Molecular Cloning, Gene Structure, Expression, and Enzyme Activity of a Serine Protease from Water Scorpion, *Laccotrephes japonensis* (Hemiptera; Nepidae)

Kwan Ho Park¹, Young Cheol Choi¹, Seong Hee Nam¹, Jae Sam Hwang¹, and Si Kab Nho^{2,*}

¹National Academy of Agricultural Science (NAAS), RDA, Suwon 441-707, Republic of Korea

²College of Agriculture and Life science, Kyungpook National University, Daegu 702-701, Republic of Korea

(Received 31 October 2012; Accepted 3 January 2013)

Serine proteases are major insect enzymes involved in the digestion of dietary proteins and in the process of blood meal digestion. In this study, cDNA was constructed using the whole body of *Laccotrephes japonensis*. The flanking sequences of the 5- and 3- end of this gene were characterized by RACE-PCR. Sequence analysis showed that this gene contained a 963-bp ORF encoding 320 amino acids. The deduced amino acid sequence showed 62% identity with the *Creontiades dilutus* serine protease, 58% with the *Lygus lineolaris* trypsin precursor, and 54% with the *Triatoma infestans* salivary trypsin. To assess the expression of the *L. japonensis* serine protease (JGsp), the JGsp gene was cloned into a baculovirus transfer vector, pBac-1, and expressed in Sf9 cells (*Spodoptera frugiperda*). SDS-PAGE and western blot analysis have shown that the JGsp recombinant protein was a monomer with a molecular weight of about 32 kDa. Recombinant JGsp has shown activity in the protease enzyme assay using gelatin as a substrate.

Key words: *Laccotrephes japonensis*, Serine proteases, Protease enzyme assay

Introduction

Proteases play two critical roles in an insect's physiology-

breaking down proteins into amino acids (aa) essential for growth, development, and inactivation of protein toxins ingested as a consequence of feeding (Terra *et al.*, 1996). Serine proteases belong to a diverse group of enzymes capable of cleaving peptide bonds and are involved in various essential processes such as intra and extra-cellular protein metabolism, blood coagulation, immune response, fertilization and developmental regulation, digestion, etc. (Barrett and Rawlings, 1995; Elvin *et al.*, 1993; Nagano *et al.*, 2003; Rao *et al.*, 1998). These enzymes have been detected in several insect orders, including Coleoptera (Zhu and Baker, 1999), Diptera (Jiang *et al.*, 1997) and Lepidoptera (Gatehouse *et al.*, 1999), and are one of the major classes of endopeptidases, strongly implicated in the digestion of proteins in the gut (Terra and Ferreira, 1994). The bugs of Nepoidea, including Belostomatidae and Nepidae, prey upon a variety of aquatic animals such as insects, snails, reptiles, anurans, and small fish in Japanese rice fields (Mori and Ohba, 2004; Ohba and Nakasuji, 2006). In addition, the *L. japonensis* adults prey upon *Kirkaldyia deyrolli* younger nymph (Ohba, 2007) and tadpole in rice fields (Ohba and Nakasuji, 2006). Under such a situation, adult *L. japonensis*, younger nymph of *Kirkaldyia deyrolli*, and anuran larva (tadpole) are regarded as intraguild predator, intraguild prey, and common prey, respectively (Ohba and Nakasuji, 2007). Predators such as *Zelus renardii* (Reduviidae) have high levels of proteolytic activity in the salivary glands as well as in the entire midgut (Cohen, 1993). Salivary protease activity was detected in the mirids *Lygus rugulipennis* (Miridae) (Laurema *et al.*, 1985) and *Creontiades dilutus* (Miridae) (Colebatch *et al.*, 2001). In *Zelus renardii* and both *Lygus hesperus* and *Lygus lineolaris* (Miridae), salivary gland protease activity is predominantly trypsin-like (Cohen, 1993; Agusti and Cohen, 2000), but in *Creontiades dilutus*, chymotrypsin-like pro-

*To whom the correspondence addressed
College of Agriculture and Life sciences, Kyungpook National University, Daegu 702-701, Republic of Korea
Tel: +82-53-950-5743; Fax: +82-53-950-5743;
E-mail: nohsk@knu.ac.kr
<http://dx.doi.org/10.7852/ijie.2012.25.2.187>

tease activity appears to predominate in the salivary glands (Colebatch *et al.*, 2002). Heteroptera midgut proteases are predominantly acidic proteases from both the cysteine and aspartic mechanistic classes (Houseman, 1978; Houseman and Downe, 1981, 1983; Overney *et al.*, 1998).

There have been no reports of cloned genes that encode proteases in *L. japonensis*. *L. japonensis* is considered to be a predator of aquatic animals. We suggest that *L. japonensis* has strong digestive enzymes because of its wide feeding habit. *L. japonensis* ingests insects or small fishes in a short time.

In this study, the serine protease gene in *L. japonensis* was cloned and characterized. The *L. japonensis* serine protease was also expressed in baculovirus-infected insect cells, and the recombinant serine protease was assayed for enzyme activity.

Materials and Methods

Insects and cell culture

L. japonensis was collected from an aquatic area in Kyungpook Province. Samples were stored at -80°C until further use or used directly for RNA extraction. Sf9 cells were maintained at 27°C in TC-100 medium (Gibco BRL, USA), supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL, USA) as described by standard methods (O'Reilly *et al.*, 1992).

RNA extraction and RT-PCR

Samples were homogenized in Trizol reagent. Total RNA was subsequently isolated according to the protocol described by the manufacturer (Invitrogen, USA). Next, 5 µg total RNA was reverse transcribed into cDNA using an oligo-dT primer and SuperScriptTMII reverse transcriptase (Invitrogen, USA).

Cloning of putative JGsp partial cDNA

The primers for the serine protease gene isolation of *L. japonensis* were designed using a highly conserved region of serine protease genes from closely related species. Two forward and one reverse primer (F1: 5-GTCATCACTGCT-GCTCACTGC-3, F2: 5-AACGACATTTCATCCTC-3, R: 5-GACTGGGCCACCGGAATCTCCCTGGC-3) were designed and PCR was performed with 2 pairs of primers (F1-R, F2-R). PCR was performed in 3 stages; denaturing at 94°C for 5 min, followed by 35 amplification cycles, i.e., 94°C for 2 min, 55°C for 30 sec, and 72°C for 30 sec, and a final extension at 72°C for 10 min. The PCR product was purified from 1% agarose gel using a Gel Extraction kit (Qiagen, USA), cloned into the pGEM-T easy vector (Promega, USA), and sequenced by Corebio Co. Ltd (Seoul, Korea).

RACE-PCR

To obtain the complete cDNA sequences, the 5' and 3' ends were amplified using the Ambion FirstChoice[®] RLM-RACE kit (Ambion, USA) according to the manufacturer's instructions and specific primers. The amplified cDNAs were cloned into the pGEM-T easy vector and recombinant clones were sequenced in both strands by Corebio Co. Ltd. Analysis of signal peptide sequences, including cleavage site localization, was carried out using SMART software at servers of the sequence analysis (<http://smart.embl-heidelberg.de/>).

Construction of recombinant virus

The full length open reading frame (ORF) of JGsp was amplified from cDNA by PCR using a forward primer adding a *Bam*HI restriction site (5-GGATCCTTGATGTTG-GAAGACTTCAT-3), which was used to clone a gene encoding a N-terminal signal peptide deleted, and a reverse primer adding a *Hind*III restriction site (5-AAGCT-TCTCGTGGCAAGTCTTGCT-3), by which the stop codon TAG was mutated to GAG. PCR amplification was carried out under the following conditions: 94°C for 5 min, 35 cycles of 94°C for 2 min, 60°C for 30 sec, 72°C for 30 sec, followed by 72°C for 10 min. The resulting fragment was cloned in the pGEM-T vector and sequenced. Plasmid DNA from the clone was digested with *Bam*HI and *Hind*III. The fragment containing the full JGsp ORF was inserted into same sites of the transfer vector (pBAC1, Novagen, Germany) to express JGsp under the control of the AcNPV polyhedrin promoter. A baculovirus expression vector system, using the AcNPV vector and the insect cell line Sf9, was employed for the production of recombinant pBAC1-JGsp protein. Then, 5 µg of the construct (pBAC1-JGsp) and 2.5 µg of the BacVector-3000 virus DNA (Novagen, USA) were co-transfected into 1.0–1.5 × 10⁶ Sf9 cells for 3 hr using the Tfx-20 reagent (Promega, USA). The transfected cells were cultivated in TC-100 medium (Gibco BRL, USA) at 27°C for 6 days. Six days post-infection, the supernatant was harvested and clarified by centrifugation at 3,000 rpm for 5 min. The supernatant was stored at 4°C until post-infection of the cells.

SDS-PAGE and western blot analysis

For the protein production of recombinant JGsp in baculovirus-infected cells, cells were grown in their respective culture media at 27°C. Then, cells were infected with pBAC1-JGsp virus stocks. After 3 days of infection, cells were harvested by centrifugation for 5 min at 3,000 rpm. The collected cells were washed with an equal culture volume of PBS, gently resuspended, and centrifuged at 3,000 rpm for 5 min. The washed cell pellets were disrupted by suspension and incubation in 150 µL I-PER

Fig. 1. Nucleotide sequence and predicted amino acid sequence of the *JGsp* gene. The stop codon (TAG) is shown. An AATAAA sequence matching the consensus polyadenylation signal in other species is boxed. Putative signal peptides are underlined. ★: Cystein residues of insects' serine protease, ☆: Active peptide site in the N-terminal region, ■: Invariant serine protease residues, △: Serine protease catalytic triad.

insect cell lysis buffer (Pierce, USA) for 10 min on ice and centrifuged at 15,000 rpm for 15 min at 4°C. For SDS-PAGE, the cells were washed twice with PBS (140 mM NaCl, 27 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4), protein sample buffer was added, and the samples were boiled for 5 min at 100°C. The total cellular lysates were subjected to 12% SDS-PAGE. After electrophoresis, gels were fixed and stained with 0.1% Coomassie brilliant blue staining solution. Following electrophoresis, the proteins were transferred to nitrocellulose transfer membranes (Schleicher & Schuell, Germany). After blotting, the membrane was blocked by incubation in 5% skim milk blocking solution, incubated with His-antibody (1:4,000 v/v) at room temperature for 1 hr, and washed in TBS-T (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% [v/v] Tween-20). The membrane was conjugated with anti-mouse 2nd antibody. After repeated washing, band visualization was achieved via chemiluminescence using the ECL reagent (Pierce Biotechnology, USA) on radiographic films.

Determination of protease activity

Samples were subjected to SDS-PAGE under non-reducing conditions using 10% polyacrylamide gels impregnated with gelatin (Sigma, USA). The samples were mixed with SDS sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM β-mercaptoethanol) and loaded onto the gel without heating and electrophoresis was performed for

3 hr at a constant current of 100 V on ice. Following electrophoresis, the gel was washed twice with 2.5% Triton X-100 and then incubated in zymogram reaction buffer (1 M Tris-HCl, pH 7.5, 1 M CaCl₂, 5 M NaCl, 2.5% Triton X-100, 0.2% NaN₃) at 37°C for 16 hr. Finally, the gel was stained with 0.1% Coomassie brilliant blue staining solution and destained in destaining solution containing 10% methanol and 5% acetic acid. Lytic bands visualized by placing the gels on a view box were then photographed.

Results

Gene cloning and sequence

To identify the coding gene of the serine protease for *L. japonensis*, we designed degenerate primers from several species related to serine proteases and trypsin-like serine proteases. *Bombyx mori* (silkworm), *Pacifastacus leniusculus* (signal crayfish), *Panulirus argus* (Caribbean spiny lobster), *Creontiades dilutus* (green mirid), *Anthonomus grandis* (boll weevil), and *Aedes aegypti* (yellow fever mosquito) serine proteases and trypsin-like serine proteases were used for primers design. An approximately 450-bp PCR product was obtained from a pair of F1 and R primers.

Characterization of the full-length *JGsp* gene

To isolate the full-length cDNA of *JGsp* from *L. japonensis*, we performed rapid amplification of cDNA ends by PCR using gene-specific primers anchored in the partial

```

Trib CDCGKKKTRIIGCHETGNEYFSMAAVDRWT----FDAPCCASIIISDRYALTAACHLLHKT--PDDFAILLVGDHMTS
Ost  CSCGYKKTNRIVGQQTGWIIEFEMAGLAHKDI----AQIKCCAVIISKRYMTAAHCLTGQS--LSNLAIVGCHVTV
JGsp -----RIVGSEETLTHEYEMAGVGVDVFTKGVSVICGGTIIVTIRHULTASHCTYGFN---EQFAGIVGDHLLFD
CreSP -----RIVGCTYYKANEYPIVGIAT--VGARGYAPFCGSGIITANH/ITAAHCTDDIIKARTRTAVLLGSHRRSR
Lygus -----RIVGSEETKWEYEMAGLIFYT----PRNVIECGGTIVTRWHVITAACHVPEVLHVPEDVQIVLGEHQS

```

```

Trib GDDTPAAVTKISNMFSPSTDQSTQLNDIAVLQTEKPEESLFGVEVCLFERYTSVNFLSQTVTAALGNGFVDVAGPKSD
Ost  G-DSPAQCEQVISAIIHPNTPSNYDYDIALKTNADITFSDRVGEVCLFEPKFNVDFTGSKLTIILGCTQFPGGPTSN
JGsp VRDTSYTR-VHNVSVTNHPGYNPTMSNDITLITHTPIEPNOKVGBACLESRLDITG--EWWKVVEGCHRRWKCQLSP
CreSP PSSDAV---INVERINQHEKYNANTIANDISILTLASSINENKLGVEVCLFPLGLDVSG--QTVRVLGMCAERFQSGAMTM
Lygus VDESPYTKVYRKEMVNHDPDELVGHHRNDIAVILSETREFENDYVGEACMFTGAETVVG--SKLRVLGMCKLSVDGPASK

```

```

Trib THQEVDTTVSTEEENATITDNP-----VTYRQICTWAPNRDACQSDSGGPIITQCPNTRRLQLLGLISMIGCA-TSRF
Ost  YHQRVDVDVISQTSRNVVPT-----LTARQICTYTEGKDACQSDSGGPIITQSSNGLLYSHGIVSNRFGAGANQP
JGsp RHNVEFLKAINVRSCARKIRQMLTVDITSTQECTNTRKDSQCGSDSGGPIITLPEPTNRYTLMGVSVSGECA-SEFP
CreSP RPKRLDTTAVSPAQCAAIWRGL--VSATNPQVCTLSKKETACQSDSGGPIVVRPQPTNRYTLIGLYSGCACT-DEKP
Lygus VMKRVNINWPIEQAKVYDRVN----TTEAKQVCTHHRKNDACQSDSGGPIITLRETNRYTLMAAVSHERGCA-BGHE

```

```

Trib AVNTRVTSYTRIVSVTEDAFYCIK-----
Ost  GVNTRVPALLSPICNTPTDASYCYK-----
JGsp GVNTHVGATLSNIEQIRRVGG-SKTC---
CreSP TVNTRVAAYLEPIKQIAATKP-AGVCTKA-
Lygus AVNTHIYVRDTEICKIIRETKPEGRICKQG

```

Fig. 2. Multiple alignment of the predicted partial amino acid sequence of the putative serine protease of *Laccotrephes japonensis*. Trib: *Tribolium castaneum* trypsin-like serine protease, Ost: *Ostrinia nubilalis* trypsin-like protease, JGsp: *Laccotrephes japonensis* serine putative protease, CreSP: *Creontiades dilutus* serine protease, Lygus: *Lygus lineolaris* trypsin precursor.

sequence obtained via reverse transcription PCR (RT-PCR) with degenerate primers. From the RACE-PCR products, the sequences were aligned and assembled to obtain a final sequence of 1,074 bp. It contains a complete single ORF of a length of 963 bp and encoding 320 aa residues. A putative polyadenylation signal, AATAAA, is located at nucleotide positions 1,044–1,049 and the N-terminal region predicted the presence of a 31-aa signal peptide. Most serine proteases have a signal peptide for secretion, which is cleaved from the mature protein by a signal peptidase. In addition, this gene has cysteine residues that were conserved in all insect serine proteases and which were located at aa positions 102, 118, 185, 229, 249, 258, 288, and 299. These cysteine residues have an important role in JGsp that was predicted to occur in disulfide bridge configurations. The serine protease catalytic triad of histidine, aspartic acid, and serine was shown at aa positions 118, 165 and 262. In addition, the activation peptide cleavage site was shown (Fig. 1). The overall deduced aa sequence of JGsp shows an overall homology with previously reported digestive protease genes; it showed 62% identity to the serine protease of *Creontiades dilutus* (green mirid), 58% to that of the *Lygus lineolaris* (tarnished plant bug) trypsin precursor LlsGP4, and 54% to the *Triatoma infestans* (assassin bug) salivary trypsin (Fig. 2).

To reveal the relationship of the *L. japonensis* serine protease with other insect serine proteases, a phylogenetic tree was constructed with serine proteases of closely related insects. Phylogenetic analysis indicated that the closest species are *Creontiades dilutus* and *Latrodectus hesperus*. This result revealed that JGsp belongs to the same clade as other *Hemipteran* insect's serine proteases (data not shown).

Fig. 3. SDS-PAGE analysis of recombinant JGsp in Sf9 cells. Sf9 cells were infected with wild-type AcNPV (wt-AcNPV), AcNPV-JGsp (pBAC1-JGsp) and mock control. Cells were collected at 3 days of post-infection. Total cellular lysates were subjected to 12% SDS-PAGE.

Expression of recombinant JGsp protein

The baculovirus transfer vector pBAC1 was used to generate a recombinant JGsp. The pBAC1-JGsp vector was constructed by inserting the signal peptide-deleted JGsp ORF under the control of AcNPV polyhedrin promoter in

Fig. 4. Western blot analysis of recombinant JGsp in Sf9 cells. Sf9 cells were infected with wild-type AcNPV (wt-AcNPV), AcNPV-JGsp (pBAC1-JGsp) and mock control. Cells were collected at 3 days of post-infection. Total cellular lysates were subjected to 12% SDS-PAGE.

Fig. 5. Zymography of recombinant JGsp in Sf9 cells. The cellular lysates were subjected to SDS-PAGE on a 10% gel containing 0.1% gelatin.

the pBAC1 vector. Recombinant AcNPV, pBAC1-JGsp, was replicated in Sf9 cells. Gene expression of the recombinant JGsp was examined by PCR analysis from the recombinant virus. As is shown in SDS-PAGE, the putative JGsp protein was expressed with a size of 32 kDa

(Fig. 3). These results were obtained by western blot analysis. Recombinant JGsp expression was detected only in cells infected with the recombinant virus, but not in cells infected with the wild-type AcNPV vector or in mock-infected cells (Fig. 4).

To identify the protease activity of recombinant JGsp, 0.1%-gelatin zymography was performed. In the SDS-PAGE containing 0.1% gelatin, the protein band with proteolytic activity was directly detected as lytic zones. On a gelatin-containing zymogram, a clear area appears when the gelatin is digested by proteases, whereas undigested gelatin is stained blue. The proteases were visualized as clear bands on a blue background (Fig. 5). The enzyme activity led to gelatin digestion and was detectable at a molecular mass of approximately 32 kDa.

Discussion

Proteolytic enzymes have a variety of functions essential for all living organisms. They are currently classified into four groups according to the catalytic mechanism: serine, cysteine, aspartic, and metalloproteases (Barrett *et al.*, 1998). Serine proteases are major insect gut enzymes involved in the digestion of dietary proteins; in general, serine proteases are expressed in the gut of insects (Barrett *et al.*, 2003; Gorman *et al.*, 2000; Lehane *et al.*, 1998; Paskewitz and Gorman, 1999; Yan *et al.*, 2001). However, little genetic information is available for serine proteases from insects and limited species comparisons are available within the insect serine proteases (Kim *et al.*, 2009).

In this report, we describe the cloning, expression and characterization of an *L. japonensis* serine protease. The deduced sequence showed that this gene contained a 963-bp ORF and encodes 320 aa. The deduced aa sequence showed 62% identity with the serine protease of *Creontiades dilutes*, 58% with that of the *Lygus lineolaris* trypsin precursor, and 54% with the *Triatoma infestans* salivary trypsin (Fig. 2). In the comparison of the serine protease family, it shows several typical structural features of serine proteases (Kraut, 1977). The product of this gene has well-conserved aa residues in the serine protease active site, as well as in the signal peptide. Most insect serine protease have a signal peptide for secretion, which is cleaved from the mature protein by a signal peptidase. The result showed that the signal peptide of the JGsp gene functions as secretion signal, presumably.

In addition, recombinant JGsp was expressed using the baculovirus expression vector system (BEVS) and revealed the protease activity of recombinant JGsp. Recent studies have also shown the functional expression of midgut serine protease using the baculovirus system

(Herrero *et al.*, 2005). The result shows that the *JGsp* gene is functionally expressed as 32 kDa-polypeptide in baculovirus-infected insect cells. Therefore, the system used here may well be applicable to other hemiptera insect serine proteases, though it still may prove difficult to express them without causing damage to the expression system. However, in the case of recombinant *JGsp*, no indications of such damage were observed (data not shown). Although the specific role of *JGsp* remains unclear, the functional expression of *JGsp* in the baculovirus-insect cell system opens a wide range of possibilities for the study of insect serine proteases.

In conclusion, the cloning and expression of a *L. japonensis* serine protease is reported in this study. The result showed that *JGsp* is functionally expressed in baculovirus-infected insect cells and has proteolytic activity. These results may provide information for manipulating the protease as a potential control agent for industrial or medical uses.

Acknowledgements

This work supported by a grant from the National Academy of Agricultural Science, Rural Development Administration, Republic of Korea (PJ0068932012) and Kyungpook National University Research Fund, 1210.

References

- Agusti N, Cohen AC (2000) *Lygus hesperus* and *L. lineolaris* (Hemiptera: Miridae), phytophages, zoophages, or omnivores: Evidence of feeding adaptations suggested by the salivary and midgut digestive enzymes. *J Entomol Sci* 35, 176-186.
- Barrett AJ, Rawlings ND (1995) Families and clans of serine peptidases. *Arch Biochem Biophys* 318, 247-250.
- Barrett AJ, Rawlings ND, Woessner JF (1998) *Handbook of Proteolytic Enzymes*. Academic Press, New York.
- Barrett AJ, Rawlings ND, Woessner JF (2003) *Handbook of Proteolytic Enzymes*. Academic Press, New York.
- Cohen AC (1993) Organization of digestion and preliminary characterization of salivary trypsin-like enzymes in a predaceous Heteropteran, *Zelus Renardii*. *J Insect Physiol* 39, 823-829.
- Colebatch G, East P, Cooper P (2001) Preliminary characterisation of digestive proteases of the green mirid, *Creontiades dilutus* (Hemiptera : Miridae). *Insect Biochem Mol Biol* 31, 415-423.
- Colebatch G, Cooper P, East P (2002) cDNA cloning of a salivary chymotrypsin-like protease and the identification of six additional cDNAs encoding putative digestive proteases from the green mirid, *Creontiades dilutus* (Hemiptera: Miridae). *Insect Biochem Mol Biol* 32, 1065-1075.
- Eldridge R, O'Reilly DR, Miller LK (1992) Efficacy of a baculovirus pesticide expressing an eclosion hormone gene. *Biol Control* 2, 104-110.
- Elvin CM, Whan V, Riddles PW (1993) A family of serine-protease genes expressed in adult buffalo fly (*Haematobia irritans-exigua*). *Mol Gen Genet* 240, 132-139.
- Gatehouse AMR, Norton E, Davison GM, Babbe SM, Newell CA, Gatehouse JA (1999) Digestive proteolytic activity in larvae of tomato moth, *Lacanobia oleracea*; effects of plant protease inhibitors in vitro and in vivo. *J Insect Physiol* 45, 545-558.
- Gorman MJ, Andreeva OV, Paskewitz SM (2000) Sp22D: a multidomain serine protease with a putative role in insect immunity. *Gene* 251, 9-17.
- Herrero S, Combes E, Van Oers MM, Vlak JM, de Maagd RA, Beekwilder J (2005) Identification and recombinant expression of a novel chymotrypsin from *Spodoptera exigua*. *Insect Biochem Mol Biol* 35, 1073-1082.
- Houseman J (1978) A thiol-activated digestive proteinase from adults of *Rhodinus prolixus* Stal (Hemiptera: Reduviidae). *Canadian J Zoology* 56, 1140-1143.
- Houseman JG, Downe AER (1981) Identification and partial characterisation of digestive proteinases from *Triatoma pallidipennis* Stal (Hemiptera: Reduviidae). *Comp Biochem Physiol* 70B, 713-717.
- Houseman JG, Downe AER (1983) Cathepsin D-like activity in the posterior midgut of hemipteran insects. *Comp Biochem Physiol* 75B, 509-512.
- Jiang QJ, Hall M, Noriega FG, Wells M (1997) cDNA cloning and pattern of expression of an adult, female-specific chymotrypsin from *Aedes aegypti* midgut. *Insect Biochem Mol Biol* 27, 283-289.
- Kim SY, Jeong EJ, Song KJ, Park KS (2009) Molecular cloning and characterization of a serine protease-like protein from silkworm (*Bombyx mori*). *Genes Genome* 31, 387-395.
- Kraut J (1977) Serine proteases: structure and mechanism of catalysis. *Annu Rev Biochem* 331-358.
- Laurema S, Varis AL, Miettinen H (1985) Studies on enzymes in the salivary glands of *Lygus rugulipennis* (Hemiptera, Miridae). *Insect Biochem* 15, 211-224.
- Lehane SM, Assinder SJ, Lehane MJ (1998) Cloning, sequencing, temporal expression and tissue-specificity of two serine proteases from the midgut of the blood-feeding fly *Stomoxys calcitrans*. *Eur J Biochem* 254, 290-296.
- Mori A, Ohba S (2004) Field observations of predation on snakes by the giant water bug. *Bull Herpetol Soc* 2004, 78-81.
- Nagano I, Wu ZL, Nakada T, Boonmars T, Takahashi Y (2003) Molecular cloning and characterization of a serine proteinase gene of *Trichinella spiralis*. *J Parasitol* 89, 92-98.
- Ohba S (2007) Notes on predators and their effect on the survivorship of the endangered giant water bug, *Kirkaldyia deyrolli* (Heteroptera : Belostomatidae), in Japanese rice fields.

- Hydrobiologia 583, 377-381.
- Ohba S, Nakasuji F (2006) Dietary items of predacious aquatic bugs (Nepoidea : Heteroptera) in Japanese wetlands. Limnology 7, 41-43.
- Ohba S, Nakasuji F (2007) Density-mediated indirect effects of a common prey tadpole on interaction between two predatory bugs: *Kirkaldyia deyrolli* and *Laccotrephes japonensis*. Popul Ecol 49, 331-336.
- Overney S, Yelle S, Cloutier C (1998) Occurrence of digestive cysteine proteases in *Perillus bioculatus*, a natural predator of the Colorado potato beetle. Comp Biochem Physiol B 120, 191-196.
- Paskewitz SM, Gorman MJ (1999) Mosquito immunity and malaria parasites. Am Entomol 80-94.
- Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol R 62, 597.
- Terra WR, Ferreira C (1994) Insect Digestive Enzymes - Properties, Compartmentalization and Function. Comp Biochem Physiol B 109, 1-62.
- Terra WR, Ferreira C, Jordao BP, Dillon, R.J. (1996) Digestive enzymes. In: Lehane, MJ, Billingsley, PF (Eds.), Biology of the Insect Midgut Chapman & Hall, London 153-194.
- Yan J, Cheng QY, Li BB, Aksoy S (2001) Molecular characterization of two serine proteases expressed in gut tissue of the African trypanosome vector, *Glossina morsitans morsitans*. Insect Mol Biol 10, 47-56.
- Zhu YC, Baker JE (1999) Characterization of midgut trypsin-like enzymes and three trypsinogen cDNAs from the lesser grain borer, *Rhyzopertha dominica* (Coleoptera : Bostrichidae). Insect Biochem Mol Biol 29, 1053-1063.