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Effect of a Bowman-Birk Proteinase Inhibitor from *Phaseolus coccineus* on *Hypothenemus hampei* Gut Proteinases In Vitro

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The coffee berry borer, *Hypothenemus hampei* (Ferrari), is an important devastating coffee pest worldwide. Both trypsin and chymotrypsin enzyme activities from *H. hampei* larval midgut can be inactivated by proteinaceous enzyme–inhibitors. A serine proteinase inhibitor belonging to the Bowman–Birk class was purified from a wild accession of *Phaseolus coccineus* L. seeds. The inhibitor (PcBBI1) is a cysteine-rich protein that is heat-stable at alkaline pH. MALDI-TOF/MS analysis showed that PcBBI1 occurs in seeds as a monomer (8689 Da) or dimer (17 378 Da). Using in vitro inhibition assays, it was found that PcBBI1 has a high inhibitory activity against *H. hampei* trypsin-like enzymes, bovine pancreatic chymotrypsin, and trypsin. According to this, PcBBI1 could be a promising tool to make genetically modified coffee with resistance to coffee berry borer.

KEYWORDS: Bowman-Birk inhibitor; *Hypothenemus hampei*; *Phaseolus coccineus*; Proteinase inhibitor

INTRODUCTION

The coffee berry borer (CBB), *Hypothenemus hampei* (Ferrari), is widely considered to be the most devastating pest of coffee crops worldwide (*1*). Both larvae and adults can penetrate the fruit berries to feed on and reproduce in the endosperm, thereby causing a severe reduction in the yield and quality of the final product (*2*). Attack by this insect also creates new sites for secondary infection by bacteria and fungi (*3*). On the basis of the feeding behavior of larval and female CBB, different control measures, including biological control and biotechnological strategies, have been used as an alternative to environmentally hazardous chemical insecticides (*4*). Proteinase inhibitors (PI) are potential defense molecules against insect pests in crop plants (*5–7*), and the introduction of PI genes into economically important crop plants has become a promising alternative for controlling such pests (*8, 9*). The report by Valencia et al. (*10*) on the trypsin and chymotrypsin activities of the *H. hampei* larval gut provides new perspectives for the use of PI genes to produce transgenic coffee plants resistant to CBB.

Proteinase inhibitors of the Bowman–Birk class (BBI), a major class in legume seeds, contain two active sites that are primarily associated with the inhibition of the digestive enzymes trypsin and chymotrypsin (*11*). In recent years, BBIs have received enormous attention because of their vital role in the defense mechanisms of plants against insect pests, especially during germination and initial seedling growth. Several roles for PIs have been suggested in plants, including the natural regulation of proteolysis, a key metabolic process that requires tight control (*12*).

Plant proteinase inhibitors have been widely studied, and their deleterious effects on Lepidoptera and Coleoptera (*13, 14*) makes them attractive agents for the biological control of insect pests (*15, 16*). Studies using transgenic plants containing PI genes have shown that cowpea BBI confers enhanced resistance

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against *Heliothis virescens* (Fabricius) in transgenic tobacco plants (*6*) and against *Diatraea saccharalis* (Fabricius) larvae in transgenic sugar cane plants (*17*). Similarly, expression of the barley trypsin inhibitor, BTI-CMe, into transgenic rice plants confers resistance to the rice weevil, *Sitophilus oryzae* L (*18*).

In this report, we describe the isolation and characterization of a proteinase inhibitor, PcBBI1, from a wild accession of *P. coccineus* seeds. PcBBI1 showed significant inhibitory activity against *H. hampei* serine proteinases in vitro, which suggests that this inhibitor could be a useful insecticidal protein for controlling important storage pests.

MATERIALS AND METHODS

Insect and *Phaseolus coccineus* Sources. A wild accession (35619) of *P. coccineus* was obtained from the University of Caldas, Manizales, Colombia. *Euschistus heros* (Fabricius) and *Anthonomus grandis* (Boheman) were obtained from Embrapa Recursos Genéticos e Biotecnologia, Brasília, Brazil. *H. hampei* (Coleoptera: Scolytidae) was obtained from the Instituto Agronômico do Paraná (IAPAR), Londrina, Paraná, Brazil.

Chemicals and Reagents. Chromatographic columns and ion exchange DEAE-cellulose (DE52) were purchased from Whatman. Size exclusion Superdex 75 HR 10/30, reverse-phase Source 5RPC ST 4.6/150 and reverse-phase Vydac C_{18} (218 TP 54) columns were obtained from GE Biosciences (Uppsala, Sweden). Bovine pancreatic trypsin (EC 3.4.21.4) and bovine pancreatic chymotrypsin (EC 3.4.21.1) were purchased from Sigma (St. Louis, MO). All other chemicals and reagents used were of analytical grade.

Purification of the Bowman−Birk Inhibitor from *P. coccineus* Seeds. Bean seeds were powdered, and the lipids were removed using three volumes of acetone. The solvent was filtered off, and the seed residue was air-dried to obtain a dry powder. Proteins from the seed powder were extracted at 4 °C with 5 volumes of 0.15 M NaCl for 5 h. The slurry was centrifuged at 10,000*g* for 30 min at 4 °C, and the proteins in the supernatant (crude extract) were precipitated with 60% ammonium sulfate. The precipitate was subsequently dissolved in 0.05 M Tris−HCl, pH 7.0, containing 0.1 M NaCl and dialyzed extensively against deionized H_2O. This fraction (3.5 mg), which had a high level of inhibitory activity against bovine pancreatic trypsin, was applied to a DEAE-cellulose column (16 × 120 mm) equilibrated with 0.025 M Tris-HCl, pH 7.5. The column was washed with this same buffer to remove unbound material, and the adsorbed proteins were eluted with a 0–0.4 M NaCl linear gradient. The elution profile was monitored at 280 nm, and the fractions showing inhibitory activity against trypsin were dialyzed against H_2O and were freeze-dried. A sample (10 μL) with a protein concentration of 10 mg ml^{-1} was applied to a Superdex 75 HR 10/30 gel filtration column equilibrated with 20% acetonitrile. The sample was eluted at a flow rate of 0.5 mL min^{-1}, and fractions containing trypsin inhibitor activity were pooled and applied (90 μg) to a reverse-phase high performance liquid chromatography (RP-HPLC) silica column (Vydac C-18), equilibrated with 0.1% trifluoroacetic acid (TFA) in ultrapure water (Milli-Q filtration system), solvent A, at a flow rate of 1.0 mL min^{-1}. Proteins were eluted with a linear gradient of 20–60% acetonitrile in 0.1% TFA, solvent B. The final purification step was done on a column of divinyl-benzene (Source 5RPC ST 4.6/150) equilibrated with 2% acetonitrile in 0.1% TFA, at a flow rate 1.0 mL min^{-1}, using a gradient of 25–35% acetonitrile in 0.1% TFA. The analytical purifications were done using an ÄKTAExplorer chromatographic system (GE Biosciences, Uppsala, Sweden), and the elution profiles were monitored at 216 and 280 nm.

Protein Determination. Protein content was measured following the Bradford method (*19*), using bovine serum albumin as the protein standard.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was done as described by Schägger and Von Jagow (*20*). The protein molecular mass markers used were lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (68 kDa), phosphorylase b (97.4 kDa), and myosin (200 kDa).

MALDI-TOF and Q-TOF Mass Spectrometric analysis. Freeze-dried fractions from HPLC were prepared for matrix-assisted laser desorption ionization-time-of-flight mass spectrometric analysis (MALDI-TOF-MS) on a Voyager-DE STR Bioworkstation (PerSeptive Biosystems, Framingham, MA). The samples were dissolved in 0.1% TFA, and the matrix α-cyano-4-hydroxycinnamic acid (a saturated solution dissolved in acetonitrile and 3% TFA, 1:1, v/v, from Sigma) was added. The solution was then vortex mixed, and 1 mL was applied to the Voyager Bioworkstation sample plate and air-dried at room temperature. The mass spectrometer, equipped with a delayed-extraction system, was operated in linear mode with internal and external calibration using the Sequazyme Standard Kit from Applied Biosystems (Framingham, MA). The analyses were also done in a Q-TOF Ultima API (Waters, Manchester, UK) mass spectrometer operating in W mode. A mixture of water/acetonitrile/acetic acid (50:50:0.1, v/v) containing the sample was applied at 2 μL min^{-1}. The capillary and cone voltages were set to 2.8 kV and 30 V, respectively.

N-Terminal Amino Acid Sequencing. The sample obtained by RP-HPLC (SOURCE column) was reduced according to Crestfield et al. (*21*) and the N-terminal sequence was determined with 2–5 pmol of the alkylated protein by automatic Edman degradation in a PPSQ-23 Sequencer (Shimadzu Co., Kyoto, Japan). The resulting sequence was used to search the Swiss Prot Databank using the Fasta 3 program of the ExPASy proteomic tools. Sequence alignments and similarity calculations were done using CLUSTALW (*22*).

Preparation of the Proteinases. Proteinases were obtained by dissecting midguts from larvae of *H. hampei* and *A. grandis* (18–20 days old) and from adults of *E. heros*, according to Terra et al. (*23*). The midguts were removed and placed in 0.05 M Tris-HCl, pH 8.0, prior to homogenization in a Potter homogenizer for 10 min at 4 °C. The homogenates were subsequently centrifuged at 10,000*g* for 30 min at 4 °C, and the crude extracts were used for proteinase and proteinase inhibitor assays.

Inhibition Assays. The proteolytic inhibitory activities were tested against chymotrypsin, trypsin, and proteinase extracts of midguts from larval and adult insects using a fluorometric assay (*24*). The substrates Z-Phe-Arg-MCA and Z-Arg-Pro-Phe-His-Leu-Leu-Val-Tyr-MCA were used for the trypsin and chymotrypsin activities, respectively. The enzymes were prepared in 0.05 M Tris-HCl, pH 8.0, containing 100 μM $CaCl_2$. Pure trypsin and chymotrypsin (0.05 μg) and insect proteinase extracts (2–10 μg) were used in the assay, in a reaction volume of 100 μL. The assays were done in the same buffer, and the preincubations containing enzyme and different concentrations of inhibitor were done at 37 °C for 30 min. The reaction was started by adding the substrate (final concentration of 0.6 mM) and then stopped after 10 min at 37 °C by adding 900 μL of 100% ethanol. The methylcumarin (MCA) released was measured using a HITACHI F2000 fluorometer, with excitation at 380 nm and emission at 440 nm. For all activity tests, appropriate blanks were used.

One relative unit corresponded to the release of 1 μmol of MCA min^{-1} mg^{-1} of protein. All fluorescence readings were corrected for the appropriate blanks. The assays were done in triplicate, and the inhibition of proteolytic activity was expressed relative to the control (no inhibitor, 100% of enzyme activity).

Thermal Stability. Thermal stability of PcBBI1 was determined in 0.05 M Tris-HCl, pH 8.0. Diluted inhibitor solutions were heated to 100 °C for 30, 60, 120, and 180 min. Aliquots were removed from the boiling water bath and immediately cooled by transferring them to an ice–water bath before measuring the residual inhibitory activity as described above (*25*).

RESULTS AND DISCUSSION

Purification of PcBBI1. Crude soluble protein extracts obtained from mature seeds of the wild bean *P. coccineus* were initially precipitated with 60% ammonium sulfate. This precipitate, which had strong inhibitory activity against bovine trypsin, was applied to a DEAE-cellulose column and resulted in three peaks (F_{ID}, F_{IID}, and F_{IIID}) (**Figure 1A**). Peak F_{IIID}, which eluted at approximately 0.2 M NaCl, completely inhibited the

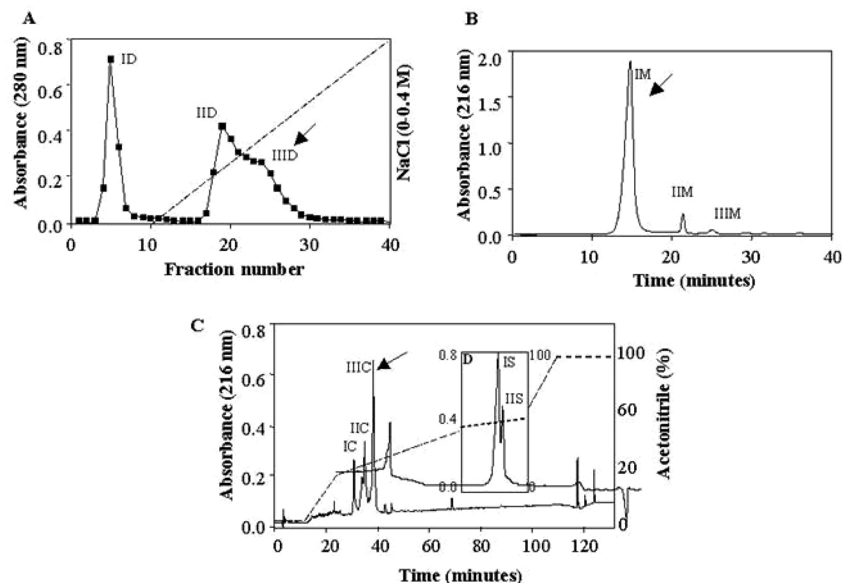

Figure 1. Elution profiles of *P. coccineus* PcBBI1 during the different purification steps. (**A**) A DEAE-cellulose ion exchange column previously equilibrated with 0.025 M Tris-HCl, pH 7.5 was loaded with approximately 3.5 mg of protein precipitated with ammonium sulfate at a concentration of 0–60%. Peaks F_{IID} and F_{IIID} were eluted with a linear gradient of 0–0.4 M NaCl in this same buffer. (**B**) Gel filtration of F_{IIID} on a Superdex 75 column equilibrated with 20% acetonitrile and eluted at a flow rate of 0.5 mL min^{-1}. (**C**) Analytical RP-HPLC of peak F_{IM} on a Vydac C_{18} analytical column equilibrated with solvent A. Peaks F_{IC}, F_{IIC}, and F_{IIIC} were eluted with a gradient (20–60%) of solvent B at a flow rate of 1.0 mL min^{-1}. (**D**, inset) Peak F_{IIIC} was chromatographed on a SOURCE divinyl-benzene column equilibrated with 2% solvent B in solvent A at a flow rate of 1.0 mL min^{-1}. All fractions were tested against trypsin. In all cases, the arrow indicates the peak with inhibitory activity.

Table 1. Purification of the Proteinase Inhibitor PcBBI1 from *P. coccineus* Seeds

peaks	total inhibitory activity	total protein (μg)	specific activity (UI/mg protein)	purification (fold)	yield (%)
F_{0-60}	31 500	3500	9	1	100
F_{IIID}	26 400	600	4.4	0.5	83.8
F_{IM}	21 990	30	733.3	82	69.8
F_{IIIC}	19 550	10	1955	217	62.1
$F_{IS\ (PcBBI1)}$	13 200	3	4400	489	41.9

The inhibitory activity in each step was assayed against bovine pancreatic trypsin by using Z-Phe-Arg-MCA as the substrate (0.6 mM final concentration). The initial inhibitor concentration used was 0.1 μg mL^{-1}. One relative unit of trypsin activity corresponded to the release of 1 μmol of MCA min^{-1} mg^{-1} of protein. The proteolytic activity inhibition was seen as a decrease in the release of MCA in the presence of PcBBI1. The assays were done in triplicate.

enzymatic activity of pancreatic bovine trypsin. This peak was applied to a Superdex 75 column (**Figure 1B**) and three new peaks were obtained (F_{IM}, F_{IIM}, and F_{IIIM}). The peak containing the trypsin inhibitor (F_{IIIM}) was chromatographed on an RP-HPLC C_{18} Vydac column (**Figure 1C**) and elution with a gradient (20–60%) of solvent B resulted in three peaks (F_{IC}, F_{IIC}, and F_{IIIC}). The largest of these peaks ($F_{IIIC,}$ which eluted with 29.4% solvent B) was applied to a reverse-phase divinyl-benzene column (**Figure 1D**, inset). The purified proteinase inhibitor, identified as PcBBI1 (peak F_{IS}), eluted with 29.2% solvent. This purification procedure resulted in a 489-fold purification of the trypsin inhibitor, with a 41.9% yield (**Table 1**).

Molecular Mass Analysis of PcBBI1. SDS-PAGE of PcBBI1 showed a single protein band with a molecular mass of approximately 8500 Da when treated with β-mercaptoethanol (**Figure 2Aa**) and a single protein band of 17 000 Da in the absence of β-mercaptoethanol (**Figure 2Ab**). This protein sample used in the SDS-PAGE assay was obtained from the divinyl-benzene column (**Figure 2B**). The lower molecular mass seen in the presence of the reducing agent indicated that this

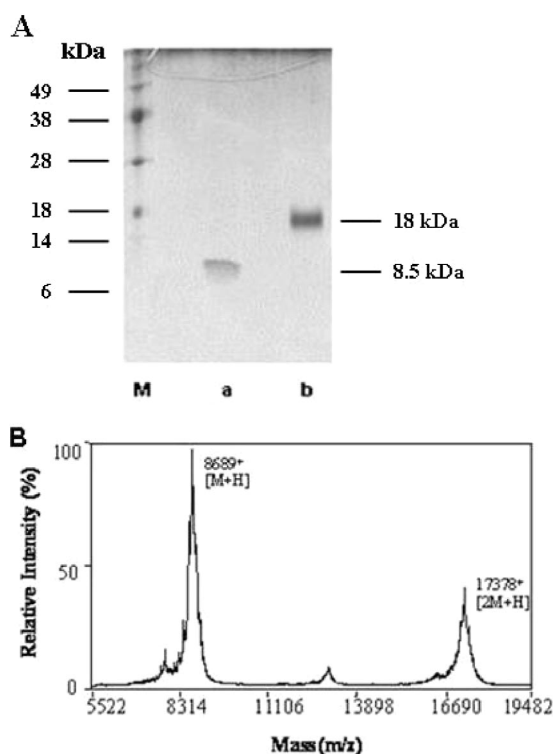

Figure 2. Molecular mass analysis of purified PcBBI1. (**A**) Molecular mass determination of PcBB1 by SDS-PAGE. Lane a, PcBBI1 in the presence of β-mercaptoethanol; lane b, PcBBI1 without β-mercaptoethanol; lane M, molecular mass markers. Ten microliters of a solution containing 1 mg of PcBBI1 mL^{-1} was loaded onto lanes a and b. (**B**) MALDI-TOF mass spectrum showing the dimeric and monomeric forms of PcBBI1.

protein consisted of two identical or very similar subunits and that disulfide bonds stabilized the dimeric conformation. A similar result was obtained by MALDI-TOF-MS, which showed a molecular mass of 8689 and 17 378 Da for the monomeric

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PcBBI1      ...................................DEPSESSKACCDHCACTKSIPPQC 23
TBPI        ..........................SGHHHHD..SSDEPSESSKACCDHCACTKSIPPQC 32
PvI-3       ..........................SGHRHESXBSTBXASXSSKPCCBHCACTKSIPPQC 34
SoyBBI      MVVLKVCLVLLFLVGGTTSANLRLSKLGLLMKSDHQHSNDDESSKPCCDQCACTKSNPPQC 60
AzukiBBI    ..........................SVHHQD..SSDEPSESSHPCCDLCLCTKSIPPQC 31
SoyBBPIDII  MVVLKVCLLLLFLVGVTAAP....MELSFFKSDQSSSYDDDEYSKPCCDLCMCTRSMPPQC 56


PcBBI1      RCSNLRLNECKHECRKCICLHRGHAHC.................. ... 51
PvI-3       RCSBLRLNSCHSECKGCICTFSIPAQCICTDTNNFCYEPCKSSHGPBBNN 85
SoyBBI      RCSDMRLNSCHSACKSCICALSYPAQCFCVDITDFCYEPCKPSEDDKEN. 110
TBPI        RCA.LRLN.CNH.CRSCICTFSIPAQCVCTDTNDFCYEPCKSGHDDDDSG 80
AzukiBBI    QCADIRLDSCHSACKSCMCTRSMPGQCRCLDTHDFCHKPCKSRDKD.... 78
SoyBBPIDII  SCEDIRLNSCHSDCKSCMCTRSQPGQCRCLDTNDFCYKPCKSRDD..... 102
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Figure 3. Comparison of the PcBBI1 sequence with other BBIs from related monocots and dicots. TBPI: P83311 (*29*), PvI-3: P81484 (*42*), SoyBBI: X68704 (*43*), AzukiBBI: P01061 (*44*), and SoyBBPID-II: CAA48657 (*45*). The trypsin and chymotrypsin binding loops are underlined (first and second regions, respectively).

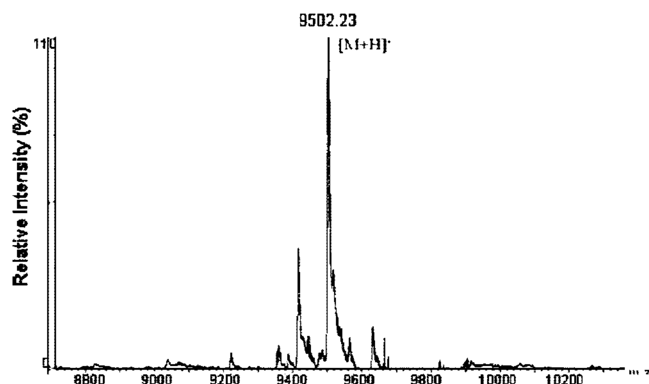

Figure 4. Mass spectrum of reduced and alkylated (with iodoacetamide) PcBBI1 obtained by Q-TOF mass spectrometry.

and dimeric forms, respectively (**Figure 2B**). Members of the Bowman−Birk family form molecular aggregates (*26, 27*), and the dimer is considered to be the active form of some inhibitors (*28*) **Figure 3**.

Sequence Analysis of PcBBI1. The N-terminal sequence of PcBBI1 was determined up to residue 51. On the basis of the sequence obtained, PcBBI1 is related to the Bowman−Birk inhibitor family because it showed 76% identity with the sequences of other members of this family. PcBBI1 contains trypsin and chymotrypsin binding loops.

The first reactive site of PcBBI1 is well-defined at Lys 18, which is a typical recognition site for trypsin binding and is conserved in most BBI sequences (*27, 29*). At the second reactive site, which recognizes chymotrypsin, there is a difference in the amino acid residue at position 48, which is His instead of the Arg, Tyr, Phe, Leu, or Ala usually present in other BBIs (*28–30*). However, this difference does not reduce the specificity of PcBBI1 for chymotrypsin. The pattern of cysteine residues seen among other BBIs is conserved in PcBBI1. Although only ten cysteines were detected by N-terminal sequencing, spectrometric analysis (**Figure 4**) of the reduced and alkylated (with iodoacetamide) protein showed the increase in mass (9.5 kDa) expected for a 14-cysteine molecule, which formed seven disulfide bonds, as reported for other BBIs (*31–33*).

In Vitro Activities of PcBBI1. The inhibitory activity of PcBBI1 toward bovine pancreatic chymotrypsin and trypsin and gut serine proteinases from *H. hampei, A. grandis*, and *E. heros* was determined by in vitro enzyme assays. **Table 2** shows that, at a concentration of 0.1 μg mL^{-1}, PcBBI1 was highly active against bovine pancreatic chymotrypsin and bovine trypsin (100% inhibition in both cases) and against *H. hampei* gut serine proteinases (80% inhibition). No significant inhibition was observed against gut serine proteinases from the insects *A. grandis* and *E. heros*, indicating some specificity of PcBBI1 against CBB. **Figure 5** shows that the inhibitory activity of PcBBI1 against trypsin occurred at all pH values that were tested. The presence of disulfide bonds could explain the high structural stability of this molecule at different pH values. At alkaline pH (8.0), the antitryptic activity of PcBBI1 was reduced to around 30% after 1 h and to approximately 10% after 3 h at 100 °C (**Figure 6**), showing a thermal stability under the conditions of our assay. This thermal stability was not tested at neutral and acidic pH. Osman et al. (*34*) demonstrated that BBIs such as TBPI and soybean BBI were heat stable at 100 °C in

Table 2. Inhibitory Activity of PcBBI1 on Bovine Pancreatic Trypsin and Chymotrypsin and Insect Midgut Proteinases

target proteases	inhibition (%)
trypsin	100 ± 0.1
chymotrypsin	100 ± 1.9
*Hh*P	80 ± 2.3
*Ag*P	4 ± 2.1
*Eh*P	3 ± 1.6

The assays were done using the substrates Z-Phe-Arg-MCA and Z-Arg-Pro-Phe-His-Leu-Leu-Val-Tyr-MCA for trypsin and chymotrypsin activities, respectively, at a final concentration of 0.6 mM. The initial inhibitor concentration used was 0.1 μg mL^{-1}. One relative unit of trypsin activity corresponded to the release of 1 μmol of MCA min^{-1} mg^{-1} of protein. The assays were done in triplicate.

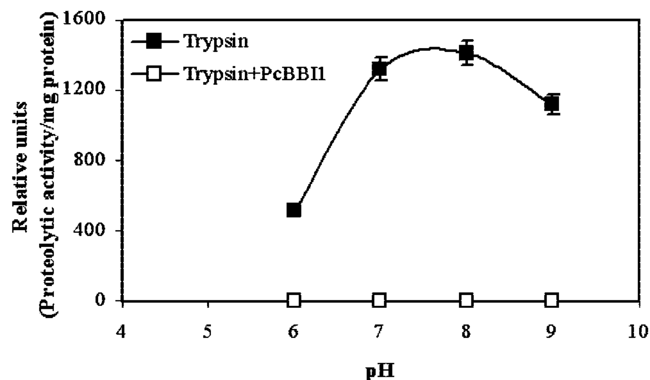

Figure 5. Inhibitory activity of PcBBI1 at different pH values. Trypsin activity was measured in the absence or presence of 0.1 μg of PcBBI1. The proteolytic enzyme was incubated with PcBBI1 for 30 min prior to assaying the enzymatic activity towards the substrate (Z-Phe-Arg-MCA). The enzymatic assays with substrate were run for 30 min before stopping the reaction with 100% ethanol. All measurements were done in triplicate, and the error bars represent the standard deviation.

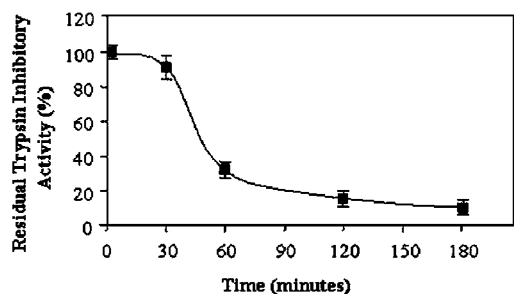

Figure 6. Thermal stability of PcBBI1. The inhibitor was incubated at 100 °C for the indicated times and then preincubated (0.1 μg) with trypsin for 30 min prior to assaying the residual enzymatic activity as described in the Methods section and in **Figure 5**. All measurements were done in triplicate, and the error bars represent the standard deviation.

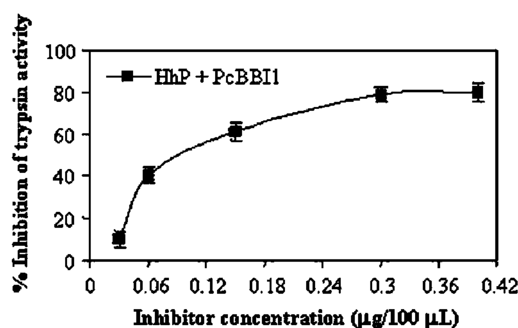

Figure 7. Inhibition of the trypsin-like proteinase activity of *H. hampei* larvae by PcBBI1. The midgut extract was preincubated for 30 min with different concentrations of PcBBI1, prior to the addition of substrate (Z-Phe-Arg-MCA, 0.6 mM). The reactions were stopped 30 min later by adding 100% ethanol. All measurements were done in triplicate, and the error bars represent the standard deviation.

neutral or acidic pH, but they were less heat stable at alkaline pH. However, DiPietro and Liener (*25*) showed that the BBI purified from *Vigna unguiculata* (L.) seeds was stable not only over a wide pH (*3–12*) but also at high temperatures. Belitz and Grosch (*35*) stated that the thermal stability of proteinase inhibitors depends on their molecular mass and on the stability of the active conformation conferred by the disulfide bonds. Since the first report by Birk et al. (*36*), many other studies have shown that BBIs are more heat stable at a variety of pH values than are Kunitz-type inhibitors, primarily because of the differences in molecular size and in the number of disulfide bonds.

At a concentration of 3 μg 1 mL^{-1}, PcBBBI1 strongly inhibited (80%) the trypsin activity of *H. hampei* larvae (**Figure 7**). The residual trypsin-like activity seen in *H. hampei* larvae could be explained by the presence of some proteinase isoforms that are resistant to the inhibitor. Several groups have reported inhibitory activity of BBIs toward gut proteinases of the aphid *Tetanops myopaeformis* (Roder) (*37*), the boll weevil *A. grandis* (*38, 39*) and in the sugar beet root maggot, *Acyrthosipon pisum* (Harris) (*40*).

Assessment of PcBBI1 specificity toward trypsin-like proteinases from *H. hampei* larvae in vitro is an important tool for screening inhibitors for their potential use in the production of transgenic coffee plants to increase the resistance against CBB. The successful implementation of PIs in transgenic applications requires information about the efficacy of these inhibitors toward insect gut proteinases in vitro and an evaluation of their ability to inhibit insect growth in bioassays (*41*). The effect of PcBBI1 on *H. hampei* growth and survival is currently being investigated by feeding laboratory-reared insects with a diet containing this inhibitor. The isolation of the gene encoding for the inhibitor is also in progress.

ABBREVIATIONS

Coffee berry borer, CBB; Bowman-Birk inhibitor, BBI; matrix-assisted laser desorption ionization-time of flight mass spectrometric analysis, MALDI-TOF-MS; *P. coccineus* Bowman-Birk inhibitor-1, PcBBI1; proteinase inhibitor(s), PI; trifluoroacetic acid, TFA; trypsin bovine pancreatic inhibitor, TBPI.

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