Article

SERINE PROTEASE FROM MIDGUT OF *Bombus terrestris* MALES

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A serine protease was isolated from midguts of the bumblebee male Bombus terrestris by a combination of precipitation procedures with column chromatography. The purified enzyme exhibited two bands with molecular masses of 25 and 26 kDa as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. These bands showed a proteolytic activity in zymography assay. Midgut enzymes showed optimum proteolytic activity at pH 9 and 35° C using N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenyl-alanine 4-nitroanilide as a substrate. The Michaelis constant (Km) and maximum reaction rate (Vmax) were 0.55 ± 0.042 mM and 0.714 ± 0.056 μ mol p-nitroalanine produced min^{-1} mg $protein^{-1}$, respectively. Inhibition was affected by trypsin inhibitor, but not by phenylmethylsulfonyl fluoride and N-tosyl-L-phenylalanine chloromethyl ketone, which indicated the trypsin-like but not chymotrypsin-like specificity. The identity of the serine protease was confirmed by nanoliquid-tandem mass spectrometry. Eleven unique peptides of the B. terrestris serine protease were found. It shows high homology to a previously reported B. ignitus serine protease covering more than 65% of the protein amino acid sequence. © *2012 Wiley Periodicals, Inc.*

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INTRODUCTION

Serine proteases play critical roles in an insect physiology. They serve as major insect gut enzymes in the digestion of dietary proteins, inactivate protein toxins, and show antimicrobial activity (Hegedus et al., 2003; Prabhakar et al., 2007; Chougule et al., 2008). Serine proteases also play critical roles in several insect biological processes such as blood coagulation, signal transduction, hormone activation, and development (Hedstrom, 2002; Clynen et al., 2005).

The catalytic function of serine proteases is accomplished via a catalytic triad (reactive serine, histidine, and aspartic acid). The degree and the type of substrate specificity is determined by many specific chemicals within the substrate-binding cleft (Perona and Craik, 1995). Serine proteases belong to a large group of endopeptidases that require a serine residue for their catalytic activity. They are equally important for biological systems (Kini, 2005; Muller et al., 2007; Yoon et al., 2007) as well as for commercial purposes (Saeki et al., 103).

Insects produce serine proteases within gut cells and release the enzymes into the gut lumen, where they digest food proteins into short peptides and amino acids suitable for their absorption by the midgut (Pauchet et al., 2008). As a defense mechanism, host plants produce proteinase inhibitors to suppress the activity of serine proteases (e.g., trypsin and chymotrypsin; Jongsma et al., 1995). Some polyphagous insects such as *Helicoverpa zea*, *Agrotis ipsilon*, and *Spodoptera frugiperda* can overcome this defense action by expressing trypsins and chymotrypsins that are insensitive to host proteinase inhibitors (Mazumdar-Leighton and Broadway, 2001; Brioschi et al., 2007). In addition to protein digestion, chymotrypsins may be involved in other physiological processes. For example, in *Manduca sexta* a chymotrypsin is involved in synthesis of chitin which is necessary for peritrophic matrix formation (Broehan et al., 2008).

The most frequently isolated protease from insects has been trypsin (Kalhok et al., 1993; Noriega et al., 1996), specifically a serine proteinase, demonstrably or presumably of the chymotrypsin-trypsin-like family. There have also been numerous reports on insect chymotrypsins (Elpidina et al., 2005; Lopes et al., 2009).

Insect species have individual digestive serine proteases. From *Locusta migratoria* (Lam et al., 2000), both trypsin and chymotrypsin have been isolated. Similarly, from the honeybee *Apis mellifica*, gut proteinases with trypsin-like and chymotrypsin-like specificities have been purified (Burgess et al., 1996). Some enzymes were present only in adult workers and drones, possibly reflecting the composition of the diets of different honeybee castes.

There is relatively little information on bumblebee digestive proteases. Here, we address this gap by characterizing the major digestive proteolytic enzyme(s) of bumblebee male *Bombus terrestris*, important glasshouse pollinators. The information helps to determine the possible function of serine proteases.

MATERIALS AND METHODS

Insect

The *B. terrestris* males (2 days old) were obtained from laboratory colonies during the winter season 2010–2011 as described by Ptáček et al. (2000). Briefly, the midguts were isolated immediately after collecting the males and kept at −18°C until use.

Preparation of Crude Enzymes

After decapitation, midgut tissues were collected in ice-cold saline solution (0.90% NaCl, w/v). Isolated midguts were stored at 0°C until use. The tissues were homogenized, at a ratio: 25 µl of saline solution per organ, using a Potter–Elvehjem homogenizer with a Teflon® pestle. The homogenate was centrifuged at 20,000 × *g* for 20 min. The pellet was resuspended in saline solution (25 µl per individual) and centrifuged at 20,000 × *g* for 20 min. The supernatants were collected and centrifuged at 20,000 × *g* for 30 min. All steps were carried out at 4°C and glycerol was added to a final concentration of 50% (w/v). The suspension was stored at –20°C until further used.

Purification

Protease activity in each fraction was monitored by the release of *p*-nitroanilide from proteinase substrate (as described below). The midgut extract was prepared from 12 g crude enzyme material obtained from 500 individuals of 2-day-old *B. terrestris* males. Solid ammonium sulfate was added to the extract to make the concentration at 70% saturation and stirred overnight at 4°C. Next day, the insoluble material was removed by centrifugation at 15,000 × *g* for 30 min. The precipitate was resuspended in 2 ml of 1 M $(NH_4)_2SO_4$ in 50 mM Tris/HCl buffer (pH 7.8) and dialyzed against the same buffer overnight using a dialysis sack. Subsequently, the dialyzed sample was applied to a Phenyl-Sepharose column (GE Healthcare Life Science, Uppsala, Sweden, formerly Amersham Biosciences) preequilibrated with the same buffer. After washing the column, proteins were eluted by a linear gradient of 50 mM Tris/HCl buffer (pH 7.8) containing $(NH_4)_2SO_4$ from 1 to 0 M at a flow rate of 1 ml/min. The active fractions were collected (5 ml) and then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and LC-MS/MS. All purification steps were carried out at 4°C.

Determination of Protein Concentration

Protein concentration was determined according to Bradford assay using bovine serum albumine as a standard (Bradford, 1976).

Enzyme Activity Assay

Serine proteinase activity of the enzyme was measured by the release of *p*-nitroanilidine from the typical serine proteinase substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA and monitored by spectrophotometry at 405 nm. The assay was performed in 50 mM Tris-HCl buffer (pH 8.2) at 25°C for 30 min in a reaction volume of 1,150 µl containing 100 ml of 0.2 mM substrate and 50 µl of the crude enzyme. One unit of protease activity (U) is defined as the amount of enzyme releasing 1 µmol of *p*-nitroaniline per minute under the given assay conditions.

Effect of Temperature and pH

The optimum temperature was determined under standard assay conditions by incubating the reaction mixtures at temperatures ranging from 25°C to 65°C. To determine thermal stability, the enzyme was preincubated for 30 min in the assay buffer at temperatures ranging from 25°C to 65°C and then chilled on ice for 10 min. The remaining enzyme

activity was then determined by the assay method. To determine the pH optimum for the enzyme activity, the assay mixtures in 50 mM of the following buffers were used: phosphate (pH 5.21–6.62), Tris-HCl (pH 7.22–8.55), and glycine-NaOH (pH 8.94–10.99). The pH stability was determined by preincubating the enzyme in the above-mentioned buffers for 30 min at 25°C. The remaining activity was then measured under standard assay conditions.

Kinetic Parameters

The kinetic parameters K_M and V_{max} were determined against the substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA. Data were obtained by measuring the initial rate of hydrolysis by incubating the enzyme with appropriate concentrations of the substrate in 50 mM Tris-HCl (pH 8.5) at 25°C. The reaction was monitored at 405 nm. Initial hydrolysis rates were determined at six different concentrations ranging from approximately 0.1 to 2.4 mM. V_{max} and K_M values were determined by using the Lineweaver–Burk plot. Reactions were run in triplicate.

Specific Inhibitors

The following compounds, all from Sigma-Aldrich (St. Louis, MO), were used to find any alteration in the proteolytic activity of the midgut of *B. terrestris* regarding to specifically used substrates in 50 mM Tris-HCl (pH 8.5) at 25°C: PMSF (phenylmethylsulfonyl fluoride, 1, 5, 10 mM); chymotrypsin inhibitor, TPCK (*N*-tosyl-*L*-phenylalanine chloromethyl ketone, 1, 5, 10 mM) and trypsin inhibitor (trypsin inhibitor from *Glycine max* [soybean], 0.05, 0.5, 1.0%). A total of 50 μl of protease extract sample and 1 ml of inhibitor were added to tubes and incubated 30 min at 25°C. Protease activity was assayed using the method described above.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed on vertical slab gels followed the methods of Laemmli, using 5 and 10% polyacrylamide for the stacking and resolving gels, respectively (Laemmli, 1970). Molecular weight standards (Protein Test Mixture from Serva [BioTech a.s., Praha, Czech Republic]) were used for protein mass determinations. Proteins were stained with Coomassie Brilliant Blue R250 (Serva) or Biosafe Coomassie Blue (Bio-Rad, Hercules, CA). Nondenaturing PAGE was performed using discontinuous SDS-PAGE gel and under nonreducing conditions (protein samples were not treated with 2-mercaptoethanol and were not heated before electrophoresis). Electrophoresis was carried out at 180 mV and 4°C for 60 min until the bromphenol blue front reached the end of the gel (Pan et al., 2011). The protein bands were then cut and subjected to in-gel digestion.

Preparation of Zymogram

SDS-PAGE (10%) was prepared according to the method of Laemmli (1970), and polymerized with casein at 0.2% (w/w) final concentration. The sample prepared under nonreducing conditions was subjected for electrophoresis using Tris (25 mM), glycine (192 mM), and SDS (0.1%) for 3 h at 180 V and 4°C. After electrophoresis, gels were washed with 5 mM sodium phosphate buffer containing 2.5% Triton X-100 with constant agitation for 20 min to completely remove SDS. The gel was incubated overnight at 37°C

in Tris-HCl buffer (50 mM) pH 7.6 containing 10 mM $CaCl_2$ and 150 mM NaCl. Gels were then stained with Coomassie Brilliant Blue R-250 (0.1%) for 30 min and finally destained with 25% ethanol in 8% acetic acid and water (30:10:60, v/v) three times for 10 min to observe the translucent activity bands.

Two-Dimensional Gel Electrophoresis (2-DE)

Lyophilized protein sample (4.65 mg) was solubilized in 130 μl of rehydration solution (7 M urea, 2 M thiourea, 2% 3-((3-cholamidopropyl)dimethylammonium)-1-propanesulfonate (CHAPS, w/v), 0.2% Bio-Lyte® ampholytes (3–10 buffer, w/v), 1% dithithreitol (w/v). Proteins were then transferred to Ready Strip™ IPG Strips (3–10 NL, 7 cm, Bio-Rad) overnight by active in-gel rehydration (50 V, 20°C).

Isoelectric focusing was carried out at 20°C with Protean® IEF cell system (Bio-Rad) under mineral oil. Proteins were focused in four steps (250 V for 20 min, linear gradient; 500 V for 1 h, linear gradient; 1,000 V for 1 h, linear gradient; 4,000 V for 20,000 Vh, rapid gradient). Prior to separation in the second dimension, the strips were equilibrated according to Görg et al. (2004). After the equilibration step, strips were rinsed in Tris-glycine buffer (pH 8.3), transferred to a homogeneous 12.5% SDS-polyacrylamide gel (with the same composition as in the previous section), and gel strips were overlaid with 0.5% (w/v) Certified™ Low Melt Agarose (Bio-Rad) in SDS-PAGE running buffer containing trace of bromophenol blue. Finally, 5 μl of Precision Plus Protein™ Standards (molecular weight range 10–250 kDa, Bio-Rad) was added at the top end of the gel. Gels were run in the Mini-Protean Tetra Cell system. The spots were then stained by Coomassie Blue, excised, and subjected to in-gel digestion. Protein bands or spots were excised from the Coomassie-stained gels, and then processed as described in Shevchenko et al. (2006). Before the in-gel digestion, the gel pieces were cooled in an ice-cold bath and swollen in a 50 μl of digestion buffer (50 mM ammonium bicarbonate) containing trypsin (30 μg/ml, type IX-S, lot 51K72501, Sigma-Aldrich, St. Louis, MO) in 50 mM ammonium bicarbonate. After 1 h of cooling at 4°C, the gel pieces were incubated overnight at 37°C.

The resulting tryptic peptides were extracted from the gel pieces by adding 150 μl of extraction buffer (5% formic acid, 30% acetonitrile in water) and sonicated for 15 min. The buffer was then replaced and the gel pieces were sonicated again for additional 15 min. After each extraction step, the solutions were spun and the supernatants withdrawn, pooled, and concentrated to dryness in a vacuum centrifuge. Dried extracts were stored at −80°C before analysis.

Analysis of Tryptic Digests by LC-MS/MS

Dried protein digests were dissolved in 20 μl of 0.1% formic acid, centrifuged, and the supernatant transferred to inserts in vials. The nano-HPLC apparatus used for protein digest analysis was a Proxeon Easy-nLC (Proxeon, Odense, Denmark) coupled to a maXis Q-TOF (quadrupole-time of flight) mass spectrometer with ultra-high resolution (Bruker Daltonics, Bremen, Germany) by nanoelectrosprayer. The nLC-MS/MS instruments were controlled with the software packages HyStar 3.2 and micrOTOF-control 3.0. The data were collected and manipulated with the software packages ProteinScape 2.0 and Data-Analysis 4.0 (Bruker Daltonics).

A total of 5 μl of the peptide mixture were injected into an NS-AC-11-C18 Biosphere C18 column (particle size: 5μm, pore size: 12 nm, length: 150 mm, inner diameter:

75 μm), with an NS-MP-10 Biosphere C18 precolumn (particle size: 5 μm, pore size: 12 nm, length: 20 mm, inner diameter: 100 μm), both manufactured by NanoSeparations (Nieuwkoop, the Netherlands). The separation of peptides was achieved via a linear gradient between mobile phase A (water) and B (acetonitrile), both containing 0.1% (v/v) formic acid. Separation was started by running the system with 5% mobile phase B, followed by gradient elution to 30% B at 70 min. The next step was a gradient elution to 50% B in 10 min, and then a gradient to 100% B in 8 min was used. Finally, the column was eluted with 100% B for 2 min. Equilibration before the next run was achieved by washing the column with 5% mobile phase B for 10 min. The flow rate was 0.25 μl/min, and the column was held at ambient temperature (25°C).

Online nanoelectrospray ionization (easy nano-ESI) was operated in positive mode. The ESI voltage was set at +4.5 kV, scan time 1.3 Hz. Operating conditions: drying gas (N_2), 1 l/min; drying gas temperature, 160°C; nebulizer pressure, 0.4 bar. Experiments were performed by scanning from 100 to 2,200 *m/z*. The reference ion used (internal mass lock) was a monocharged ion of $C_{24}H_{19}F_{36}N_3O_6P_3$ (*m/z* 1,221.9906). Mass spectra corresponding to each signal from the total ion current chromatogram were averaged, enabling an accurate molecular mass determination. All LC-MS/MS analyses were done in duplicates.

Bioinformatic Analysis

Data were processed using ProteinScape software. Peptides were identified by correlating tandem mass spectra to the SwissProt database, using the MASCOT searching engine (www.matrixscience.com). Trypsin was chosen as the enzyme parameter. One missed cleavage was allowed, and an initial peptide mass tolerance of ±10.0 ppm was used for MS and ±0.05 Da for MS/MS analysis. Cysteines were assumed to be carbamidomethylated, proline and lysine to be hydroxylated, and methionine was allowed to be oxidated. All these possible modifications were set to be variable. Monoisotopic peptide charge was set 1+, 2+, and 3+. The Peptide Decoy option was selected during the data search process to remove false-positive results. Only significant hits with score ≥80 were accepted. In the next stage of the database searching, amino acid sequence corresponding to the gene for *B. terrestris* serine protease was processed using the MASCOT searching engine. Other parameters remained the same.

RESULTS

The serine protease was extractable from midgut homogenate in buffer. Digestive protease from the midgut was purified by a multiple-step procedure (Table 1). After the final step,

***Table 1. Purification of Serine Protease from Midgut of Male* Bombus terrestris**

Purification step	*Total activity (U)*	*Protein (mg)*	*Specific activity (U/mg)*	*Purification factor*
Homogenate	0.18	3,885.0	9.4	1
Dialyzate	0.07	2,568.0	5.7	0.6
$(NH_4)_2SO_4$ precipitation	0.25	2,539.0	19.3	2.1
Dialyzate	0.28	2,025.0	27.8	3.0
Phenyl-Sepharose	0.11	26.8	787.6	83.9

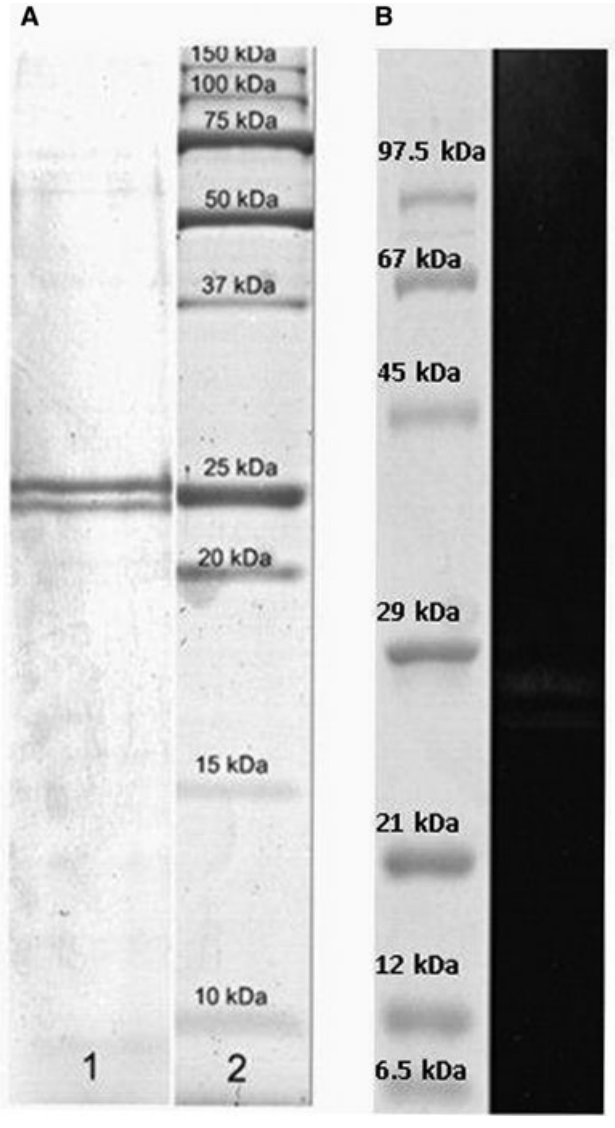

Figure 1. Electrophoretic analysis of the purified serine protease sample. (A) SDS-PAGE (on 12% polyacrylamide gel). Lane 1: protease sample, Lane 2: broad-range molecular weight standards. (B) Broad-range molecular weight standards (on 8% polyacrylamide gel); Zymogram (on 8% polyacrylamide gel co-polymerized with casein as substrate).

the enzyme was purified 83.9-fold with specific activity of 787.6 U/mg. Two intensive bands with molecular masses of 25 and 26 kDa were observed (Fig. 1). Both bands were identified by nLC-MS/MS analysis as *B. ignitus* serine protease (gi|94549034), the most abundant digestive protein present in the bumblebee midgut. We registered four spots with apparent molecular masses in the region of ca 24 kDa to ca 27 kDa, and a p*I* of ca 9–10 (Fig. 2). Spot no. 1 is large and it likely contains additional isomers, but due to the technical limits of 2-DE and the lack of the sample, the spot was excised and processed as one protein sample only. Database searching confirmed the numbered spots to be

Figure 2. Two-dimensional electrophoresis of the purified serine protease sample. The numbered spots (1–4) correspond to the serine protease isoforms as identified by nLC-MS/MS analysis.

Table 2a. Parameters of the Matched Peptides

Peptide	*m/z meas.*	*Δm/z [ppm]*	*z*	*MASCOT score (total ion score)*	*[ppm]*	*Calculated pI for peptide*	*Range*
1	492.2738	−1.85	2	63.3	2.54	9.76	35–42
2	554.5870	0.88	3	77.5	1.94	6.74	43–57
3	895.4732	0.78	2	97.8	1.77	6.91	58–73
4	773.3847	1.03	3	95.1	2.99	6.47	74–95
5	802.9545	0.64	2	122.3	2.42	5.84	101–115
6	685.9125	1.67	2	74.6	2.39	10.00	119–130
7	1,158.5333	1.43	2	116.0	1.15	5.99	131–152
8	966.0167	1.46	2	123.9	1.37	6.05	157–173
9	662.3224	1.02	2	84.1	2.04	8.18	181–191
10	516.5931	0.73	3	89.5	3.34	8.20	218–231
11	691.8514	0.39	2	102.8	2.75	5.81	232–243

identical with *B. ignitus* serine protease. Eleven unique peptides with MASCOT score higher than 60 were identified (Tables 2a, b). The peptide mass measurement accuracy was lower than ±1.5 ppm.

No specific peptides for *B. terrestris* serine protease were detected, because all peptides matched well to the sequence of *B. ignitus* serine protease covering more than 65% of their sequence (Fig. 3).

Enzyme activity increased with reaction temperature to an optimum at 35°C then decreased slowly at higher temperatures. Serine protease was stable up to 55°C and lost activity at 60°C. The *B. terrestris* serine proteases displayed a broad pH range of activity (from 6 to 9). The proteases were stable in a pH range of 6–10. We studied the effect of proteinase inhibitors phenylmethanesulfonyl fluoride (PMSF), trypsin inhibitor from

Table 2b. Parameters of the Matched Peptides

Peptide	*Sequence*	*Modification*[a]	*Spot no.*[b]
1	K.FPHQVSLR.Q		1,2,3
2	R.QNGNHFCGGSIIDSR.H	7	1,2
3	R.HILTAAHCVEGLNNLK.S	8	1,2
4	K.SITVHAGTNQLNTNGQSYGVEK.A		1,2
5	K.GFSSVTLVNDIALIR.V		1,2,3,4
6	K.NIAFNNLVKPIK.L		1,2,3
7	K.LATGSNNYEGSNCVLSGWGTTK. L	13	1,2,4
8	R.APNNLQYINLLVESQSK.C		1,2,3,4
9	R.VQSSHICTYTK.V	7	1,2
10	F.GQPCAVGKPDVYTR.V	4	1,2
11	R.VSSFVSWIDSQK.S		1,2,3

[a]Modification: carbamidomethyl.
[b]Peptide found in spot no.

1	MFTLSASFVL	CLAVAAFGFP	EGQIVGGKDA	PIGK**FPHQVS**	**LRQNGNHFCG**	50
51	**GSIIDSRHIL**	**TAAHCVEGLN**	**NLKSITVHAG**	**TNQLNTNGQS**	**YGVEK**AVAHK	100
101	**GFSSVTLVND**	**IALIR**VNKNI	**AFNNLVKPIK**	**LATGSNNYEG**	**SNCVLSGWGT**	150
151	**TK**LGGR**APNN**	**LQYINLLVES**	**QSK**CKQAHWR	**VQSSHICTYT**	**K**VGEGACHGD	200
201	SGGPLIHDDV	QIGVVSF**GQP**	**CAVGKPDVYT**	**RVSSFVSWID**	**SQK**SVSYERN	250

Figure 3. Sequence of the *B. ignitus* serine protease. The matched peptides found in this study in *B. terrestris* serine protease sample are in the bold. These peptides are identical with the peptides included in this sequence. The downward arrow indicates the sites of the serine protease peptide bonds trypsin cleavage observed in this study.

Table 3. Effect of Specific Inhibitors on Serine Protease Activity from Midgut of Male* Bombus terrestris. *The Values Are Means ± Standard Deviation of Three Separate Experiments

Inhibitor	*Inhibitor concentration*	*Relative activity (% of control)*
None	-	100 ± 1.3
PMSF	1 mM	100.1 ± 0.8
PMSF	5 mM	96.3 ± 1.9
PMSF	10 mM	90.7 ± 1.4
TPCK	1 mM	102.8 ± 2.1
TPCK	5 mM	86.2 ± 1.1
TPCK	10 mM	28.6 ± 0.9
SBTI	0.05%	16.6 ± 1.2
SBTI	0.5%	14.1 ± 0.8
SBTI	1.0%	9.5 ± 0.6

Glycine max (soybean, SBTI), and *N-p*-tosyl-*L*-phenylalanine chloromethyl ketone (TPCK) on the *B. terrestris* serine proteases activity. The serine proteases were strongly inhibited by SBTI, but not affected by PMSF inhibitor. TPCK which irreversibly inhibits the serine protease α-chymotrypsin, showed negligible inhibition (Table 3). The values of the kinetic parameters, *Km* and V_{max}, were 0.55 ± 0.042 mM and 0.714 ± 0.056 μmol *p*-nitroalanine

produced mg min^{-1} $protein^{-1}$, respectively. Concentration–velocity curve followed the Michaelis–Menten kinetic model.

DISCUSSION

The major digestive enzymes include serine proteases such as trypsins and chymotrypsins, cysteine proteases, elastase-like enzymes, and various carboxypeptidases or aminopeptidases (Hegedus et al., 2003; Prabhakar et al., 2007; Chougule et al., 2008). Serine proteases are typical for lepidopteran larvae (Chougule et al., 2008). The first study of the mechanism of enzyme secretion in Lepidoptera was done using larval *Bombyx mori* and indicated membrane-bound trypsin-like proteases. Some of the trypsin-like proteases are incorporated in the peritrophic membrane (Kuriyama and Eguchi, 1985; Terra and Fereira, 1994). On the contrary, we found the serine protease in midgut of 2-day-old *B. terrestris* males was extractable from midgut homogenate in buffer. Similar findings were reported by Choo et al. (2007), who described the cDNA cloning and characterization of midgut-specific serine protease of *B. ignitus*. The gene was specifically expressed in the midgut of *B. ignitus* queens, males, and workers, suggesting that the BiSP is a gut enzyme involved in the digestion of dietary proteins (Choo et al., 2007).

The termostability of the *B. terrestris* serine protease was comparable to results obtained with crab digestive lipase and pancreatic lipases for which the presence of the substrate increases the thermostability of the lipolytic enzymes (Cherif et al., 2007; Verger, 1984). Insect serine protease pH optima are always alkaline (most between 8 and 9), irrespective of the pH prevailing in midguts from which the enzymes were isolated (Reeck et al., 1999). Nevertheless, trypsins isolated from lepidopteran insects have higher pH optima, corresponding to the higher pH values found in their midguts (Sasaki and Suzuki, 1982). The proteolytic enzymes of *B. terrestris* were fully active at pH 9 as reported for chymotrypsin-like proteases from scallop (*Pecten maximus*; Le Chevalier et al., 110) and yellow mealworm, *Tenebrio monitor* (Elpidina et al., 2005). However, trypsin from the gut fluid of *B. mori* (Sasaki et al., 1993) had maximum activity about pH 11.

Our results show that bumblebee serine protease is actually a mixture of several isotypes with the same molecular mass, but different p*I*s. Only one isotype has a bit higher molecular mass. A couple of hypotheses may explain the presence of multiple spots identified as serine protease in the 2-DE gel (Fig. 2). The observed spots could be due to alternative splicing or to a combination of posttranslational modifications. Also, there may be small differences in amino acid composition between the serine proteases.

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