Characterization of the molecular features and expression patterns of two serine proteases in *Hermetia illucens* (Diptera: Stratiomyidae) larvae

Wontae Kim1,2, Sungwoo Bae1, Ayoung Kim1, Kwanho Park1, Sangbeom Lee1, Youngcheol Choi1, Sangmi Han1, Younghan Park3 & Youngho Koh2,*

1National Academy of Agricultural Science, Rural Development Administration, Suwon 441-707, 2Iłsong Institute of Life Science, Hallym University, 3Department of Obstetrics and Gynecology, Hallym University Sacred Heart Hospital, Anyang 431-060, Korea

To investigate the molecular scavenging capabilities of the larvae of *Hermetia illucens*, two serine proteases (SPs) were cloned and characterized. Multiple sequence alignments and phylogenetic tree analysis of the deduced amino acid sequences of Hi-SP1 and Hi-SP2 were suggested that Hi-SP1 may be a chymotrypsin- and Hi-SP2 may be a trypsin-like protease. Hi-SP1 and Hi-SP2 3-D homology models revealed that a catalytic triad, three disulfide bonds, and a substrate-binding pocket were highly conserved, as would be expected of a SP. *E. coli* expressed Hi-SP1 and Hi-SP2 showed chymotrypsin or trypsin activities, respectively. Hi-SP2 mRNAs were consistently expressed during larval development. In contrast, the expression of Hi-SP1 mRNA fluctuated between feeding and molting stages and disappeared at the pupal stages. These expression pattern differences suggest that Hi-SP1 may be a larval specific chymotrypsin-like protease involved with food digestion, while Hi-SP2 may be a trypsin-like protease with diverse functions at different stages. [BMB reports 2011; 44(6): 387-392]

INTRODUCTION

Polyphagous insects have the ability to synthesize several different kinds of proteolytic enzymes for digesting the diverse proteins obtained from a wide range of foods. Biochemical characteristics of proteolytic enzymes including carboxypeptidase A, carboxypeptidase B, aminopeptidase, and members of the serine protease family (including a trypsin-like enzyme, a chymotrypsin-like enzyme, and trypsin) have been widely studied (1). These major digestive enzymes play important roles in protein digestion and absorption (2). Much research has been focused on midgut enzymes of insects and, special attention has been directed toward trypsin and chymotrypsin, which belong to trypsin subfamily (3). Trypsin and chymotrypsin are the major proteases in most insect species, and these proteases take part in a number of physiological processes such as coagulation, immunity, fibrinolysis, embryonic development, and digestion (4). However, recent studies have reported that the mechanisms regulating expression and post-transcriptional modification of these digestive enzymes are quite variable among different insect species (4).

The black soldier fly (BSF), or *Hermetia illucens* L. (*H. illucens*), belongs to the polyphagous insect group. These flies can devour large amounts of garbage and food scraps in just a few hours, converting the waste into organic material that can be used as fertilizer (3). Despite many studies on the BSF, the biochemical and molecular features of the proteolytic enzymes of the BSF are unknown. Studies aimed at understanding the biochemical, cellular and molecular properties of their enzymes may reveal the basis of the superior scavenging abilities of the BSF. In this study, we cloned and characterized a trypsin- and a chymotrypsin-like protease from the BSF. We also examined the spatial and temporal expression patterns of these proteases during larval development.

RESULTS

cDNA cloning and sequence analysis of Hi-SP1 and Hi-SP2

The full length cDNA of *H. illucens* serine protease 1 (Hi-SP1) is 895 bp with an open reading frame (ORF) of 804 bp. The Hi-SP1 cDNA encodes a polyptide of 267 amino acids (aa), consisting of a 16 aa signal peptide and a trypsin domain (34-263 aa) (Fig. 1A). In contrast, the full length cDNA of Hi-SP2 is 863 bp with an ORF of 744 bp. The Hi-SP2 ORF encodes a 247 aa protein with a 16 aa signal peptide and a trypsin domain (25-243 aa) (Fig. 1A). In addition, both cDNAs had poly-adenylation tails.

Multiple alignments of deduced aa sequences of Hi-SP1 and Hi-SP2 revealed that Hi-SP1 had between 23% and 40%
Characteristics of serine proteases in the BSF
Wontae Kim, et al.

Fig. 1. Multiple sequence alignment and phylogenetic tree analysis of *Hermetia illucens*-SP1 and Hi-SP2 with chymotrypsins and trypsins of different insect species. (A) The GENBANK accession numbers for the sequences analyzed are *H. illucens* SP1 (HQ424574), *Aedes aegypti* Chy (AF487334.1), *Anopheles gambiae* Chy1 (Z18888.1), *A. gambiae* Chy2 (Z18888.1), *Drosophila melanogaster* Chy (AE014297.2), *Helicoverpa armigera* Chy (Y12287.1), *Lucilia cuprina* Chy (U03760.1), *Manduca sexta* Chy (Z18888.1), *D. melanogaster* Try (X190632.1), *C. pipiens pallens* Try (AY034060.1), *D. melanogaster* Try (X02989.1), *Closina moristan* Try (AF252869.1), *L. cuprina* Try3 (L155632.1) and *L. cuprina* Try4 (L155632.1). The abbreviations used are as follows: Chy: chymotrypsin; and Try: trypsin. (A) The identical residues are shadowed in black. Six conserved Cys residues are marked with circles. The catalytic triads are indicated with an asterisk and the substrate binding residues are marked with a black solid triangle on the top. Missing amino acid residues are indicated with a “-” in the alignment. (B) The numbers above the branches of the phylogenetic tree denote the bootstrap values as a percentage. The scale bar represents the number of substitutions per site.

Phylogenetic tree analysis with trypsin and chymotrypsin from several species were revealed that they were clustered into two major groups (Fig. 1B). Group 1 included two subgroups: 1) trypsins from the Diptera including Hi-SP2 and 2) chymotrypsins from Lepidoptera (Fig. 1B). Hi-SP1 was clustered together with group 2 including chymotrypsins from Lepidoptera (Fig. 1B). These multiple sequence alignment and phylogenetic analysis results suggest that Hi-SP1 and Hi-SP2 may be a chymotrypsin-like protease and a trypsin-like protease, respectively, in the BSF.

The 3-D structural features of chymotrypsin and trypsin were conserved in Hi-SP1 and Hi-SP2
Multiple alignments of aa sequences of Hi-SP1 and Hi-SP2, together with those of trypsins and chymotrypsins from Diptera and Lepidoptera, revealed that several important residues in a trypsin domain were completely conserved. We found that 6 cysteine (Cys) residues forming 3 disulfide bonds shared sequence identity compared to other chymotrypsins, and Hi-SP2 had between 37% and 52% shared sequence identity compared to other trypsins from various insect species (Fig. 1A).

Phylogenetic tree analysis with trypsin and chymotrypsin from several species were revealed that they were clustered into two major groups (Fig. 1B). Group 1 included two subgroups: 1) trypsins from the Diptera including Hi-SP2 and 2) chymotrypsins from Lepidoptera (Fig. 1B). Hi-SP1 was clustered together with group 2 including chymotrypsins from Lepidoptera (Fig. 1B). These multiple sequence alignment and phylogenetic analysis results suggest that Hi-SP1 and Hi-SP2 may be a chymotrypsin-like protease and a trypsin-like protease, respectively, in the BSF.

The 3-D structural features of chymotrypsin and trypsin were conserved in Hi-SP1 and Hi-SP2
Multiple alignments of aa sequences of Hi-SP1 and Hi-SP2, together with those of trypsins and chymotrypsins from Diptera and Lepidoptera, revealed that several important residues in a trypsin domain were completely conserved. We found that 6 cysteine (Cys) residues forming 3 disulfide bonds...
The residues of the Hi-SP1 substrate binding site were Ser213, Ser238 and glycine (Gly)240. In contrast, those residues of the Hi-SP2 substrate binding site were Asp198, Ser219 and Gly221.

Structural conservation was further confirmed by the 3-D structures of Hi-SP1 and Hi-SP2, obtained by 3-D homology modeling analysis. Similar to other 3-D structures of trypsin structures of Hi-SP1 and Hi-SP2, obtained by 3-D homology modeling analysis. Two α-helix structures were localized on the outskirts of the 3-D structures (Fig. 2A and B). Six Cys residues in Hi-SP1 and Hi-SP2 formed 3 disulfide bonds, as indicated by blue bars between two Cys residues (Fig. 2A and B). These disulfide bonds may stabilize 3-D structures of the proteases. Modeling substrate binding revealed the substrate binding sites of Hi-SP1 and Hi-SP2. In case of SP1, benzamidine, a reversible competitive inhibitor of trypsin, was placed within a substrate binding pocket and closely positioned with 3 substrate binding sites (Fig. 2C). A molecule of arginine (Arg), a substrate for trypsin-like protease activity, respectively, after IPTG induction (Fig. 3C and D). These results suggest that Hi-SP1 and Hi-SP2 may act as a chymotrypsin and a trypsin in the BSF larvae.

**Tissue-specific and developmental expression profiles of Hi-SP1 and Hi-SP2**

Because our results suggest that Hi-SP1 and Hi-SP2 may have different preferences for their substrates (Fig. 2 and 3), we examined whether there were differences in the spatial and temporal expression patterns of Hi-SP1 and Hi-SP2 in the BSF. To examine spatial expression patterns, the amounts of Hi-SP1 and Hi-SP2 transcripts in the various tissues of 5th instar larvae were measured by performing semi-quantitative RT-PCR (Fig. 3). The amount of Hi-SP2 transcripts in cDNAs was 3 fold greater than the amount of Hi-SP1 (Fig. 3A). While both Hi-SP1 and Hi-SP2 transcripts were detected strongly in the thorax, and midgut samples isolated from adult BSFs (data not shown).

To investigate temporal expression patterns of Hi-SP1 and Hi-SP2 transcripts, total RNA was isolated from the mid-gut of 4th, 5th, and 6th instar larvae with feeding or molting stages, Hi-SP2 transcripts, total RNA was isolated from the mid-gut of 4th, 5th, and 6th instar larvae with feeding or molting stages, Hi-SP1 and Hi-SP2 transcripts were detected strongly in the thorax, and midgut samples isolated from adult BSFs (data not shown).

Because our results suggest that Hi-SP1 and Hi-SP2 may have different preferences for their substrates (Fig. 2 and 3), we examined whether there were differences in the spatial and temporal expression patterns of Hi-SP1 and Hi-SP2 in the BSF. To examine spatial expression patterns, the amounts of Hi-SP1 and Hi-SP2 transcripts in the various tissues of 5th instar larvae were measured by performing semi-quantitative RT-PCR (Fig. 3). The amount of Hi-SP2 transcripts in cDNAs was 3 fold greater than the amount of Hi-SP1 (Fig. 3A). While both Hi-SP1 and Hi-SP2 transcripts were detected strongly in the thorax, and midgut samples isolated from adult BSFs (data not shown).

Because our results suggest that Hi-SP1 and Hi-SP2 may have different preferences for their substrates (Fig. 2 and 3), we examined whether there were differences in the spatial and temporal expression patterns of Hi-SP1 and Hi-SP2 in the BSF. To examine spatial expression patterns, the amounts of Hi-SP1 and Hi-SP2 transcripts in the various tissues of 5th instar larvae were measured by performing semi-quantitative RT-PCR (Fig. 3). The amount of Hi-SP2 transcripts in cDNAs was 3 fold greater than the amount of Hi-SP1 (Fig. 3A). While both Hi-SP1 and Hi-SP2 transcripts were detected strongly in the thorax, and midgut samples isolated from adult BSFs (data not shown).

Because our results suggest that Hi-SP1 and Hi-SP2 may have different preferences for their substrates (Fig. 2 and 3), we examined whether there were differences in the spatial and temporal expression patterns of Hi-SP1 and Hi-SP2 in the BSF. To examine spatial expression patterns, the amounts of Hi-SP1 and Hi-SP2 transcripts in the various tissues of 5th instar larvae were measured by performing semi-quantitative RT-PCR (Fig. 3). The amount of Hi-SP2 transcripts in cDNAs was 3 fold greater than the amount of Hi-SP1 (Fig. 3A). While both Hi-SP1 and Hi-SP2 transcripts were detected strongly in the thorax, and midgut samples isolated from adult BSFs (data not shown).

**Characteristics of serine proteases in the BSF**

Wontae Kim, et al.

---

**Fig. 3.** *E. coli* expressed mature polypeptide of Hi-SP1 and Hi-SP2 displays chymotrypsin- and trypsin-like protease activities. The mature polypeptides of Hi-SP1 (A) and Hi-SP2 (B) were expressed in *E. coli*. After IPTG induction, bands corresponding to Hi-SP1 and Hi-SP2 (each indicated by a black arrow) were significantly increased. The chymotrypsin-like protease activity of Hi-SP1 (C) and the trypsin-like protease activity of Hi-SP2 (D) were significantly greater than the protease activity of control *E. coli*. Statistical analysis consisted of performing a t-test using the SAS program. Asterisks indicate *P* < 0.05.
Fig. 4. Spatial and temporal expression patterns of Hi-SP1 and Hi-SP2. (A) The relative amounts of Hi-SP1 transcripts and Hi-SP2 transcripts were compared using cDNA from the total midgut sample of 5th instar larvae. Transcripts of Actin in both Hi-SP1 and Hi-SP2 cDNA samples were used as loading controls. (B) The spatial expression patterns of Hi-SP1 were quantitated. Hi-SP1 transcripts were detected only from cDNAs derived from the total gut sample and the total midgut sample. (C) Similarly, Hi-SP2 transcripts were only detected from cDNAs derived from the total gut sample and the total midgut sample. (D, E) The temporal expression patterns of Hi-SP1 (D) and Hi-SP2 (E) in the midgut of H. illucens are depicted. Statistical analysis consisted of using a t-test to generate P values. The abbreviations used are as follows: 4F: 4th instar feeding stage; 4M: 4th instar molting stage; 5F: 5th instar feeding stage; 5M: 5th instar molting stage; 6F: 6th instar feeding stage; 6M: 6th instar molting stage; PP: pre-pupal stage; P: pupal stage.

DISCUSSION

Compared to other insect scavengers or microorganisms, the superior capabilities of the BSF larvae for devouring garbage and food scraps and converting them into fertilizer, suggested the importance of investigating the molecular features of the SPs of the BSF. SPs are among the most abundant proteins in insects and are known to participate many important biological processes (7). We identified the full length cDNAs of two SPs, Hi-SP1 and Hi-SP2, from H. illucens total gut cDNA. The two cDNAs had an open reading frame encoding a signal peptide and a trypsin domain (Fig. 1) and a long polyadenylation tail. These features suggested that the cDNAs were generated from mature mRNAs that are actively translated into enzymes, which are secreted into the digestive tract or other tissues in the BSF. In addition, multiple sequence alignment and phylogenetic tree analysis of Hi-SP1 and Hi-SP2 with other SPs from other insect species suggested that Hi-SP1 may be a chymotrypsin-like protease while Hi-SP2 may be a trypsin-like protease (Fig. 1). Even though the Hi-SP1 and Hi-SP2 aa sequence identities were within 23% to 50% of other characterized insect trypsins and chymotrypsins, the important residues known to be critical for serine protease activity and the 3-D structures were highly conserved. Because experimental 3-D structures of the target proteins were not available, 3-D structures of trypsin and chymotrypsin were well documented (9). Trypsin is known to hydrolyze peptide chains primarily at the carboxyl side of a positively charged aa residue such as lysine (Lys) or Arg. In contrast, chymotrypsin preferentially digests peptide amide bonds before bulky hydrophobic aa residues, including tyrosine, tryptophan, phenylalanine, leucine, or methionine. The 3-D models of Hi-SP1 and Hi-SP2 may be very close to their physiological structures. The differences in the biochemical and structural features of trypsin and chymotrypsin are well documented (9). Trypsin is known to hydrolyze peptide chains primarily at the carboxyl side of a positively charged aa residue such as lysine (Lys) or Arg. In contrast, chymotrypsin preferentially digests peptide amide bonds before bulky hydrophobic aa residues, including tyrosine, tryptophan, phenylalanine, leucine, or methionine. The 3-D models of Hi-SP1 and Hi-SP2 may be very close to their physiological structures. The differences in the biochemical and structural features of trypsin and chymotrypsin are well documented (9). Trypsin is known to hydrolyze peptide chains primarily at the carboxyl side of a positively charged aa residue such as lysine (Lys) or Arg. In contrast, chymotrypsin preferentially digests peptide amide bonds before bulky hydrophobic aa residues, including tyrosine, tryptophan, phenylalanine, leucine, or methionine. The 3-D models of Hi-SP1 and Hi-SP2 may be very close to their physiological structures. The differences in the biochemical and structural features of trypsin and chymotrypsin are well documented (9). Trypsin is known to hydrolyze peptide chains primarily at the carboxyl side of a positively charged aa residue such as lysine (Lys) or Arg. In contrast, chymotrypsin preferentially digests peptide amide bonds before bulky hydrophobic aa residues, including tyrosine, tryptophan, phenylalanine, leucine, or methionine. The 3-D models of Hi-SP1 and Hi-SP2 may be very close to their physiological structures. The differences in the biochemical and structural features of trypsin and chymotrypsin are well documented (9). Trypsin is known to hydrolyze peptide chains primarily at the carboxyl side of a positively charged aa residue such as lysine (Lys) or Arg. In contrast, chymotrypsin preferentially digests peptide amide bonds before bulky hydrophobic aa residues, including tyrosine, tryptophan, phenylalanine, leucine, or methionine. The 3-D models of Hi-SP1 and Hi-SP2 may be very close to their physiological structures. The differences in the biochemical and structural features of trypsin and chymotrypsin are well documented (9). Trypsin is known to hydrolyze peptide chains primarily at the carboxyl side of a positively charged aa residue such as lysine (Lys) or Arg. In contrast, chymotrypsin preferentially digests peptide amide bonds before bulky hydrophobic aa residues, including tyrosine, tryptophan, phenylalanine, leucine, or methionine. The 3-D models of Hi-SP1 and Hi-SP2 may be very close to their physiological structures. The differences in the biochemical and structural features of trypsin and chymotrypsin are well documented (9). Trypsin is known to hydrolyze peptide chains primarily at the carboxyl side of a positively charged aa residue such as lysine (Lys) or Arg. In contrast, chymotrypsin preferentially digests peptide amide bonds before bulky hydrophobic aa residues, including tyrosine, tryptophan, phenylalanine, leucine, or methionine. The 3-D models of Hi-SP1 and Hi-SP2 may be very close to their physiological structures. The differences in the biochemical and structural features of trypsin and chymotrypsin are well documented (9). Trypsin is known to hydrolyze peptide chains primarily at the carboxyl side of a positively charged aa residue such as lysine (Lys) or Arg. In contrast, chymotrypsin preferentially digests peptide amide bonds before bulky hydrophobic aa residues, including tyrosine, tryptophan, phenylalanine, leucine, or methionine. The 3-D models of Hi-SP1 and Hi-SP2 may be very close to their physiological structures. The differences in the biochemical and structural features of trypsin and chymotrypsin are well documented (9). Trypsin is known to hydrolyze peptide chains primarily at the carboxyl side of a positively charged aa residue such as lysine (Lys) or Arg. In contrast, chymotrypsin preferentially digests peptide amide bonds before bulky hydrophobic aa residues, including tyrosine, tryptophan, phenylalanine, leucine, or methionine. The 3-D models of Hi-SP1 and Hi-SP2 may be very close to their physiological structures. The differences in the biochemical and structural features of trypsin and chymotrypsin are well documented (9). Trypsin is known to hydrolyze peptide chains primarily at the carboxyl side of a positively charged aa residue such as lysine (Lys) or Arg. In contrast, chymotrypsin preferentially digests peptide amide bonds before bulky hydrophobic aa residues, including tyrosine, tryptophan, phenylalanine, leucine, or methionine. The 3-D models of Hi-SP1 and Hi-SP2 may be very close to their physiological structures. The differences in the biochemical and structural features of trypsin and chymotrypsin are well documented (9). Trypsin is known to hydrolyze peptide chains primarily at the carboxyl side of a positively charged aa residue such as lysine (Lys) or Arg. In contrast, chymotrypsin preferentially digests peptide amide bonds before bulky hydrophobic aa residues, including tyrosine, tryptophan, phenylalanine, leucine, or methionine. The 3-D models of Hi-SP1 and Hi-SP2 may be very close to their physiological structures. The differences in the biochemical and structural features of trypsin and chymotrypsin are well documented (9). Trypsin is known to hydrolyze peptide chains primarily at the carboxyl side of a positively charged aa residue such as lysine (Lys) or Arg. In contrast, chymotrypsin preferentially digests peptide amide bonds before bulky hydrophobic aa residues, including tyrosine, tryptophan, phenylalanine, leucine, or methionine. The 3-D models of Hi-SP1 and Hi-SP2 may be very close to their physiological structures. The differences in the biochemical and structural features of trypsin and chymotrypsin are well documented (9). Trypsin is known to hydrolyze peptide chains primarily at the carboxyl side of a positively charged aa residue such as lysine (Lys) or Arg. In contrast, chymotrypsin preferentially digests peptide amide bonds before bulky hydrophobic aa residues, including tyrosine, tryptophan, phenylalanine, leucine, or methionine. The 3-D models of Hi-SP1 and Hi-SP2 may be very close to their physiological structures. The differences in the biochemical and structural features of trypsin and chymotrypsin are well documented (9). Trypsin is known to hydrolyze peptide chains primarily at the carboxyl side of a positively charged aa residue such as lysine (Lys) or Arg. In contrast, chymotrypsin preferentially digests peptide amide bonds before bulky hydrophobic aa residues, including tyrosine, tryptophan, phenylalanine, leucine, or methionine. The 3-D models of Hi-SP1 and Hi-SP2 may be very close to their physiological structures. The differences in the biochemical and structural features of trypsin and chymotrypsin are well documented (9).
peptides of Hi-SP1 and Hi-SP2 showed significantly increased chymotrypsin- and trypsin-like protease activities (Fig. 3). Collectively, our evidence strongly supports that Hi-SP1 is a chymotrypsin-like protease and Hi-SP2 is a trypsin-like protease in the BSF.

The physiological roles of Hi-SP1 and Hi-SP2 in the BSF were further examined by investigating the spatial and temporal expression patterns. Similar to proteases of Trichoplusia ni (10), Helicoverpa armigera (11), Stomoxys calcitrans (12), and Glossina morsitians morsitians (1), transcripts of Hi-SP1 and Hi-SP2 were specifically expressed in the midgut but not in the foregut, hindgut, salivary glands, malphigian tubules, muscle, fat body, or integument during the feeding stage (Fig. 4). These results suggest that one of major functions of Hi-SP1 and Hi-SP2 is digestion. In this study, we also found that the temporal expression patterns of Hi-SP1 were different from those of Hi-SP2. While high levels of Hi-SP2 transcripts were detected throughout development and decreased at the pupal stage, the expression of Hi-SP1 transcripts fluctuated between feeding and non-feeding larvae and decreased at the detection level in the pupae. These expression pattern differences and a 3.26 fold increase in Hi-SP2 levels compared to Hi-SP1 (Fig. 3A) suggest that Hi-SP2 may have additional functions. These additional functions may include activation of other serine proteases by digesting them. It is a well-known phenomenon that the activation of chymotrypsin requires trypsin digestion (13). In contrast, the roles of Hi-SP1 might be restricted to larval digestion, because Hi-SP1 expression levels significantly decreased during the molting stages and completely disappeared during pupal development. Furthermore, the transcripts of Hi-SP1 were not detected in adult tissues. Thus, it is possible that Hi-SP1 may be a larval specific gene, similar to chymotrypsins reported from the cotton bollworm (14) and the Hessian fly (15).

In conclusion, two SPs, Hi-SP1 and Hi-SP2, were cloned for the first time in this study. Several lines of evidence suggest that Hi-SP1 may be a larval specific chymotrypsin-like protease involved with digestion. We also have found evidence that Hi-SP2 may be a trypsin-like protease with several functions in larval, pupal, and adult stages in H. illucens, in addition to digestion.

MATERIALS AND METHODS

Insects
Hermetia illucens larvae were maintained at 30 ± 1°C, with a light: dark cycle of 14:10 and 60 ± 5% relative humidity, and reared on standard Drosophila media (16).

Cloning of full-length Hi-SP1 and Hi-SP2 cDNAs
Total RNA from 10 total gut samples of 5th instar H. illucens larvae were extracted with Trizol® (Invitrogen, Carlsbad, CA). After treating with DNase I, cDNA were synthesized from 2 μg of total RNA, using cDNA synthesis kits (Invitrogen). To amplify the partial cDNA fragments of the H. illucens SP1 (Hi-SP1) and the Hi-SP2, four degenerate primers (5’-gtntacngngcncaytg-3’ and 5’-amggncncnrwtncwrc-3’ for Hi-SP1 and 5’-ssaycargktcstcgtc-3’ and 5’-gccwrcgagtcw-cytrg-
ac-3’ for Hi-SP2) were used. Rapid amplification of cDNA ends (RACE) was carried out with the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA). Based on the partial cDNA fragments for Hi-SP1 and Hi-SP2, the 3’ ends of the Hi-SP1 and Hi-SP2 genes were obtained using gene specific primers (5’-gcttgccgctcacacaaagctcgg-3’ for Hi-SP1 and 5’-accctgccatgctgaaagc-3’ for Hi-SP2). The 5’ ends of the Hi-SP1 and Hi-SP2 genes were obtained using gene specific primers (5’-cagctgttggtggtcactcctccc-3’ for Hi-SP1 and 5’-gtctggtggtggtcactcctccc-3’ for Hi-SP2). All PCR reactions were performed with an AccuPower PCR premix (Bioneer, Daejeon, Republic of Korea). The nucleotide sequences of the two SPs were submitted to GenBank and have the following accession numbers: Hi-SP1: HQ424574; and Hi-SP2: HQ424575.

Bioinformatics analysis and phylogenetic tree analysis
Translation of the genes and prediction of the aa sequences were performed using the BioEdit program (http://www.mbio.ncsu.edu/ Bioedit/bioedit.html). The signal sequence and motifs were predicted by ScanProsite (http://www.u.expasy.org/prosite) and SMART (http://www.smart.embl-heidelberg.de). Multiple sequence alignments were performed with the ClustalW and DNAstar programs (http://www.dna star.com). The phylogenetic tree was produced using the neighbor-joining method in MEGA 4.1 (http://www.megasoftware.net).

3-D homology modeling of Hi-SP1 and Hi-SP2
Three-dimensional (3-D) homology models of Hi-SP1 and Hi-SP2 were generated using the Swiss Model workspace (http://swissmodel.expasy.org/workspace; (8, 17)). Hi-SP1 and Hi-SP2 3-D homology models were generated based on a 3-D structure of salmon trypsin (PDB ID = 1HBJ) containing 36.3% aa sequence identity with SP1 and a 3-D structure of Fusarium oxysporum Trypsin (PDB ID = 1PQ7; (6)) containing 49.1% aa sequence identity with SP2, respectively. The high aa sequence identities between Hi-SP1 and Hi-SP2 and their PDB templates suggested that the 3-D structures generated in this study are consistent with those results obtained from experiments.

Assay of enzymatic activity
To characterize the protease activities of Hi-SP1 and Hi-SP2, the DNA sequences of the mature polyepitides were cloned individually into the E. coli (BL21-DE3) expression vector, pET41a (Merck, Darmstadt, Germany). To induce expression of Hi-SP1 and Hi-SP2, 1 M isopropyl-D-thiogalactoside (IPTG) was added, after which E. coli were harvested every hour. Protein extracts from E. coli expressing Hi-SP1 were incubated with N-succinyl-alaa-alaa-pro-phe-p-nitroanilide (AAPF),
a substrate for chymotrypsin-like protease, to assay Hi-SP1 enzymatic activity. Protein extracts from E. coli expressing Hi-SP2 were incubated with N-α-benzoyl-L-Arg-p-nitroanilide (L-BAPNA), a substrate for trypsin-like protease, to assay Hi-SP2 enzymatic activity. Enzymatic activity was determined using the protocol with a modification (18). Briefly, a solution of 180 μl of 0.05M phosphate buffer (pH 7.2) and 10 μl of each substrate was pre-incubated at 39°C for 10 min. After pre-incubation, 10 μl of enzyme was added to start the reaction. The reaction was incubated at 37°C for 20 min, and the absorbance of the reaction solution was monitored at 410 nm for chymotrypsin-like protease activity and at 405 nm for trypsin-like protease activity.

**Semi-quantitative RT-PCR**

Total RNA from 10 salivary glands, 1 total gut, 10 malphigian tubules, 1 sample of muscle including fat body and integument, 10 foreguts, 1 midgut, and 5 hindguts of 5th instar BSF larvae and midguts at feeding and molting stages was extracted using the Trizol® (Invitrogen). After treatment with DNase I, cDNA were synthesized from 2 μg of total RNA using cDNA synthesis kits (Invitrogen). The cDNA were used as a template in PCR reactions with gene specific primers: 5’-atgagacctgcaattttttgg-3’ and 5’-aatcctctccttaatccaaat-3’ for Hi-SP1 and 5’-atgctccctcttgaatctc3’ and 5’-tgtgttctccttaaccacag-3’ for Hi-SP2. An actin gene fragment was also amplified as control, with the primers ActinF (5´-aaggactcgtacgtgggtg-3´) and ActinR (5´-catctctccttaatccaaat-3´). Both strands of the amplified PCR fragments were sequenced to confirm the specificity of the primers. The experiments were repeated three times with three independent cDNA samples. Statistical analysis was completed using a t-test to compute P values in the SAS program.

**Acknowledgements**

This work was supported by a grant from the National Academy of Agricultural Science, Rural Development Administration, Republic of Korea (PJ006893202010).

**REFERENCES**


