JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2002,** *50,* 3849−3855 **3849**

Trypsin-Like Proteinase Produced by *Fusarium culmorum* Grown on Grain Proteins

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The fungal disease Fusarium head blight occurs on wheat (*Triticum* spp.) and barley (*Hordeum vulgare* L.) and is one of the worldwide problems of agriculture. It can be caused by various *Fusarium* species. We are characterizing the proteinases of *F. culmorum* to investigate how they may help the fungus to attack the grain. A trypsin-like proteinase has been purified from a gluten-containing culture medium of *F. culmorum.* The enzyme was maximally active at about pH 9 and 45 °C, but was not stable under those conditions. It was stabilized by calcium ions and by the presence of other proteins. The proteinase was most stable at pH 6−7 at ambient temperatures, but was quickly inactivated at 50 °C. It was strongly inhibited by *p*-amidino phenylmethylsulfonyl fluoride (*p*-APMSF), and soybean trypsin and Bowman−Birk inhibitors, and it preferentially hydrolyzed the peptide bonds of the protein substrate β-purothionin on the C-terminal side of Arg (mainly) and Lys residues. These characteristics show that it is a trypsin-like proteinase. In addition, its N-terminal amino acid sequence was 88% identical to that of the *F. oxysporum* trypsin-like enzyme. The proteinase hydrolyzed the D hordein and some of the C hordeins (the barley storage proteins). This enzyme, and a subtilisin-like proteinase that we recently purified from the same organism, possibly play roles in helping the fungus to colonize grains.

KEYWORDS: ***Fusarium*; cereal; barley (*Hordeum vulgare* L.); trypsin; proteinase**

INTRODUCTION

Fusarium head blight (FHB, scab) of wheat and barley has long caused serious problems and economic losses for producers and cereal industries in the Americas, Europe, and Asia (*1*). Occasional infestations have also occurred in Australia and Africa. This disease reduces crop yields and both seed and grain quality (*2*−*4*). It is caused by several *Fusarium* species and occurs in regions that have high humidities, or heavy rainfalls or dew (*2, 3*). Plants are most susceptible to the fungal attack at anthesis and during the early stages of kernel development, but the mechanism(s) of how these fungi invade the kernels is not fully understood. It seems likely that the degradation of grain components by fungal enzymes figures prominently in pathogenesis.

It has been suggested that the fungi synthesize proteases in infested grain, because the levels of solubilized protein and free amino nitrogen (amino acids and small peptides) of worts made from *Fusarium*-contaminated malts are greater than those made from clean grain (*5*). In addition, the storage protein matrix of FHB-diseased wheat kernels is often destroyed (*6, 7*). However, the fungal enzymes that degrade the grain proteins have not previously been identified. It has been shown that *F. culmorum* and *F. graminearum* both produced alkaline proteinases when they were grown in a gluten-containing growth medium or on autoclaved barley grain, but not when grown on protein-free medium (*8*). The fungi may also produce these proteinases when colonizing developing grain. Proteinases of various plant pathogenic fungi have been detected in infected host plant tissues (*9*−*18*), but in most cases it is not clear what impact these enzymes have on the development of disease.

To study the roles of fungal proteinases in the development of FHB-disease, two *F. culmorum* proteinases have been isolated and purified from a gluten-containing culture medium. A study of one of them, a subtilisin-like enzyme, was reported recently (*19*). In this paper we describe the purification and characterization of the second enzyme.

MATERIALS AND METHODS

Proteinase Assay. The azogelatin assay method of Jones et al. (*20*), as slightly modified in Pekkarinen et al. (*19*), was used for analyzing the proteinase activities during the purification and characterization of the enzyme. Unless indicated otherwise, the final reaction mixtures contained 1% azogelatin in 80 mM, pH 6.0, Na citrate and were carried out at 40 °C. In the characterization assays, the enzyme concentrations of the reaction mixtures were generally about 0.5 μg/mL. Each assay was performed in duplicate. The activity was expressed as arbitrary

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10.1021/jf020027x CCC: $22.00
Published on Web 05/22/2002

units (U), one unit being the change in absorbance at 440 nm per min multiplied by 100.

Protein Assays. The protein concentrations of the solutions reported in the purification table were measured with the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA) (*21*). Bovine serum albumin (BSA, Pierce, Rockford, IL) was used to prepare a standard curve. In all of the other cases, the protein concentrations were calculated from the absorbances of the preparations at 280 nm, presuming that 1 absorbance unit corresponded to 1 mg/mL protein.

Proteinase Purification. The proteinase was produced by *F. culmorum* (strain VTT-D-80148) that was grown in a gluten-containing medium and purified as described earlier (*19*). The enzyme was purified from concentrated culture medium devoid of fungal mycelium by using size exclusion (Bio-Gel P30, Bio-Rad), and cation exchange (CM52, Whatman) chromatographies and carboxymethyl (CM)-HPLC (Shodex IEC CM-825 column, Phenomenex, Torrance, CA). The enzyme reported in this paper is the second major protein that was separated by the CM-HPLC step with an NH_4HCO_3, pH 8, gradient (*19*). The absorbance values of all of the chromatography fractions were measured at 280 nm and their proteinase activities were analyzed at pH 8.9 in 80 mM Tris−HCl with the azogelatin method.

Determining the Molecular Mass and N-Terminal Amino Acid Sequence of the Enzyme. The size of the proteinase was determined by both SDS−PAGE and mass spectrometric analyses. For the electrophoretic analysis, a CM-HPLC preparation was boiled for 1.5 min with SDS sample buffer and separated on a 12% SDS−PAGE gel (*22*) that was then stained with Coomassie brilliant blue R-250 (Sigma, St. Louis, MO) using the method described previously (*19*). A Precision Protein Standard (Bio-Rad) protein mixture sample was used to calibrate the gel. The mass spectrometric analysis was carried out at the University of Wisconsin Biotechnology Center by using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) method with a Bruker Biflex III (Bruker Daltonics, Billerica, MA) mass spectrometer. The N-terminal sequence was analyzed using the Edman method with an ABI 420/H amino acid analysis system at the University of Texas Medical Branch Cancer Center Protein Chemistry Laboratory, Galveston, Texas.

Effects of pH and Temperature on the Enzyme Activity. The activities of the proteinase were measured in 80 mM buffers: Na acetate (pH 3.7, 4.1, 4.4, 5.1, and 5.5), Na citrate (pH 5.5, 6.0, 6.6, and 7.0), Tris−HCl (pH 7.0, 7.5, 8.0, 8.5, 8.8, and 9.2), and CAPS (pH 9.2, 9.7, 10.1, and 10.6). Because the reactions were not quite linear at pH values higher than 6.6, the activities were calculated from the absorbance values of the reaction mixture samples after 20 min of incubation. The effect of temperature on the proteinase activity was studied at 20, 30, 35, 40, 45, and 50 °C at pH 6.0 in 80 mM Na citrate and pH 8.7 in 80 mM Tris−HCl.

Effects of pH and Temperature on the Enzyme Stability. For studying the stability of the proteinase at different pH values, about 2.5 μg/mL of the purified enzyme was incubated in 30 mM buffers: Na acetate, pH 4.1 and 5.0; Na citrate, pH 6.0 and 6.6; or Tris−HCl, pH 7.9 and 8.6. The activity of each sample was measured immediately after mixing the enzyme with buffer and after 90 min of incubation at 40 °C. The thermal stability of the enzyme was studied by incubating the purified proteinase (∼2.5 μg/mL) at 24, 40, 50, or 60 °C in 30 mM Na citrate, pH 5.9, for 50 min and assaying its remnant activity at 40 °C. The remaining activities were compared to the initial activity of the preparation, as measured immediately after the enzyme and incubation buffer were mixed.

Effect of Azogelatin Concentration on the Enzyme Activity. To ascertain the effect of the azogelatin substrate concentration on the proteinase activity, assays were carried out with substrate concentrations between 0.5 and 10 mg/mL in either 80 mM, pH 6.0, Na citrate or 80 mM Tris−HCl, pH 8.7. The enzyme concentrations in the pH 6.0 and pH 8.7 reactions were approximately 0.4 or 0.2 μg/mL, respectively.

Assays with Various Serine Class Proteinase Inhibitors. As reported earlier (*19*), the activity of a mixture of the two *Fusarium* enzymes was only slightly affected by any class specific inhibitors other than those that influence serine class proteinases. To more precisely determine the class-specific properties of the purified enzyme, its activities were analyzed with the azogelatin assay at pH 6.0 in the presence of 1.0 mM phenylmethylsulfonyl fluoride (PMSF), 1.0 mM *p*-amidino phenylmethylsulfonyl fluoride (*p*-APMSF), 33 μM chymostatin (CST), 2.5 μM soybean Bowman−Birk inhibitor (BBI), or 1.0 μM soybean trypsin inhibitor (STI, Type II-S) (*19*). The PMSF, *p*-APMSF, and CST were dissolved in dimethyl sulfoxide (DMSO). Control reactions were carried out in the presence of solvents (water or 4% DMSO). All of the inhibitors were purchased from Sigma.

Effects of Calcium and Protein on the Enzyme Activity and Stability. To determine whether calcium or extraneous protein affected or stabilized the proteinase activity, the azogelatin assays were carried out in the presence of 0−20 mM Ca acetate in either 80 mM Na citrate, pH 6.0 (0, 5, and 20 mM Ca^{2+}) or 80 mM NH_4 acetate, pH 5.4 (0, 1, 5, and 20 mM) buffers, or in pH 6.0, 80 mM, Na citrate that contained 0, 1, or 5 μg/mL bovine serum albumin (BSA).

To study the enzyme stability effects, the purified proteinase was incubated for 90 min at 40 °C in solutions containing 0−100 mM Ca acetate in either 30 mM NH_4 acetate, pH 5.2, or 30 mM Tris−HCl, pH 8.6, or in a solution of 0−10 μg/mL BSA in 30 mM Na citrate, pH 5.9. The activities of the incubated samples were then analyzed at pH 6.0 and compared with those measured prior to the 90-min incubation. To ensure that none of the observed effects were due to differing calcium or BSA levels in the reaction mixtures, the Ca acetate and BSA concentrations of the reaction mixtures were adjusted to 20 mM or 4 μg/mL, respectively.

Determining the Hydrolytic Specificity of the Enzyme. *Protein Hydrolysis*. The sites at which the purified proteinase cleaved a purified substrate protein were determined by using the method of Mak and Jones (*23*). The purified enzyme (∼4 ng) was incubated with 100 μg/mL of reduced and pyridylethylated (R&P) β-purothionin (*23*) in 700 μL of 50 mM NH_4 acetate, pH 5.5 buffer at 40 °C. Aliquots (120 μL each) were removed from the reaction mixture after 0, 15, 30, 60, and 120 min of incubation, immersed in boiling water for 10 min to stop the reactions, and freeze-dried.

For analysis, the freeze-dried aliquots were dissolved in 0.3 mL of 0.1% trifluoroacetic acid (TFA), filtered through 3-mm, 0.45-μm, PTFE filters, and the hydrolysis peptides were separated by reversed-phase (RP-) HPLC (Zorbax SB-C18 4.6 mm × 7.5 cm column, MAC-MOD Analytical, Inc., Chadds Ford, PA) with a 17-mL linear gradient that ran from 3% to 32% acetonitrile. Both the 3% and 32% acetonitrile solutions contained 0.1% TFA. The separated peptides were individually collected and their masses were determined, at the University of Wisconsin Biotechnology Center, by MALDI-TOF analysis using a Bruker Biflex III mass spectrometer. The compositions of the peptides were computed by comparing their masses with those of peptides that could have been formed, given the known amino acid sequence of R&P-β-purothionin (*24*). Analogous reactions were carried out in the presence of 1.0 μM STI to ensure that only the trypsin-like activity was being detected.

Studies with Synthetic Substrates. The specificity of the proteinase was also studied using the following synthetic substrates, all purchased from Sigma: Nα-benzoyl-L-Arg pNA (BApNA), N-benzoyl-Val-Gly-Arg pNA (BVGRpNA), N-succinyl-Ala-Ala-Pro-Phe pNA (SAAPF-pNA), N-succinyl-Ala-Ala-Pro-Leu pNA (SAAPLpNA), or N-glutaryl-L-Phe pNA (GPpNA). The assays were carried out in duplicate, as described previously (*19*). Each reaction mixture contained 5.0 mM substrate in 175 mM Tris−HCl, pH 9.0 in 4% DMSO. The enzyme concentrations in the reaction mixtures were 0.16 μg/mL (BVGRpNA) or 6.5 μg/mL (BApNA, SAAPFpNA, SAAPLpNA, and GPpNA). The K_m value of the proteinase for BVGRpNA was determined by analyzing the activity of approximately 13 ng/mL of proteinase in 6−800 μM substrate dissolved in pH 6.0, 175 mM, Na citrate that contained 4% DMSO. The K_m value under alkaline conditions was measured in the same way, but with approximately 7 ng/mL of enzyme and with between 1 and 100 μM BVGRpNA in 4% DMSO and 175 mM Tris−HCl, pH 9.0, buffer. The kinetic constants were calculated by fitting the data to the Michaelis−Menten equation by using nonlinear regression analysis.

To study the effects of CST and STI on the enzymatic activity with the substrate BVGRpNA, the enzyme (∼0.7 μg/mL in 20 mM NH_4 acetate, pH 4.9) was incubated on ice for 5 min with either 10 μM STI or 16.5 μM CST (in 20% DMSO), and the activities were measured at

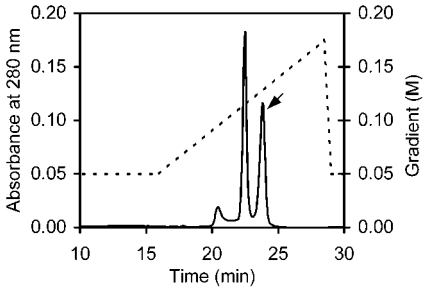


Figure 1. Separation of two *F. culmorum* proteinases by cation exchange HPLC: (—), protein absorbance at 280 nm; (- - -), elution gradient, molarity of pH 8 NH_4HCO_3. The proteinase that is described in this report eluted in the peak area indicated by the arrow.

28 °C, with 0.5 mM BVGRpNA in 4% DMSO and 175 mM Tris−HCl at pH 9.0. As control reactions, the enzyme was preincubated with either water or 20% DMSO, as appropriate. The final concentrations of STI and CST in the reaction mixtures were 1.0 μM and 1.7 μM, respectively.

Hordein Hydrolysis. Barley (*Hordeum vulgare* L. cv. Morex) grains were ground with a U/D Cyclone sample mill (UDY, Ft. Collins, CO) to pass a 0.5-mm screen, and hordeins were extracted from the ground sample with 55% 2-propanol/2% β-mercaptoethanol (*25*). A substrate suspension was prepared by mixing 80 μL of the hordein extract and 200 μL of 50 mM, pH 6.0, Na citrate. A purified enzyme sample of ~18 μg/mL was incubated for 10 min on ice in the presence or absence of 150 μM STI. The substrate solution was heated to 40 °C and 40 μL of the enzyme mixture or buffer was added, as appropriate. After 0, 25, and 100 min, 50-μL aliquots of the reaction mixtures were removed, mixed with 13 μL of 5 × SDS sample buffer, and the reactions were stopped by heating them at 100 °C for 3 min. The hordein proteins and hydrolysis products were separated with 12% SDS−PAGE and the gels were stained with Coomassie brilliant blue R-250 (*19*).

RESULTS

Purification of the Proteinase. The proteinase was purified using the method of Pekkarinen et al. (*19*), which included separations with a P-30 size separation column, with an open column of carboxymethyl cellulose (CMC) and a final HPLC ion exchange column. Until the HPLC step the enzyme eluted concomitantly with the previously reported subtilisin-like proteinase. **Figure 1** shows this final separation of the two *Fusarium* proteinases and the arrow indicates the peak that represents the proteinase of interest. The purified enzyme comprised 8% of the original proteolytic activity of the *F. culmorum* growth medium concentrate (**Table 1**). After the penultimate, CMC, step its specific activity was about 6.7 times higher than that in the crude extract. However, this activity decreased about 25% during the final HPLC separation step because of removal of the contaminant subtilisin-like proteinase (*19*) from the CMC preparation.

Mass and N-Terminal Amino Acid Sequence of the Proteinase. The final proteinase preparation contained a 24.8 kDa protein and a very small amount of a 19.6 kDa protein that was detected by Coomassie staining after an SDS−PAGE separation (**Figure 2**). Mass spectrometric analysis indicated that, in a preparation that was free of contaminants, the protein had a molecular mass of 22 398 ± 35 Da. An amino acid sequence analysis indicated that the N-terminal sequence of the protein was IVGGTSASAGDFPFIVSISRNGGPW. A BLAST search of protein sequence databases indicated that, of the sequences listed, this protein shared the highest homology with the trypsin-like proteinase from *Fusarium oxysporum* (*26*, Swiss-Prot P35049), because the two proteinases had 88% identical N-terminal amino acids. This N-terminal sequence was 64−68% identical with proteinases from the plant pathogenic fungi *Cochliobolus carbonum* (*Bipolaris zeicola*) (*27*, TrEMBL Q00344) and *Phaeosphaeria nodorum* (*Stagonospora nodorum*) (*9*, TrEMBL O74696), and two proteinases from the entomopathogenic fungus *Metarhizium anisopliae* (*28*, TrEMBL Q01136; TrEMBL Q9Y842).

Activity Properties. The initial activity of the proteinase increased with temperature up to about 45 or 50 °C at both pH 6.0 and 8.7 (**Figure 3**). The activities were measured at these pH values because the pH of a crushed grain sample was ~6 and because the enzyme was most active at a pH of ~9. Most of the activities at pH 8.7 showed an initial lag period, after which the activity rates (change in absorbance per minute) increased with time (**Figure 3B**). This lag effect was negligible at pH 6.0 (**Figure 3A**). At 50 °C (pH 6.0) or 45 and 50 °C (pH 8.7), the activities started out very high, but dropped off after a few minutes, presumably because the enzyme was inactivated at these high temperatures. The enzyme was inactivated in less than 10 min at 50 °C and pH 8.7. Because of this inactivation, the maximal activities over extended time periods were at 45 °C (pH 6.0) and 40 °C (pH 8.7). The pH optimum of the proteinase was at approximately 9 (**Figure 4**). However, the enzyme was activated during the reactions at pH values above 6.6, and sigmoidal curves were obtained at pH values 9.7 and higher (corresponding effects are seen in **Figure 3B**).

The presence of calcium ions did not affect the proteinase activities in either Na citrate or NH_4 acetate buffers at 40 °C. To ascertain whether extraneous protein influenced the enzyme activity, either 1 or 5 μg/mL BSA was added to the azogelatin hydrolysis reactions. The activities decreased by about 15%, but this reduction was probably not significant.

Stability of the Proteinase. When the stability of the enzyme to temperature was tested at pH 5.9, 90% and 72% of its activity remained after 50 min of incubation at 24 and 40 °C respectively, but it was totally inactivated at 50 °C (results not shown). The proteinase was most stable at pH 6−7, where 58% of its activity was retained after 90 min of incubation at 40 °C (**Table 2**). As shown in **Figure 5**, calcium stabilized the enzyme at both pH 5.2 and 8.6. This Ca^{2+} effect was stronger in alkaline than in acidic solution, as the lowest Ca^{2+} concentration tested (5 mM) increased the enzyme stability almost to the maximal level. The presence of extraneous protein also protected the enzyme from inactivation. In the presence of an equivalent amount of BSA (2.5 μg/mL of both BSA and proteinase), the proteinase maintained its full activity for at least 90 min at pH 5.9 and 40 °C.

Substrate Inhibition Occurs with Azogelatin. When the relationship between substrate (azogelatin) concentration and activity was measured at pH 6.0, the activities dropped when the azogelatin concentration exceeded 3 mg/mL, indicating that substrate inhibition was taking place (**Figure 6**). At pH 8.7 the initial activities were all identical at the lowest substrate concentrations (0.5−2.0 mg/mL) and substrate inhibition occurred when the azogelatin concentrations were 3.0 mg/mL or higher (results not shown). In addition, the pH 8.7 activities increased with reaction time, leading to sigmoidal reaction rates such as those that were previously found in the 30−45 °C reactions of **Figure 3B**.

Mechanistic Class. As reported earlier (*19*), when various inhibitors that are specific for each of the four classical proteinase groups were added to a semi-purified mixture that contained approximately equal amounts of this enzyme and the subtilisin-like *Fusarium* proteinase, only the serine proteinase inhibitors caused any significant diminution of activity, indicat-

Table 1. Purification of the *F. Culmorum* Proteinase: Activities Were Measured with the Azogelatin Assay at pH 8.9, all Values are Based on 26.5 mL of Crude Preparation

preparation	total protein (mg)	total activity (U[a])	yield (%)	specific activity (U/mg protein)	purification (fold)
crude	4.7	1 400	100	300	1.0
P30 pool	1.9	1 300	93	680	2.3
CMC pool	0.33	660	47	2 000	6.7
CM-HPLC	0.07	110	7.9	1 600	5.3

[a] $U = \Delta A_{440} \times 100 \times$dilution factor/min.

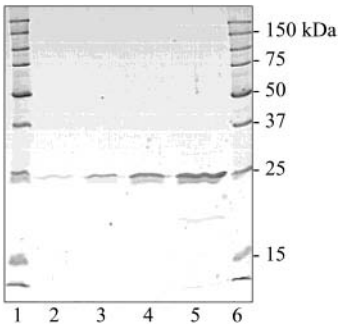


Figure 2. SDS–PAGE analysis of the purity of the proteinase. The gel was stained with Coomassie brilliant blue R-250. Lanes 1 and 6 were loaded with Bio-Rad Precision molecular weight standards; lanes 2–5 were loaded with 0.07, 0.20, 0.70, and 2.0 μg of the proteinase, respectively.

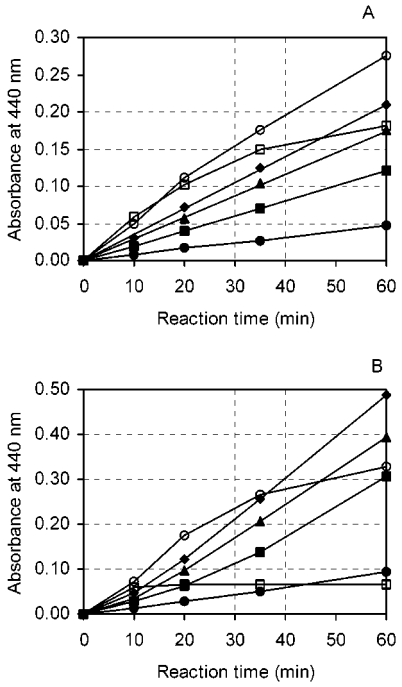


Figure 3. Effects of temperature on the proteinase activities at (A) pH 6.0 and (B) pH 8.7. The analysis temperatures were (●) 20 °C, (■) 30 °C, (▲) 35 °C, (◆) 40 °C, (○) 45 °C, or (□) 50 °C.

ing that both this and the subtilisin-like enzyme were serine class proteinases. For this reason, only the effects of various serine-class protease inhibitors were tested on this proteinase. When azogelatin was used as substrate, the activity was strongly inhibited by *p*-APMSF, STI, and BBI, but not by CST, indicating that the enzyme was similar to trypsin (**Table 3**). A corresponding effect was observed when the activities were measured using the synthetic substrate BVGRpNA in the presence of STI or CST (**Table 3**).

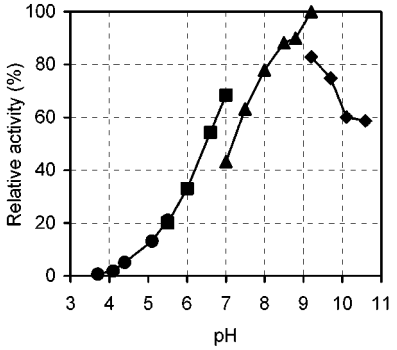


Figure 4. Activity of the proteinase at various pH levels. The activity was measured using the azogelatin method. All buffers were 80 mM and contained (●) Na acetate, (■) Na citrate, (▲) Tris–HCl, or (◆) CAPS.

Table 2. Activities of Proteinase that Had Been Heated for 90 min at 40 °C at Various pH Levels

pH	remaining activity (%)
4.1	50
5.0	41
6.0	58
6.6	58
7.9	42
8.6	24

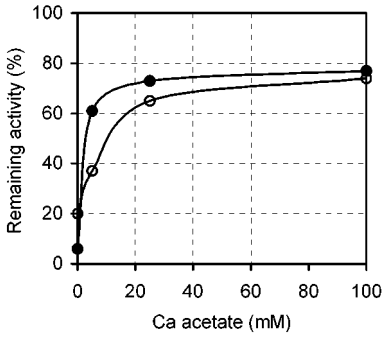


Figure 5. Effect of Ca^{2+} ions on the proteinase stability at (○) pH 5.2 and (●) pH 8.6. The samples were incubated at 40 °C for 90 min in the presence of Ca acetate.

Substrate Specificity. To ascertain which peptide bonds the purified proteinase hydrolyzed preferentially, it was used to hydrolyze a reduced and pyridylethylated low-molecular-weight wheat protein called β-purothionin. The resulting peptides were separated by RP-HPLC and those separations are shown in **Figure 7A**. The substrate β-purothionin was completely digested within 60 min, with the concomitant formation of smaller peptides. Small amounts of peptides were present even after 0 min of hydrolysis, because some hydrolysis occurred before the enzyme became thermally inactivated. The eluted peptides were collected and their molecular masses were measured (**Figure**

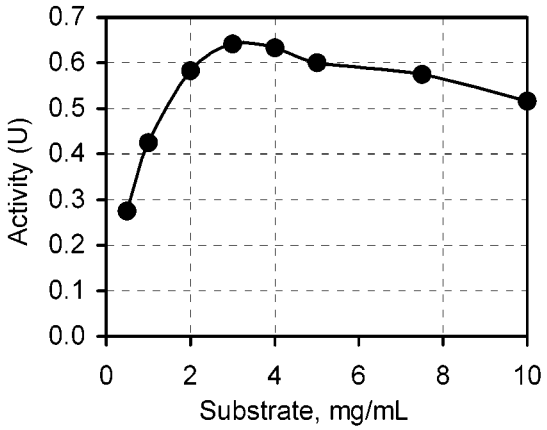


Figure 6. Effect of azogelatin concentration on the proteinase activity at pH 6.0 and 40 °C.

Table 3. Inhibition of the Purified Enzyme by Various Serine Class Proteinase Inhibitors

inhibitor	concentration (μM)	inhibition (%)
p-APMSF	1 000	100
PMSF	1 000	22
chymostatin	33	2
	1.7	–8[a]
soybean trypsin inhibitor	1.0	83
		79[a]
Bowman–Birk	2.5	94

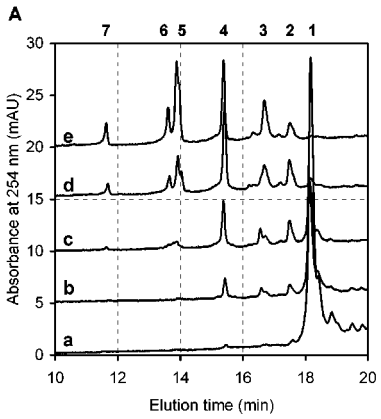
[a] Measured with BVGRpNA at pH 9.0 and 28 °C. All other assays were with azogelatin at pH 6.0 and 40 °C.

7B). These were compared with the calculated molecular masses of the peptides that could have been released, given the sequence of the intact pyridylethylated β-purothionin (*24*). As indicated by the arrows in **Figure 7C**, the enzyme primarily hydrolyzed peptide bonds that were on the C-terminal side of Arg and at the Lys5 residue. Small amounts of peptides that resulted from cleavage on the C-terminal side of Lys23 and Lys41 were also detected (results not shown). The hydrolysis was essentially completely inhibited by STI; the peptide separation pattern of the sample that was incubated for 2 h with the purified enzyme and STI was identical to that of the 0 min hydrolysis ('b') of **Figure 7A**.

The hydrolytic activities of the proteinase (in nkat/mg protein, *19*) with selected synthetic substrates at pH 9.0 are listed in **Table 4**. These results also show that the enzyme preferred to hydrolyze bonds adjacent to the Arg residue. However, the small trypsin-specific substrate BApNA was not readily hydrolyzed, even though it is commonly used to study bovine and other trypsins.

Kinetic Constants for the Hydrolysis of Synthetic Substrates. The K_m value and maximal velocity (V_{max}) for the hydrolysis of BVGRpNA by the purified enzyme at pH 9.0, its pH optimum, were 21.9 ± 3.4 μM and 3 520 ± 230 nkat/mg protein, respectively. When measured at pH 6.0, the corresponding values were 107 ± 9 μM and 2 020 ± 50 nkat/mg protein.

Hordein Hydrolysis. An SDS−PAGE separation of hordeins that were incubated with the *Fusarium* proteinase at pH 6.0 showed that the enzyme hydrolyzed the D and the two largest C class hordeins (**Figure 8**). The smallest C hordein and a 25 kDa protein were also degraded after extended periods (not shown). STI inhibited the hordein hydrolyses, but only when present in great excess.



B

Peak #	$Mass_{ms}$	$Mass_c$	Peptide[a]	Peak #	$Mass_{ms}$	$Mass_c$	Peptide[a]
1	5770.3	5770.0	1-45	4y	1609.2	1610.1	6-17
2x	3417.7	3417.1	18-45	5x	1094.4	1095.5	11-17
2y	3190.3	3189.9	20-45	5y	1599.5	1599.8	18-30
3x	3954.3	3952.8	1-30	6	1372.0	1372.7	20-30
3y	1834.4	1835.2	31-45	7	1292.3	1293.6	1-10
4x	2369.4	2370.8	1-17				

[a] The amino acid residues that comprised the peptide.

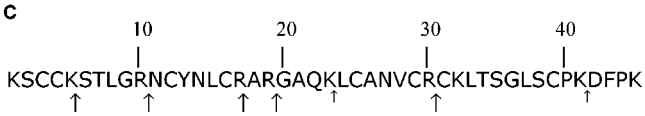


Figure 7. Hydrolysis of reduced and pyridylethylated β-purothionin by the purified *F. culmorum* proteinase. (A) Reversed-phase HPLC separations of the peptides produced. The substrate was incubated at pH 5.5 and 40 °C: a, without enzyme for 120 min; b–e, with the proteinase for 0, 15, 60, and 120 min, respectively. (B) Identification of the HPLC-separated peptides (peaks 1–7) and their molecular masses (ms, mass spectrometric analysis; c, calculated on the basis of their amino acid compositions). Several peaks contained two major peptides, which are designated x and y. (C) The primary enzymatic cleavage sites are indicated by arrows on the β-purothionin amino acid sequence. Cleavage at the peptide bonds indicated by the shorter arrows occurred slowly.

Table 4. Hydrolytic Activities of the Proteinase Measured at pH 9.0 with Various Synthetic Substrates

substrate	activity[a]
Nα-benzoyl-L-Arg pNA	22.3 ± 1.4
N-benzoyl-Val-Gly-Arg pNA	3 710 ± 215
N-succinyl-Ala-Ala-Pro-Leu pNA	5.5 ± 0.9
N-succinyl-Ala-Ala-Pro-Phe pNA	19.5 ± 1.7
N-glutaryl-L-Phe pNA	0.3 ± 0.0

[a] nkat/mg protein.

DISCUSSION

This *Fusarium* proteinase is categorized as a trypsin-like enzyme because it was inhibited by soybean trypsin inhibitor and, as is typical of trypsins, was inhibited more strongly by *p*-APMSF than by PMSF. Its substrate specificity was similar to that of the trypsins. Bovine trypsin favors the hydrolysis of peptide bonds adjacent to Lys over those of Arg but, like the trypsin from *Fusarium oxysporum* (*26*, *29*), this enzyme more readily hydrolyzed bonds adjacent to the Arg residue. In addition, its N-terminal amino acid sequence was highly homologous with that of trypsin-like enzymes of *F. oxysporum*

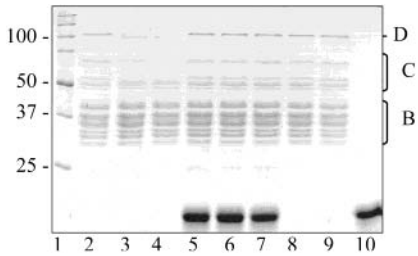


Figure 8. Hydrolysis of hordeins by the purified *F. culmorum* proteinase. The hordein hydrolysis products were separated with SDS–PAGE. The reactions contained the following: lanes 2–4, enzyme, no STI; lanes 5–7, enzyme + STI; lanes 8–9, no enzyme, no STI; lane 10, enzyme + STI, no hordein. Hydrolysis times were as follows: lanes 2, 5, 8, and 10, 0 min; lanes 3 and 6, 25 min; and lanes 4, 7, and 9, 100 min. Protein molecular weight standards were loaded on lane 1.

and several other fungi (*9*, *26*−*28*). The characteristics of the enzyme, and possibly its structure, are very similar to those of the *F. oxysporum* trypsin (*26*). Unlike bovine or *Streptomyces griseus* trypsins, the *F. oxysporum* enzyme does not have a Ca-binding site, as determined by X-ray crystallography (*26*). Calcium did not affect the activity of the *F. culmorum* trypsin, but it must have interacted with the enzyme, because the stability of the proteinase increased in the presence of Ca^{2+} ions.

Some plant pathogenic fungi synthesize trypsin- and subtilisin-like enzymes concomitantly (*26*, *27*, *30*). A subtilisin-like enzyme has been purified from the same culture medium as this *F. culmorum* trypsin-like enzyme (*19*). Under the conditions where the proteinase activity was monitored throughout the purification (pH 8.9, 40 °C), the rate of azogelatin hydrolysis per mg of trypsin proteinase was about 30% slower than that of the subtilisin-like enzyme. This may at least partially explain why the specific activity of the trypsin preparation decreased during the CM-HPLC separation. Both of these enzymes were maximally active at approximately pH 9 and 40−45 °C. The trypsin-like proteinase was generally more stable than its subtilisin-like counterpart, but it was more sensitive to elevated temperatures. Both enzymes were stabilized by the presence of added protein (BSA), which may protect the enzymes from either autolysis (*31*) or from surface-catalyzed inactivation (*32*).

Several plant pathogens besides the *Fusarium* spp., such as *Cochliobolus carbonum* (*27*) and *Verticillium* spp. (*30*, *33*), synthesize trypsin-like proteinases when grown in artificial growth media. The trypsin-like proteinases of *Stagonospora nodorum* (*9*) and *Ustilago* sp. (*10*) and nontrypsin proteinases of various other fungal pests (*11*−*18*) are present in infected host plants, but in most cases no direct evidence has been presented to confirm that they play a role in the pathogenesis. Urbanek and Yirdaw (*34*) have reported that *F. culmorum* growing on maize seedlings synthesized an acid proteinase, but the *F. culmorum* strain that was used for our study produced very little acid proteinase (*8*).

Both the trypsin- and subtilisin-like *F. culmorum* proteinases hydrolyzed hordeins and other barley proteins in vitro, indicating that they probably play a role in rendering the barley proteins accessible for use by the fungus. Now it is important to establish whether these enzymes are present in infected grains and, if so, what their purposes are.

ABBREVIATIONS USED

p-APMSF, *p*-amidino phenylmethylsulfonyl fluoride; BAp-NA, Nα-benzoyl-L-Arg *p*-nitroanilide; BBI, Bowman−Birk inhibitor; BSA, bovine serum albumin; BVGRpNA, N-benzoyl-Val-Gly-Arg *p*-nitroanilide; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; CMC, carboxymethyl cellulose; CM-HPLC, carboxymethyl-HPLC; CST, chymostatin; DMSO, dimethyl sulfoxide; GPpNA, N-glutaryl-L-phe *p*-nitroanilide; PMSF, phenylmethylsulfonyl fluoride; R&P, reduced and pyridylethylated; SAAPFpNA, N-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide; SAAPLpNA, N-succinyl-Ala-Ala-Pro-Leu *p*-nitroanilide; STI, soybean trypsin inhibitor; TFA, trifluoroacetic acid.

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Received for review January 8, 2002. Revised manuscript received April 17, 2002. Accepted April 17, 2002. We greatly appreciate the financial support that we received from the Tor-Magnus Enari Fund, the Finnish Food Research Foundation, the American Malting Barley Association, and Anheuser-Busch, Inc.

JF020027X