH6 cDNA was synthesized with oligo(dT) primers using total RNA extracted from etiolated barley seedlings. Gene-specific primers BTH6DraIfor and BTH6strepRevBam were used to amplify the sequence coding for the proprotein from the cDNA by PCR and to introduce the restriction sites for DraI and BamHI. The amplicon was subsequently digested with XbaI and BamHI and ligated into the original pETtrx_1a vector digested with XbaI and BamHI and cloned into pETtrxMyc_1a to produce pETtrxMycBTH6strep.

The plasmid pETtrxMycBTH6strep was transformed into the E. coli expression strain SHuffle C3030 (21). Induction was performed with 1 mM isopropyl-β-D-galactopyranoside for 16 h at 16 °C. Cells were harvested by centrifugation and target protein was purified using a nickel-nitriacetic acid 1-ml HisTrap FF column on an Äkta purifier 10 system. Bound proteins were eluted by increasing the imidazole concentration from 25 mM MES, 100 mM NaCl, 10 mM CaCl₂, pH 6.5, using Amicon Ultra 10K filtration centrifugal filters (Millipore) according to the manufacturer’s recommendations. The purified myc-BTH6-strep proprotein was confirmed by LC-ESI-MS.

**Purification of BTH6 from Barley Seedlings**—Etiolated barley seedlings were frozen in liquid nitrogen and ground to a fine powder. Protein extraction was performed with 10% acetic acid, and the solution was desalted with a gravity flow BAKERbond SPE octyl solid phase extraction column (Mallinkrodt Baker). Elution of bound compounds was performed with 70% acetonitrile. The eluate was dried in a centrifugal evaporator and resuspended in 10 ml of 20 mM sodium acetate, pH 5. A Mono S HR 16/10 column (GE Healthcare) was used to capture the thionin fraction. Bound proteins were eluted with a linear NaCl gradient against 25 mM MES, 100 mM NaCl, 10 mM CaCl₂, pH 6.5, using Amicon Ultra 10K filtration centrifugal filters (Millipore) according to the manufacturer’s recommendations. The purified peptide was sequenced and analyzed by MALDI-TOF and applied to the reverse phase chromatography column Source C15 RPC ST 4.6/100 (GE Healthcare). Thionin-containing fractions were determined by MALDI-TOF, dried in a centrifugal evaporator, resuspended in 0.1% TFA, and applied to a µRPC C2/C18 column. Bound peptides were eluted with a linear NaCl gradient (0–100%). Thionin-enriched fractions were identified using Amicon Ultra 10K filtration centrifugal filters (Millipore). The resulting peptides were separated and analyzed by LC-ESI-MS.
room temperature with constant shaking. Destaining was performed three times in 10% acetic acid for 1 h each time, and results were documented with a digital camera.

**Carboxymethylation of Cysteines**—Recombinant thionin proproteins were dried in a centrifugal evaporator (MI-VAC Quattro Concentrator, GeneVac) and reconstituted in 100 mM Tris/HCl, pH 8.5, to a final concentration of 1 mg/mL. Reduction of cystines was performed by adding 10 μL of 1 M DTT (final concentration 10 mM) followed by incubation at 37 °C for 2 h. The samples were cooled to room temperature, and 25 μL of 1 M iodoacetic acid (prepared in 1N NaOH) was added. The solution was incubated for 30 min at room temperature in the dark. 20 μL of 1 M DTT was added to quench the iodoacetic acid. The reduced and cysteine-carboxymethylated protein samples were dialyzed against TPPE digestion buffer (25 mM MES, 100 mM NaCl, 10 mM CaCl₂, pH 6.5) using Amicon Ultra 10K ultrafiltration centrifugal filters (Millipore) according to the recommendations of the manufacturer.

**LC-ESI-MS/MS Analysis**—Peptides were dried in a centrifugal evaporator (MI-VAC Quattro Concentrator, GeneVac) for 20 min, reconstituted in water, and applied to HPLC in combination with an electrospray tandem mass spectrometer (LC-ESI-MS/MS, Dionex Ultimate 3000, Bruker maXis 4G ETD). Separation of trypsin-digested peptides was performed using a reverse phase C18 column (Thermo BioBasic C18, 150 × 0.32 mm, 5 μm packing) with peptide elution achieved by using a gradient of 55 min from 5% acetonitrile (solvent A, 0.1% formic acid) to 80% acetonitrile. Data generated by mass spectrometry were processed using Data Analysis 4 (Bruker Daltonics) and MASCOT (24). Deconvolution was performed using MaxEnt software (Micromass, Manchester, UK).

**MALDI-TOF-MS Analysis**—Thionin-containing fractions were determined by MALDI-TOF-MS. Samples were desalted and concentrated with Bond Elut OMIX C4 tips (Agilent) according to manufacturer’s recommendations and eluted from the tips with elution buffer (50% (v/v) acetonitrile, 0.1% TFA). 1 μL of desalted peptide samples were spotted on the target plate (MTP 384 target ground steel TF, Bruker Daltonics). The probes were dried under vacuum, and 1 μL of matrix (45% (v/v) acetonitrile, 1% (v/v) α-cyano-4-hydroxycinnamic acid) was applied to the dried peptides. Peptides were ionized by a 355 nm Smartbeam II laser at a frequency of 1 kHz, and time of flight (TOF) was measured in linear modus. Data were processed using FlexAnalysis (Bruker Daltonics).

**Results**

**Isolation of BTH6 from Barley Seedlings**—In earlier work we and others have obtained genomic and cDNA sequences for several leaf thionin precursors. Comparison with other thionins, e.g. the endosperm hordothionins, which have been purified from plants and sequenced, indicated that the 3’ end of leaf thionins would most likely be -CPSDYPK (Fig. 1). To unambiguously identify the C terminus of the leaf thionin BTH6, the native peptide was purified from etiolated barley leaves using ion exchange chromatography and two reverse phase chromatographic purification steps. BTH6-containing fractions were identified using MALDI and ESI MS/MS mass spectrometry. To obtain amino acid sequences, the purified peptide was carboxymethylated with iodoacetamide and digested with trypsin. The resulting peptides were separated and analyzed by LC-ESI-MS/MS. The masses and corresponding sequences that were found are shown in Table 2. The peptides shown in Table 2 were fractionated, and amino acid sequences were obtained. These results confirmed that the purified leaf thionin BTH6 has a native C terminus ending with lysine as predicted by sequence alignments.

**Purification and Identification of a Barley TPPE**—The protease database MEROPS collects information about known proteases of all kingdoms, including possible cleavage sites. A search of the database (Release 9.8) with the putative cleavage sites for thionin proproteins did not reveal any hit (results not shown). The authors of an earlier report used a barley leaf thio-

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**TABLE 2**

| m/z        | Sequence                           |
|------------|------------------------------------|
| 984.86     | KSCCKDTRLARNCYNCTCRFAGGSRPVCAGAC  |
| 575.31     | RCKIISGPCKPSDYPK                   |
| 614.38     | IIGSGP                            |
| 866.37     | CPSDYPK                           |
| 494.18     | NCYNTCR                           |
| 733.34     | FAGSRSVPVCAGAC                    |

**FIGURE 1. Alignment of barley leaf thionin proproteins deduced from DNA sequences.** Arrows indicate the suggested end of the thionin domain. DB4, DC4, and DG3 are from cDNAs, and BTH6 and BTH7 are from genomic DNA clones. References are given in parentheses.
nin proprotein labeled with $^{35}$S to detect the activity of TPPE with the help of autoradiography (17). Because this is a laborious procedure, we used a fluorogenic peptide covering the sequence between thionin and the acidic domain in the BTH6 proprotein to identify the TPPE protease. As starting material, etiolated barley seedlings were used. It has previously been shown that leaf thionins are strongly expressed in etiolated seedlings (18), and it could be assumed that these would also contain the corresponding TPPE. The initial steps followed the original procedure and consisted of gel filtration, cation exchange, and anion exchange chromatography as described under “Experimental Procedures.” Fractions from the anion exchange column with high protease activity (F8, F14, F24, and F32) were concentrated and separated on SDS-PAGE. Bands in the 66-kDa range were cut out from the gel (Fig. 2) followed by reduction, carboxymethylation, and tryptic in-gel digestion. Extracted peptides were analyzed by LC-ESI-MS/MS. Peptides with homology to a subtilisin protease (gi/182382494) from *Triticum aestivum* were identified in a total of seven bands in the 66-kDa range (Fig. 2, A–G) and in all four fractions (data not shown). No complete homologous DNA sequence for *H. vulgare* was available at the time but was published shortly after (25). The data generated by LC-ESI-MS/MS for band A were then re-analyzed by MASCOT using the updated NCBInr database. Six distinctive peptides were found to match the sequence of the BAJ93208 gene, confirming the presence of the protease in the purified fractions. The peptides found by the MASCOT analysis of sample A are shown in Fig. 3. The published mRNA sequence with the GenBank™ accession number BAJ93208 (GI:326526063) included 2701 nucleotides, coding for 784 amino acids (25).

**FIGURE 2.** SDS-PAGE analysis of fractions with protease activity. Protein extracts from etiolated barley seedlings were purified and fractions F8, F14, F24, and F32 were separated on a SDS-polyacrylamide gel. Proteins appearing in bands A–G were extracted from the gel and digested, and peptides were sequenced by LC-ESI-MS/MS.

**FIGURE 3.** Sequence and structure of BAJ93206. A, schematic domain representation according to the architecture prediction program SMART. B, amino acid sequence of BAJ93208. Amino acids forming the catalytic triad His-227, Asn-331, and Ser-556 are highlighted in green. C, domains of BAJ93208. Signal peptide, red; inhibitor I9, blue; peptidase domain, black; PA, green; and fibronectin 3 domain, orange.
In Silico Analysis of BAJ93208—The identified protease BAJ93208 has been annotated as a subtilisin-like protease comprising 784 amino acids (Fig. 3). The domain architecture prediction program SMART (Simple Modular Architecture Research Tool (26)) was used to identify known structures. A signal peptide was found to be present at the N terminus according to the web-based signal peptide prediction program SignalP (27). The predicted signal peptide cleavage site was determined to be between Ala-23 and Ala-24. An intramolecular inhibitor domain (I9) was identified between Ala-24 and His-116. A peptidase family S8 subfamily A domain is located between Thr-136 and Ala-501. The catalytic domain is interrupted by a protease-associated domain (PA domain, Ala-379–Val-474). A fibronectin 3 domain is present at the C terminus. Additionally, a short SPI motif is present. The amino acid sequence SPI in the C terminus of SBT3 was found to be involved in sorting within the secretory pathway (28). The catalytic triad consisting of His-227, Asn-331, and Ser-556 is highlighted. The structure modeling program PHYRE (29) was used to predict a structure for BAJ93208. This model showed that the PA domain and the FN3 domain extend from the intrinsic subtilase domain (Fig. 4).

Cleavage of the Fluorogenic Peptide—As predicted, the intramolecular inhibitor domain is cleaved off, together with an additional 19 amino acids as has been shown recently for the precursor protein that was expressed in E. coli (30). The activated recombinant BAJ93208 protease was also able to cleave the fluorogenic peptide. The digestion products were analyzed by LC-ESI-MS. The protease was found to cleave the peptide at the C-terminal side of KLN releasing the peptides (ANA)-SDYPKLN and LLPK-(MCA) with the masses 999.429 and 684.361 Da, respectively. Both peptides were identified by MS, and no other digestion products were found (Fig. 5). Even with longer incubation times (>3 h), no additional products were detected (data not shown). However, this cleavage site has not been predicted as the C terminus of the mature BTH6 thionin, which would end with the amino acid sequence ... DYPK (Fig. 1).

Production of Recombinant BTH6 Proprotein—Because the fluorogenic peptide was not cleaved as expected, we produced a recombinant BTH6 proprotein in E. coli to use it as a substrate. To investigate the destiny of the mature thionin and the acidic
domain after cleavage, the BTH6 proprotein was produced with an N-terminal Myc tag and C-terminal strep tag in *E. coli* (Fig. 6A). To overcome solubility problems during the expression in *E. coli*, the proprotein was further expressed as a fusion protein with thioredoxin, which is known to enhance disulfide bond formation and solubility for its fusion partner during expression in the cytoplasm (20, 31). A His tag was included for purification of the fusion protein. After expression and purification, the fusion protein was cleaved from the proprotein by TEV (Fig. 6B), and the tagged proprotein was purified by reverse phase chromatography to apparent homogeneity (Fig. 7). To confirm that the purified proprotein contained the seven disulfide bridges, the mass of the purified myc-BTH6-strep proprotein was measured by LC-ESI-MS. The calculated mono-isotopic mass for the molecule with the 14 cysteines in the oxidized form was calculated as 14,312.57 Da. We measured \([M+H]^+ = 14,313.47\) which corresponded to the monoisotopic mass of the myc-BTH6-strep proprotein in the oxidized form. These results demonstrated that none of the cysteines were in the reduced form and that all seven disulfide bridges were formed.

After incubation with the activated recombinant BAJ93208 protease, two smaller fragments appeared on SDS-polyacrylamide gels, although attempts to detect the thionin domain or the acidic domain by Western blots using either an anti-Myc or an anti-strep antibody consistently failed (data not shown). The digestion products were therefore separated by reversed phase chromatography and analyzed by mass spectrometry (Fig. 8). The cleavage sites identified for the oxidized proprotein are outlined in Fig. 9A. The protease cleaved the acidic domain of the barley thionin proprotein at least at six positions, preferentially after asparagine and glutamine residues. The overall preferred cleavage site within the proprotein was within the acidic domain, i.e. two residues after the mature BTH6 thionin. In agreement, this corresponded to the cleavage site in the fluorogenic peptide (see above). Data generated by time-dependent measurements of the digestion products (Fig. 10) indicated that the two amino acids leucine and asparagine are removed in a second single proteolytic step, releasing the mature thionin with the wild-type C terminus. Although the TPPE cleaved several times within the acidic domain and even within the Myc tag, no cleavage occurred within the thionin domain. Thionins have a general three-dimensional structure, which is composed of two \(\alpha\)-helices and two \(\beta\)-sheets and stabilized by three or four disulfide bridges (32). The same compact structure was
predicted for the BTH6 thionin by PHYRE with high accuracy (Fig. 11) and might protect possible cleavage sites. This was tested by opening the disulfide bridges of the thionin proprotein by carboxymethylation of all cysteines of the recombinant BTH6 proprotein before digestion. The calculated monoisotopic mass for the molecule with 14 carboxymethylated cysteines was calculated as 15,139.65 Da (average neutral mass 15,148.87 Da). The isotopically resolved MS spectrum of the modified precursor showed a mass of $[\text{M + H}]^+ = 15,138.65$ which corresponded to the monoisotopic mass of the proprotein with 14 carboxymethylated cysteines, confirming that all disulfide bridges had been reduced and the cysteines carboxymethylated. The reduced precursor was digested with the recombinant BAJ93208 protease as before, and the products were also analyzed by MS. Opening of the disulfide bridges resulted in additional cleavage sites within the precursor as shown in Figs. 9B and 12. The acidic domain was digested at additional sites, and also the thionin domain was digested.

FIGURE 8. TPPE releases the mature thionin from oxidized pro-BTH6. LC-ESI-MS analysis of the digestion of myc-proBTH6-strep with recombinant TPPE. The acidic domain is cleaved several times, although the thionin is not cleaved. The masses shown correspond to cleavage products of the BTH6 precursor (compare Fig. 9A).

FIGURE 9. Cleavage of myc-proBTH6-strep by TPPE. Cleavage sites were identified by LC-ESI-MS after incubation of recombinant myc-proBTH6-strep with recombinant activated His$_6$-BAJ93208-strep protease. A, BTH6 proprotein in oxidized form with intact disulfide bridges. TPPE cleaved the acidic domain and the Myc tag. The preferred cleavage site in the acidic domain corresponds to the cleavage site in the fluorogenic peptide (see Fig. 5), but fragments with the authentic thionin end were also identified. Note that there was no cleavage within the thionin domain. B, precursor in a reduced form with open disulfide bridges. The acidic domain is cleaved at additional sites, and the thionin domain is also cleaved. Green, Myc tag; yellow, acidic domain; red, strep tag.
Thionin Proprotein Processing

These results confirmed that the three-dimensional structure of the mature BTH6 thionin, which is stabilized by the disulfide bridges, is responsible for preventing proteolysis by the TPPE.

Discussion

Thionins are a group of plant-specific antimicrobial peptides that have been isolated from different plant species as peptides with ~45 amino acids (5, 6). Various cDNA and genomic DNA sequences showed that thionins are produced as preproteins. Although many fully processed thionins have been isolated from plants such as mistletoe and barley, a peptide corresponding to the acidic domain was never found. A first attempt by Romero et al. (17) to identify a TPPE from barley was not successful. Since then, the sensitivity and accuracy of mass spectrometry methods have been improved, and large numbers of genomic and transcriptome sequences are available in public databases, which would allow the identification of proteins once partial peptide sequences have been obtained. For detection of TPPE, we designed a protease substrate probe, namely a fluorogenic peptide covering the flanking region between the thionin domain and the acidic domain. Fluorogenic substrates have been used previously to study the substrate preferences and characteristics of different proteases (34–36). This assay was fast and easy to perform. We used etiolated barley seedlings for the purification of the TPPE because it was known that these contain large amounts of leaf-specific thionins (18). *Arabidopsis* also contains thionins, but in lower amounts, and therefore represents a less promising starting material because it could be assumed that the TPPE might also be present in lower amounts.

Activity of the recombinant barley TPPE was tested by using two different substrates, the fluorogenic peptide and the recombinant BTH6 proprotein produced in *E. coli*. After digestion, the products were analyzed by mass spectrometry. The fluorogenic peptide was found to be cleaved with high efficiency by the protease at the position (ANA)-SDYPKLN↓LLPK-(MCA) and no other products were detected even after 3-h incubations. This site was also the preferred cleavage site in the recombinant BTH6 proprotein. But in contrast to the fluorogenic peptide, the same sequence in the recombinant BTH6 proprotein was processed further, and the two amino acids leucine and asparagine were cleaved off in one step to generate the authentic C terminus of the mature thionin. These differences indicate that the protease might have a rather large substrate binding pocket that recognizes more than seven amino acids. Similarly, substrate length has also been shown to be important for the subtilase C1 where at least nine amino acid residues are required for highest cleavage efficiency (37). Another possible explanation might be that the N-terminal quencher group inhibited further cleavage of the peptide.

Because the preferred cleavage site of the TPPE does not correspond to the authentic C terminus of the mature thionin, it cannot be excluded that *in planta* the TPPE might be assisted by a dipeptidase. In further work it will therefore be important to analyze the processing of thionin proproteins *in planta*. This, however, would be difficult in barley but could be done in *Arabidopsis*, which also contains four thionin genes.

The acidic domain of the recombinant barley BTH6 proprotein is cleaved by the recombinant barley TPPE several times, finally releasing the mature BTH6 thionin with a C terminus as it is found naturally in barley leaves. This finding also explains why an acidic domain peptide has never been isolated from any plant species. Considering all cleavage sites within the BTH6 proprotein revealed that asparagine and glutamine are the preferred amino acids at the P1 position, cleavages at other sites also occurred. A similar preference has been shown for cucumisin with preferences for leucine, asparagine, and glutamine at the P1 position (38). However, after opening of the disulfide bridges, additional sequences with different amino acids at the P1 position were also cleaved. All in all, the information about preferred substrates is limited, and a study using a greater variety of substrates would be necessary to test the substrate preferences of TPPE in more detail.

The acidic domain was cleaved several times, and unexpectedly, the Myc tag was also cleaved, which explained why the anti-Myc antibody did not detect the thionin domain on West-
ern blots. The strep tag was not cleaved, but because the fragments attached to the strep tag were rather small after cleavage, these were also missed on Western blots aimed at detecting the strep tag (data not shown). A similar situation was found for the strep tag attached to the recombinant TPPE (30). The thionin domain itself was protected from cleavage by TPPE by its three-dimensional structure (Fig. 11). For many different thionins, the structure has been determined either by NMR or x-ray analysis, and in all cases this has been found to consist of two $\beta$-sheets and two $\alpha$-helices (33). Thus, the three-dimensional structure of thionins is not only important for activity (39) but is also important to prevent cleavage by the TPPE.

**In silico** sequence analysis revealed that BAJ93208 is a serine protease belonging to the pyrolysins within the subtilase family. The sequence showed a typical plant pyrolysin architecture, including the signal peptide, the inhibitor domain, the catalytic domain, the PA domain, and a C-terminal FN3 extension. A similar protease from tomato, SISBT3, is also synthesized as a proproenzyme, and autocatalytic processing of the prodomain in the endoplasmic reticulum has been shown to be a prerequisite for passage through the secretory pathway (28). The structure of SISBT3 was determined by x-ray diffraction analysis and showed that the PA domain and the FN3 domain extend from the intrinsic subtilase domain (40). A similar structure is predicted for BAJ93208 (Fig. 4) by the program PHYRE (29). However, experimental data will be needed to confirm this structure for BAJ93208.

Two similar enzymes have previously been isolated from young barley leaves, and the question arose whether TPPE is one of them. The enzymes are called hordolisin and SEP-1, respectively, and only short amino acid sequences were published. Hordolisin has been purified and characterized by Terp et al. (41). The authors obtained a short amino acid sequence at the N terminus (TTRTPFLGDLAREALFESN). A recent BLAST (Basic Local Alignment Search Tool) search among all currently known protein sequences in *H. vulgare* revealed that the two proteins have an identity of 47.9% and thus encode two different enzymes. SEP-1 has been purified and characterized by Fontanini and Jones (42). The authors published two short sequences of their enzyme (EFPADAILGDGVYR and YVTTDPVPTATISF). A BLAST analysis with all currently known protein sequences in *H. vulgare* revealed no consensus sequence. An amino acid comparison between the TPPE and the two SEP-1 peptides identified here revealed three mismatches in the sequence, indicating that the two described enzymes are encoded by different genes (Fig. 13). It can be concluded that, hordolisin, SEP-1, and TPPE are three different enzymes. No clear role has been assigned yet to hordolisin and SEP-1, but it cannot be excluded that they might also be able to process thionin proproteins.

In *Arabidopsis*, several pyrolysins have stringent substrate specificities. The *Arabidopsis* protease AtS1P (AtSBT6.1) is an ortholog of the mammalian S1P and is a component of the signaling pathway that mediates salt stress responses. AtS1P releases a part of the endoplasmic reticulum membrane-localized AtbZIP17 transcription factor that is subsequently translocated into the nucleus to promote the transcription of salt stress genes (43). In addition, AtS1P is able to process the precursor of the plant peptide hormone AtRALF23 at the recognition site RRIL↓ (44). Moreover, AtS1P is likely involved in maturation of a pectin methyl esterase by cleaving after dibasic sites such as RRLL↓ within the proproteins [45]. The *Arabidopsis* growth factor AtPSK4 that is synthesized as a proproteins is processed by the subtilase AtSBT1.1. In this case, the enzyme cleaves at the C-terminal side of the amino acid sequence RRSLVI↓ (46). Additional subtilases take part in plant-specific developmental processes; for example, the *Arabidopsis* enzyme SDD1 (AtSBT1.2) is involved in the regulation of stomatal density. Indeed, T-DNA knock-out of the gene led to increased appearance of stomata (47). The enzyme AtSBT1.7 is involved in mucilase release from *Arabidopsis* seed coats during seed germination (48). Another pyrolysin, AtSBT5.4, appears to play a role in shoot meristem development by interacting with the clavata pathway (49).
Digestion of the reduced BTH6 proprotein has shown that the TPPE would be able to cleave a variety of different protein sequences. The expression and activity of this enzyme therefore need to be tightly controlled in the plant. One level of control would thus be the regulation of transcription. It might be expected that TPPE is only expressed in those tissues that express thionins. The subcellular location of TPPE is not known, but because thionins have been found in the apoplast and the vacuole of barley (50), one might speculate that TPPE is also secreted. This is supported by the identification of a signal peptide in the TPPE precursor by SignalP (Fig. 3). However, if processing of the thionin precursors takes place in the endoplasmic reticulum, the Golgi, in vacuoles or the apoplast, needs to be addressed in subsequent work. Furthermore, barley contains in addition to leaf thionins another group of thionins in the endosperm (51). If the TPPE that we have identified here is also responsible for processing of endosperm thionins or even the endosperm (51), one might speculate that TPPE is only expressed in those tissues that would thus be the regulation of transcription. It might be expected that TPPE is only expressed in those tissues that express thionins. The subcellular location of TPPE is not known, but because thionins have been found in the apoplast and the vacuole of barley (50), one might speculate that TPPE is also secreted. This is supported by the identification of a signal peptide in the TPPE precursor by SignalP (Fig. 3). However, if processing of the thionin precursors takes place in the endoplasmic reticulum, the Golgi, in vacuoles or the apoplast, needs to be addressed in subsequent work. Furthermore, barley contains in addition to leaf thionins another group of thionins in the endosperm (51). If the TPPE that we have identified here is also responsible for processing of endosperm thionins or even other unrelated precursor proteins is currently unknown.

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