Available online at www.sciencedirect.com

SCIENCE DIRECT®

Virology 321 (2004) 154–162

VIROLOGY

www.elsevier.com/locate/yviro

Antiviral activity of a serine protease from the digestive juice of *Bombyx mori* larvae against nucleopolyhedrovirus

Hiroshi Nakazawa,[a] Eiko Tsuneishi,[b] Kangayam M. Ponnuvel,[a] Seiichi Furukawa,[a] Ai Asaoka,[a] Hiromitsu Tanaka,[a] Jun Ishibashi,[a] and Minoru Yamakawa[a,b,*]

[a] *Innate Immunity Laboratory, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, 305-8634 Japan*
[b] *Institute of Agriculture and Forestry, University of Tsukuba, Tsukuba, Ibaraki, 305-8572 Japan*

Received 14 October 2003; returned to author for revision 10 December 2003; accepted 16 December 2003

Abstract

A protein showing strong antiviral activity against *Bombyx mori* nucleopolyhedrovirus (BmNPV) was purified from the digestive juice of *B. mori* larvae. The molecular mass of this protein was 24 271 Da. Partial N-terminal amino acid sequence of the protein was determined and cDNA was cloned based on the amino acid sequence. A homology search of the deduced amino acid sequence of the cDNA showed 94% identity with *B. mori* serine protease so the protein was designated *B. mori* serine protease-2 (BmSP-2). Analysis of BmSP-2 gene expression showed that this gene is expressed in the midgut but not in other tissues. In addition, BmSP-2 gene was shown to not be expressed in the molting and wandering stages, indicating that the gene is hormonally regulated. Our results suggest that BmSP-2, an insect digestive enzyme, can be a potential antiviral factor against BmNPV at the initial site of viral infection.

Keywords: *Bombyx mori*; BmSP-2; Antiviral

Introduction

Many antiviral molecules other than interferons and antibodies have been reported in vertebrates (Asano et al., 2002; Murphy et al., 2000; Nozaki et al., 2003; Reading et al., 1998; Siciliano et al., 1999; Singh et al., 1999). On the other hand, immune reactions against viral pathogens in invertebrates remain obscure. As invertebrates possess no acquired immunity and the occurrence of interferons has not been reported from invertebrates, it is speculated that invertebrates have other immune factors to fight viral pathogens.

Tachyplesin I, a cationic peptide composed of 17 amino acid residues from horseshoe crab, *Tachypleus tridentatus*, hemocytes was found to show antiviral activity against vesicular stomatitis virus (Murakami et al., 1991) and human immunodeficiency virus (HIV) (Morimoto et al., 1991) although the horseshoe crab is not a host for these viruses. In insects, a fraction of culture medium from mosquito, *Aedes albopictus*, cells persistently infected with Banzi virus possessed the ability to suppress the replication of Banzi virus and contained two proteins of similar molecular weight (Lee and Schloemer, 1981). However, no amino acid sequence analysis of these antiviral factors was conducted. In addition, a protein that showed antiviral activity against Semiliki Forest virus was purified to homogeneity from *A. albopictus* cultured cells (Luo and Brown, 1993). This 3.2-kDa antiviral protein was extremely hydrophobic and the amino-terminus blocked (Luo and Brown, 1993). Although the effect of the 3.2-kDa mosquito antiviral protein on virus replication was studied, the amino acid sequence and gene expression patterns of this protein were not analyzed. Insect antimicrobial peptides melittin and cecropin were shown to inhibit replication of HIV by suppressing viral gene expression (Wachinger et al., 1998) and alloferon from the blow fly *Calliphora vicina* showed antiviral activity against influenza viruses A and B (Chernysh et al., 2002).

The silkworm *Bombyx mori* has also been used to study antiviral factors because viral infection often causes large losses in silk production. The presence of antiviral sub-

* Corresponding author. Innate Immunity Laboratory, National Institute of Agrobiological Sciences, Owashi, 1-2, Tsukuba, Ibaraki 305-8634, Japan. Fax: +81-29-838-6028.
E-mail address: yamakawa@nias.affrc.go.jp (M. Yamakawa).

0042-6822/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2003.12.011

stances against *B. mori* nucleopolyhedrovirus (BmNPV) in the gut juice of the silkworm has been reported (Aizawa, 1962; Funakoshi and Aizawa, 1989a; Hayashiya et al., 1971; Hayashiya et al., 1968), but these substances were not identified. A viral inhibitory factor (VIF) produced in *B. mori* hemolymph was also detected and this fatty acid-based VIF inactivated BmNPV in vitro but did not show prophylactic or curative properties when injected into infected *B. mori* pupae (Funakoshi and Aizawa, 1989b). Therefore, the primary function of this compound may not be antiviral. A red fluorescent protein (RFP) in *B. mori* gut juice was first reported by Mukai et al. (1969) and found to possess antiviral activity against BmNPV (Hayashiya et al., 1968). The RFP was demonstrated to be produced in vitro from three components: a protein from the midgut of larvae, chlorophyll-a, and a basic protein from the chloroplast of green leaves (Hayashiya et al., 1976a). Chlorophyllide-a derived from chlorophyll-a rather than a protein consisting of the RFP was suggested to play an essential role in antiviral activity against BmNPV (Hayashiya et al., 1976a, 1976b). A different RFP (65 kDa) having antiviral activity against BmNPV was also purified (Nagaraja-Sethuraman et al., 1993). A substance detected in an extract of silkworm faeces was shown to markedly suppress production of Sendai virus, herpes simplex virus type-1, and HIV (Hiraki et al., 1996). The active substance was partially purified and sugars were also detected with lectin blotting (Hiraki et al., 1996). In addition, a chlorophyll-like substance from silkworm faeces was demonstrated to have clear antiviral activity against Sendai virus, herpes simplex virus, and HIV (Hiraki et al., 1997). It has also been shown that the hemagglutinating activity of *B. mori* hemolymph increased when silkworms were infected

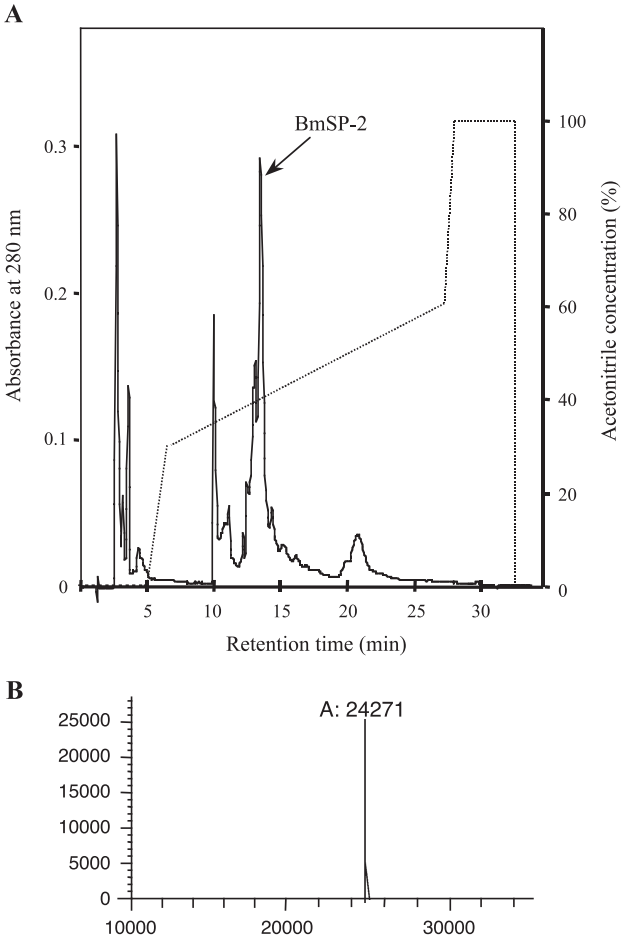

Fig. 1. (A) Final HPLC purification profile of BmSP-2. BmSP-2 was eluted with 49.5% acetonitrile/0.05% TFA by reverse-phase HPLC. The arrow indicates the position of BmSP-2. (B) The LC-MS spectrum with molecular mass of BmSP-2.

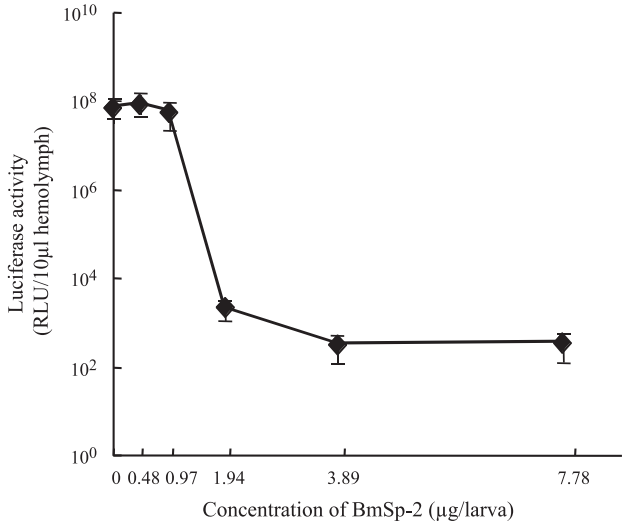

Fig. 2. Effect of BmSP-2 dose on BmNPV-ODV infectivity. ODV infectivity was examined after treatment of ODV (860 ng per larva) with different concentration of BmSP-2. Data shown are means ± SD of results from five experiments. Luciferase activity was measured at 136 hpi. Luciferase activity of hemolymph samples from nontreated *B. mori* larvae was determined as a background and the level of relative light units (RLU) per 10 µl of hemolymph was 120 ± 3.4.

Table 1
Effect of ODV treated with BmSP-2 on mortality and pupation of *B. mori* larvae

Experiment	Luciferase activity[a] (RLU/10 µl hemolymph)		Mortality[b] (%)		Pupation (%)	
	I	II	I	II	I	II
None	1.04×10^2	2.50×10^2	0	0	100	100
Control ODV	1.01×10^8	9.95×10^7	100	100	0	0
ODV + BmSP-2	1.29×10^2	6.76×10^2	0	0	100	100

RLU: relative light units. None: non-treated larva. Control ODV: ODV (22.5 ng per larva) treated with 40 mM phosphate buffer, pH 7.4, was used as a control. ODV + BmSP-2: ODV (22.5 ng per larva) was incubated with BmSP-2 (2.2 µg per larva). Five larvae were used for each mortality and pupation test. Experimental conditions are described in detail in Materials and methods.

[a] Luciferase activity was measured at 136 hpi and is expressed as the mean of activities from 30 individual larvae.

[b] Mortality was checked at 168 hpi.

with *B. mori* cytoplasmic polyhedrovirus (Mori et al., 1989).

To date, the primary structure, antiviral specificity, and gene expression of antiviral proteins from invertebrates in immune reactions against viral pathogens have not been fully elucidated. Thus, we aimed to identify antiviral proteins from the digestive juice of *B. mori* larvae to analyze their role in the immune responses against viral pathogens at an early stage of viral infection. Here, we report the purification, cDNA cloning, and gene expression of a digestive enzyme that shows strong antiviral activity against BmNPV-occlusion-derived virus (ODV).

Results

Purification and antiviral activity of an antiviral protein

An antiviral protein factor against BmNPV-ODV was partially purified from the digestive juice of *B. mori* larvae by 40% ammonium sulphate fractionation. The ammonium sulphate fraction was further purified by gel-filtration and reverse-phase high performance liquid column chromatography (HPLC). Antiviral activity was monitored using BmNPV containing a luciferase reporter gene driven by a p10 promoter (Tomita et al., 1995). An aliquot of each

```
ATTCTACTCGAGGGAACCTGTAACTGGTAGTAGCCATGAAGGTCTTCGCAGCAGTACTGA    60
                                      M  K  V  F  A  A  V  L  M     9

TGGCGTTGGCGGCCGTGGTCGTGGCAGAAGAGGCTATCGAACTTGACTACCACACCAAGA   120
  A  L  A  A  V  V  V  A  E  E  A  I  E  L  D  Y  H  T  K  I    29

TCGGTATCCCCCGGGCCGAGAGTCTTAAGCGCGCCGAGGAAGCCGCTGACTTCGACGGTA   180
  G  I  P  R  A  E  S  L  K  R  A  E  E  A  A  D  F  D  G  T    49

CCAGGATTGTGGGTGGTTCTGCCGCCAACGCTGGTGCTCACCCCCATCTTGCTGGACTTG   240
  R  I  V  G  G  S  A  A  N  A  G  A  H  P  H  L  A  G  L  V    69

TGATCGCACTCACGAATGGCAGAACTTCAATCTGCGGAGCTTCCTTACTGACCAACACCC   300
  I  A  L  T  N  G  R  T  S  I  C  G  A  S  L  L  T  N  T  R    89

GCTCTGTGACCGCTGCTCACTGCTGGAGGTCCAGGGATGCCCAGGCTCGTCAGTTCACCC   360
  S  V  T  A  A  H  C  W  R  S  R  D  A  Q  A  R  Q  F  T  L   109

TCGCTTTTGGCACAGCTAACATCTTCTCCGGAGGCACCAGGGTCACCACCTCCAGTGTCC   420
  A  F  G  T  A  N  I  F  S  G  G  T  R  V  T  T  S  S  V  H   129

ATATGCACGGCAGCTACAACATGAACAACCTCCACAATGACGTCGCCGTCATCAACCACA   480
  M  H  G  S  Y  N  M  N  N  L  H  N  D  V  A  V  I  N  H  N   149

ACCATGTTGGCTTCAACAACAACATTCAGCGCATCAACCTAGCCAGTGGAAGCAACAACT   540
  H  V  G  F  N  N  N  I  Q  R  I  N  L  A  S  G  S  N  N  F   169

TTGCTGGTACTTGGGCCTGGGCTGCCGGCTTCGGCAGAACTTCCGATGCTGCTTCGGGAG   600
  A  G  T  W  A  W  A  A  G  F  G  R  T  S  D  A  A  S  G  A   189

CCAACAACCAACAAAAACGCCAAGTCAGCCTTCAGGTCATTACTAACGCCGTCTGCGCCC   660
  N  N  Q  Q  K  R  Q  V  S  L  Q  V  I  T  N  A  V  C  A  R   209

GGACTTTTGGAAACACTCTGATCATTGGCTCCACCCTCTGTGTTGACGGCTCTAACGGTC   720
  T  F  G  N  T  L  I  I  G  S  T  L  C  V  D  G  S  N  G  R   229

GCAGCACCTGCAGGGGAGACTCCGGCGGCCCTCTCACCATCGGCAGCGGCGGAGGCCGTC   780
  S  T  C  R  G  D  S  G  G  P  L  T  I  G  S  G  G  G  R  Q   249

AGCTGATCGGTATCACATCGTTCGGATCAGCCCAAGGCTGCCAGAGAGGCTTCCCCGCCG   840
  L  I  G  I  T  S  F  G  S  A  Q  G  C  Q  R  G  F  P  A  A   269

CCTTCGCCAGAGTCACATCTTTTAACTCCTGGATCCGGGCTAGAATTTAAATGACGACTG   900
  F  A  R  V  T  S  F  N  S  W  I  R  A  R  I  *               284

GCGAATGACTCATTAATAATAAAATAATTTCATTTAC(A)n                       938
```

Fig. 3. Nucleotide and deduced amino acid sequences of BmSP-2 cDNA. The putative signal sequence of purified BmSP-2 is underlined. An asterisk shows the termination codon (TAA). The polyadenylation signals are underlined. The nucleotide sequence of BmSP-2 has been deposited with the DDBJ, EMBL, and GenBank Data Libraries under accession number AB117641.

fraction was incubated first with BmNPV-ODV containing luciferase gene and *B. mori* larvae were orally inoculated with this mixture. Hemolymph was collected from larvae at 136 h postinfection (hpi) and luciferase activity derived from budded virus (BV) was measured. Note that virus particles present in the hemolymph are BVs originated from ODVs containing the luciferase gene. Two main antiviral peaks were detected at 13.5 and 21 min in the final reverse-phase HPLC (Fig. 1A). The antiviral peak eluted at 21 min was previously analyzed and found to be lipase (Ponnuvel et al., 2003). Thus, another antiviral peak eluted at 13.5 min with 39.5% acetonitrile/0.05% trifluoroacetic acid (TFA) was analyzed in this work. The purity of the antiviral substance in this peak fraction was examined by both liquid chromatography-mass spectrometry (LC-MS) and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). The results showed this fraction contained a single protein and its molecular mass determined by LC-MS and MALDI-TOFF-MS was 24271 Da (Fig. 1B).

Effect of the antiviral protein dose on BmNPV-ODV infectivity was analyzed. Results showed that the infectivity of ODV treated with 0.48 or 0.97 μg per larva of this antiviral protein was very high but dramatically deceased when the virus was treated with high concentration of this protein (more than 1.94 μg per larva) (Fig. 2). Infectivity of ODV treated with more than 3.89 μg/ml of the protein was at a background level.

Mortality and pupation were also examined after ODV was treated with the purified antiviral protein. The purified protein (2.2 μg per larva) was incubated with ODV (22.5 ng per larva) and *B. mori* larvae were inoculated orally with the mixture to investigate the antiviral activity. Luciferase activity derived from budded virus (BV) was measured after hemolymph was collected. If the purified protein does not show antiviral activity against ODV, the luciferase activity level in the hemolymph should be comparable with that of the sample from larvae inoculated with control ODV treated with phosphate buffer. The results showed the luciferase activity was at a background level (Table 1), whereas the control ODV sample revealed very high luciferase activity (Table 1). All fifth-instar larvae inoculated with ODV treated with this protein entered the pupal stage, although all control larvae inoculated with control ODV died within 168 hpi (Table 1). These results indicate the purified protein has strong antiviral activity against ODV.

cDNA cloning and comparison of the amino acid sequence

The purified protein was subjected to analysis of N-terminal amino acid sequence. The amino acid sequence determined was NH_2-IVGGSAANAGAHPHLAGLVI. Homology search of this amino acid sequence was conducted using *B. mori* expressed-sequenced tag (EST) database. Four EST clones (mg0688, mg103, mg564, and mg994) were found to have an identical sequence. Full-length nucleotide sequences of these clones were determined and the complete amino acid sequence deduced (data not shown). Proteins deduced from these four clones showed the amino acid sequences have high similarity, suggesting they are isoforms (data not shown). Of these four clones, mg103 was found to encode 284 amino acid residues and 50

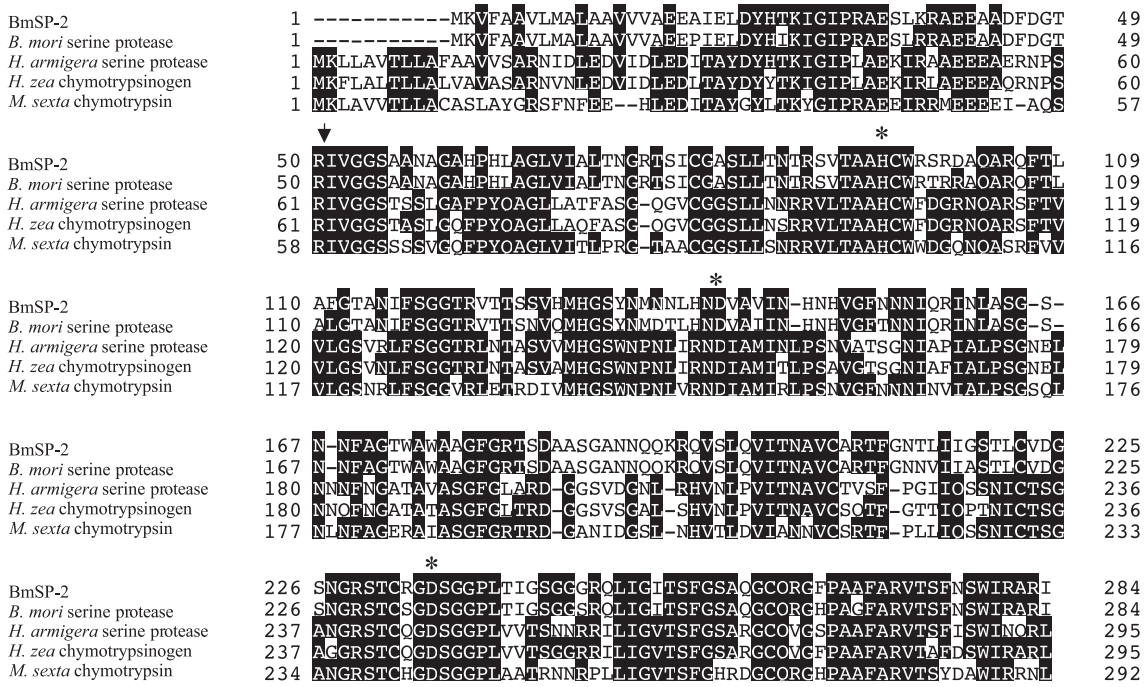

Fig. 4. Comparison of the amino acid sequences between BmSP-2 and other enzymes. Identical amino acid residues, in which more than three out of five residues show identity, are boxed in black. The active site of the enzymes is shown with asterisks. An arrow indicates the cleavage site for mature proteins.

amino acid residues at the N-terminus were assumed to be removed to produce the mature protein (Fig. 3). Molecular mass of this mature peptide was calculated to be 24276, coincident with the measured masses by LC-MS and MALDI-TOF-MS. Molecular masses of the mature proteins deduced from mg0688, mg564, and mg994 clones were different from that of the purified protein. Thus, we concluded the mg103 clone is derived from mRNA encoding the purified antiviral protein. Computer-aided homology search for the deduced amino acid sequence of this protein showed that it had 94% identity with a *B. mori* serine protease (Kotani et al., 1999) (Fig. 4). In addition, this protein showed a strong relationship to the primary structure of cotton bollworm (*Helicoverpa armigera*) chymotrypsin-like protease (Bown et al., 1997), corn earworm (*Helicoverpa zea*) chymotrypsinogen (Mazumdar-Leighton and Broadway, 2001), and tobacco hornworm (*Manduca sexta*) chymotrypsin (Peterson et al., 1995). Active sites of these proteins are completely conserved (Fig. 4). In addition, protease activity of the purified antiviral protein was confirmed (data not shown). The purified protein was thus designated *B. mori* serine protease-2 (BmSP-2) based on these results.

Gene expression of BmSP-2

Tissue specificity of BmSP-2 gene expression was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). BmSP-2 gene was expressed exclusively in the midgut and no expression of this gene was detected in the fat body, hemocyte, Malpighian tubule, silk gland, and trachea (Fig. 5A). The midgut was further separated into three portions and BmSP-2 gene expression examined. BmSP-2 gene was expressed in the anterior, middle, and posterior portion of the midgut (Fig. 5B). BmSP-2 gene expression in fourth- and fifth-instar larvae was analyzed by Northern blotting. The results showed that gene expression strongly declines at the molting stage between fourth and fifth instars and at the wandering stage just before pupation (fifth instar 7 days) (Fig. 5C). Inducibility of BmSP-2 gene

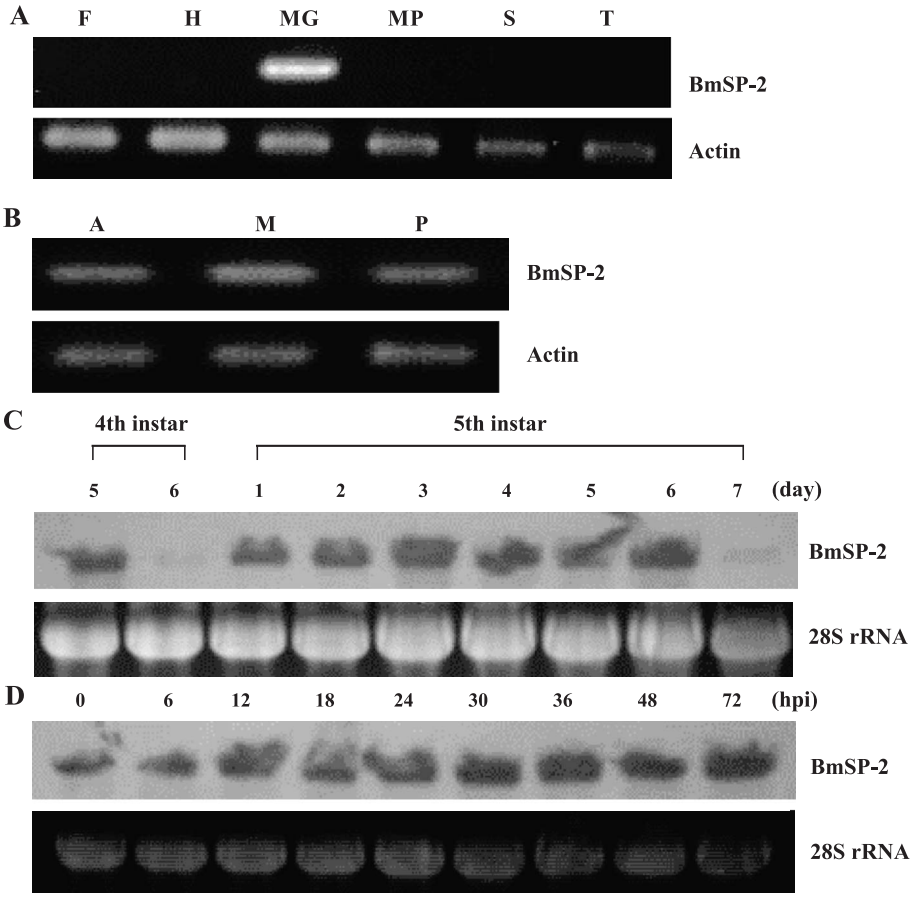

Fig. 5. (A) Tissue-specific gene expression of BmSP-2. BmSP-2 gene expression was analyzed by RT-PCR. Total RNA was extracted from the fat body (F), hemocyte (H), midgut (MG), Malpighian tubule (MP), silk gland (S), and trachea (T). As an internal marker, actin gene was also analyzed. (B) Analysis of BmSP-2 gene expression in the different portions of the midgut by RT-PCR. Total RNA was extracted from the anterior (A), middle (M), and posterior portion (P) of the midgut. (C) BmSP-2 gene expression in fourth- and fifth-instars. Northern blotting was conducted. As an internal marker, 28S rRNA was visualized by ethidium bromide staining. Fourth-instar larvae on days 5 and 6 are the molting stages. Fifth-instar day 7 is the wandering stage. (D) Effects of BmNPV infection on BmSP-2 gene expression. BmSP-2 gene expression was analyzed at different time intervals after BmNPV infection by Northern blotting.

expression by BmNPV infection was also analyzed by Northern blotting. The results showed that BmSP-2 gene is expressed regardless of BmNPV infection (Fig. 5D).

Discussion

BmNPV was chosen as a model virus to examine insect immune mechanisms against viral infection. NPVs are double-stranded DNA viruses belonging to the family Baculoviridae. NPVs require two phenotypically different but genetically identical virions to complete their life cycle (Engelhard and Volkman, 1995; Maeda, 1989; Trudeau et al., 2001). Both forms perform a different role during pathogenesis. An ODV initiates primary infection within the midgut of hosts following ingestion. The second virion phenotype, BV, buds out of the midgut cells and transmits infection from cell to cell within the host. Therefore, immune mechanisms in hosts against ODV and BV are speculated to be different.

We purified a protein showing strong antiviral activity against BmNPV-ODV from the digestive juice of *B. mori* larvae. A cDNA encoding this protein was cloned to deduce the full amino acid sequence. BmSP-2 showed 94% amino acid sequence identity with a serine protease from *B. mori* digestive juice (Kotani et al., 1999). The serine protease had an isoelectric point of pH 10–11 and the pH optimum for degradation activity of sucinyl-Leu-Val-Tyr-MCA was about 10 (Kotani et al., 1999). The pH optimum of BmSP-2 was also determined to be about 11 showing this enzyme is a highly basic protease (data not shown). A sequence comparison of BmSP-2 with other proteases indicated that BmSP-2 is a mammalian-type serine protease with a catalytic triad composed of His 45, Asp 92, and Ser 186 (Fig. 3). A large number of Arg may contribute to the stability and function of BmSP-2 by remaining charged at a high pH as suggested by Kotani et al. (1999).

A final yield of the pure BmSP-2 from 200 larvae was estimated to be 116.8 μg, showing that a larva contains approximately 0.58 μg BmSP-2. On the other hand, the LD_{50} of ODV was determined to be 12.0 ng per larva with 95% confidence limits between 10.6 and 13.7 ng per larva (Fig. 6). The LD_{50} was also confirmed by a mortality of 100% in *B. mori* larvae infected with 22.5 ng ODV (Table 1). The effect of BmSP-2 concentration on a fixed amount of ODV (860 ng per larva) was examined. The results showed that ODV treated with more than 3.89 μg of BmSP-2/larva cannot propagate at all (Fig. 2). These data suggest the physiological concentration of BmSP-2 cannot protect against infection with 22.5 ng of ODV, although the same amount of ODV can be completely inactivated by 2.2 μg of BmSP-2 (Table 1). Nonetheless, BmSP-2 is speculated to inactivate a portion of ODV when a larva is infected with less than 22.5 ng of ODV. In fact, the mortality was 20% for larvae infected with 11 ng of ODV per larva and no larvae died when infected with less than 5.5 ng of ODV per larva (Fig. 6). We recently demonstrated a lipase designated Bmlipase-1 from *B. mori* digestive juice showing a strong antiviral activity against BmNPV-ODV (Ponnuvel et al., 2003), suggesting that digestive enzymes such as BmSP-2 and Bmlipase-1 contribute as physiological barriers to inactivation of BmNPV-ODV at the initial infection site in *B. mori* larvae.

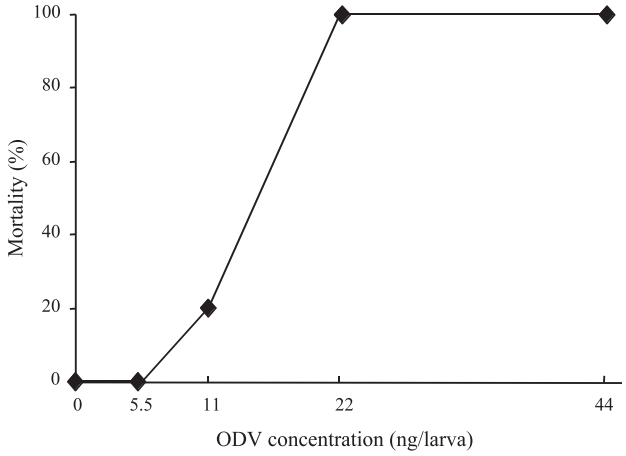

Fig. 6. Effect of different concentrations of BmNPV-ODV on the mortality of *B. mori* larvae. Each fifth-instar larva just after ecdysis was orally infected with the indicated concentration of ODV. Mortality was checked at 168 hpi. The data shown are the typical result of three independent experiments.

BmSP-2 gene was shown to be expressed only in the midgut (Fig. 5). As described above, at least five serine protease isoforms including BmSP-2 are assumed to occur in *B. mori*. Moreover, the results of Southern blotting indicate that *B. mori* has more serine protease isoforms (data not shown), suggesting they form a gene family. It seemed to be difficult to distinguish expression of each gene from this family by Northern blotting used in this work. Thus, RT-PCR using BmSP-2 specific primers was conducted and the positive signals in Northern blotting were confirmed (data not shown). Results of Northern blot analysis suggest that expression of the family genes is regulated simultaneously. The family genes were constitutively expressed regardless of BmNPV infection but gene expression strongly diminished both at the molting stage and the wandering stage just before pupation (Fig. 5), suggesting the family genes including BmSP-2 gene are hormonally regulated. However, a possibility that mRNA for BmSP-2 is simply unstable at the molting stage and wandering stage cannot be ruled out at present. The BmSP-2 gene expression pattern analyzed by RT-PCR is the same as for Bmlipase-1 gene (Ponnuvel et al., 2003), except for the expression pattern in the posterior portion of the midgut, suggesting that both genes have different regulatory mechanisms in the posterior portion of the midgut. Our gene expression analysis suggests the main physiological role of both digestive enzymes is food digestion. Our results, however, also suggest that these digestive enzymes have potential to inactivate BmNPV.

Although the antiviral mechanisms of BmSP-2 and Bmlipase-1 against ODV remain unclear at present, it is conceivable that inactivation of ingested viruses by digestive enzymes at the initial infection site before they propagate in the midgut and spread into hemocoel is an efficient strategy for host defense in *B. mori*. Thus, suppression of ODV attachment to midgut epithelial cells by digestive enzymes in the alimentary canal may provide an attractive mechanism to protect larvae from BmNPV infection. Analysis of antiviral mechanisms of these digestive enzymes against BmNPV-ODV may shed light on the insect immune response against viruses at the initial infection site.

Materials and methods

Insects

All *B. mori* larvae used in this study were collected from the silkworm germplasm station of the National Institute of Agrobiological Sciences, Kobuchisawa, Yamanashi, Japan. *B. mori* larvae of the Daizo race were reared on mulberry leaves, whereas larvae of Tokai × Asahi race were fed on an artificial diet (Nihonnosanko). The silkworms were maintained in a silkworm-rearing room under controlled environmental conditions at 27 °C.

Purification of BmSP-2

The purification procedure depended on previously published methods by Ponnuvel et al. (2003). Briefly, ammonium sulphate was added to the collected digestive juice from Daizo race larvae by mild electric shock to give 40% saturation. The precipitate was suspended in 40 mM phosphate buffer at pH 7.4 and treated with 50% *n*-butanol (final concentration) overnight at 4 °C. The lower aqueous layer was collected after centrifugation for 30 min at 10 000 × *g*. Half-volume ice-cold acetone was added to the solution to precipitate proteins in the aqueous layer. The precipitated proteins were collected by centrifugation for 30 min at 10 000 × *g* and dried. The proteins dissolved in 40 mM phosphate buffer, pH 7.4, were applied to a Superdex 200 HR 10/30 column attached to a FPLC system (Pharmacia) and equilibrated with the same buffer. Antiviral activity in each fraction was tested. Fractions showing antiviral activity were further purified through a reverse-phase column, Sephasil C8 SC 2.1/10 attached to a SMART system (Pharmacia) with a linear gradient of acetonitrile/0.05% TFA.

Antiviral activity of BmSP-2 against BmNPV-ODV

The antiviral activity was assayed using BmNPVp10luc, which contains a luciferase reporter gene driven by a p10 promoter (Tomita et al., 1995). The recombinant BmNPV was obtained from Dr. Shuichiro Tomita from our institute. The recombinant virus expresses a luciferase reporter gene at 15 hpi. *B. mori* Tokai × Asahi race was used to quantify the infection and mortality levels. The ODV was purified by ultracentrifugation on sucrose gradient (Summers and Smith, 1978). The ODV suspension was mixed with BmSP-2 dissolved in 40 mM phosphate buffer at pH 8.0 and incubated at 30 °C for 1 h. Different concentrations of BmSP-2 were incubated with ODV (860 ng per larva) to examine the dose effect on ODV infectivity. The ODV (22.5 ng per larva) was mixed with BmSP-2 (2.2 μg per larva) to examine the mortality and pupation rates of *B. mori* larvae. Fifth-instar larvae just after ecdysis were used to orally inoculate the ODV mixture (5 μl). Ten microliters of hemolymph collected at 136 hpi was added to 50 μl of luciferase assay buffer (Promega) and luciferase activity was measured by a Luminocounter 700 (Microtech-Nition).

cDNA cloning and nucleotide sequencing of BmSP-2

The expressed-sequenced tag (EST) database of *B. mori* (silkbase; http://www.ab.a.u-tokyo.ac.jp/silkbase/) was screened taking advantage of the N-terminal amino acid sequence of the purified protein. Clones designated mg0688, mg103, mg564, and mg994 from the midgut cDNA library showed homology with this protein. These clones were kindly provided by Dr. Kazuei Mita from our institute. The nucleotide sequences of these clones were determined and found to lack the sequence of the 5′ region. mRNA extracted from the midgut and purified by a Quick Prep mRNA Kit (Amersham Pharmacia Biotech) was used to obtain a complete nucleotide sequence by a First Choice RLM-RACE Kit (Ambion). The nucleotide sequence was determined by dye-terminator cycle sequencing using a DNA sequencer (ABI 373A).

Analysis of BmSP-2 gene expression

Tissues such as the midgut, hemocyte, silk gland, trachea, fat body, and Malpighian tubules were prepared from fifth-instar 2-day larvae. The midgut was further separated into anterior, middle, and posterior portions. Total RNA from each tissue was isolated with ISOGEN (Nippon Gene). First-strand cDNA was synthesized with 5 μg of total RNA using a First-Strand cDNA Synthesis Kit (Amersham). RT-PCR was conducted under the following conditions: 94 °C for 1 min and then 15 cycle reactions (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s). The following BmSP-2 specific primers were used: 5′-ACTGCTGGAGGTCCAGGGAT-3′ (forward primer) and 5′-GGTGGAGCCAATGATCAGAG-3′ (reverse primer). As an internal control, actin gene was also analyzed using the following primers: 5′-AGCAGGAGATGGCCACC-3′ (forward primer) and 5′-TCCACATCTGCTGGAAGG-3′ (reverse primer). Northern blotting (Sambrook et al., 1989) was conducted with 20 μg total RNA and screened with a Digoxygenine (DIG)-labeled BmSP-2 probe prepared by PCR DIG Labeling Mix

(Roche). BmSP-2 gene expression was analyzed at different time intervals in the life cycle or after BmNPV infection.

Effect of increasing amounts of BmNPV-ODV on the mortality of B. mori larvae

Each fifth-instar larva just after ecdysis was orally inoculated with different amounts of ODV and the mortality was determined at 168 hpi. LD_{50} of ODV was calculated by the computer program PriProbit (version 1.63) by M. Sakuma.

Acknowledgments

We thank Dr. Toru Arakawa and Dr. Yoji Furuta in our institute for their technical advice. This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences of the Bio-Oriented Technology Research Advancement Institution, Japan, and by Grants-in-Aid (Bio Design Program and Insect Technology Program) from the Ministry of Agriculture, Forestry and Fisheries of Japan.

References

Aizawa, K., 1962. Antiviral substance in the gut-juice of the silkworm, *Bombyx mori* (Linnaeus). J. Insect Pathol. 4, 72–76.

Asano, A., Ko, J.H., Morozumi, T., Hamashima, N., Watanabe, T., 2002. Polymorphisms and the antiviral property of Mx1 protein. J. Vet. Med. Sci. 64, 1085–1089.

Bown, D.P., Wilkinson, H.S., Gatehouse, J.A., 1997. Differentially regulated inhibitor-sensitive and insensitive protease genes from the phytophagous insect pest, *Helicoverpa armigera*, are members of complex multigene families. Insect Biochem. Mol. Biol. 27, 625–638.

Chernysh, S., Kim, S.I., Bekker, G., Pleskach, V.A., Filatova, N.A., Anikin, V.B., Platino, V.G., Bullet, P., 2002. Antiviral and antitumor peptides from insects. Proc. Natl. Acad. Sci. U.S.A. 99, 12628–12632.

Engelhard, E.K., Volkman, L.E., 1995. Developmental resistance in forth instar *Trichoplusia ni* orally inoculated with *Autographa californica* M nuclear polyhedrosis virus. Virology 209, 384–389.

Funakoshi, M., Aizawa, K., 1989a. Antiviral substance in the silkworm gut juice against a nuclear polyhedrosis virus of the silkworm, *Bombyx mori*. J. Invertebr. Pathol. 53, 135–136.

Funakoshi, M., Aizawa, K., 1989b. Viral inhibitory factor produced in the hemolymph of the silkworm, *Bombyx mori*, infected with a nuclear polyhedrosis virus. J. Invertebr. Pathol. 54, 151–155.

Hayashiya, K., Nishida, J., Matsubara, F., 1968. Inactivation of nuclear polyhedrosis virus in the digestive juice of silkworm larvae, *Bombyx mori* L: I. Comparison of anti-viral activities in the digestive juice of larvae reared on natural and artificial diets. J. Appl. Entomol. Zool. 12, 189–193 (in Japanese).

Hayashiya, K., Matsubara, F., Nishida, J., Kawamoto, F., 1971. Comparative experiments with the silkworm larvae reared on mulberry leaves and artificial diets: I. Comparison of antiviral activities in the digestive juice of larvae reared on natural and artificial diets. Bull. Fac. Text. Sci. Kyoto Univ. Ind. Arts Text. Fibers 6, 87–100.

Hayashiya, K., Nishida, J., Uchida, U., 1976a. The mechanism of formation of the red fluorescent protein in the digestive juice of silkworm larvae—The formation of chlorophyllide-a. J. Appl. Entomol. Zool. 20, 37–43 (in Japanese).

Hayashiya, K., Uchida, U., Nishida, J., 1976b. Comparison of anti-viral activities of the silkworm larvae reared in light and in darkness in relation to the formation of red fluorescent protein (RPF). J. Appl. Entomol. Zool. 20, 139–143 (in Japanese).

Hiraki, A., Ikuno, Y., Kim, J., Ueda, S., 1996. Suppression of enveloped virus production with a substance from silkworm faeces. Cell Struc. Funct. 21, 501–514.

Hiraki, A., Yukawa, M., Kim, J., Ueda, S., 1997. Antiviral substance from silkworm faeces: purification and its chemical characterization. Biol. Pharm. Bull. 20, 547–555.

Kotani, E., Niwa, T., Tokizane, M., Suga, K., Sugimura, Y., Oda, K., Mori, M., Furusawa, T., 1999. Cloning and sequence of a cDNA for a highly basic protease from the digestive juice of the silkworm, *Bombyx mori*. Insect Mol. Biol. 8, 299–304.

Lee, C.H., Schloemer, R.H., 1981. Identification of the antiviral factor in culture medium of mosquito cells persistently infected with Banzi virus. Virology 110, 445–454.

Luo, T., Brown, D.T., 1993. Purification and characterization of a Sindbis virus-induced peptide which stimulates its own production and blocks virus RNA synthesis. Virology 194, 44–49.

Maeda, S., 1989. Expression of foreign genes in insects using baculovirus vectors. Annu. Rev. Entomol. 34, 351–372.

Mazumdar-Leighton, S., Broadway, R.M., 2001. Identification of six chymotrypsin cDNAs from larval midguts of *Helicoverpa zea* and *Agrotis ipsilon* feeding on the soybean (Kunitz) trypsin inhibitor. Insect Biochem. Mol. Biol. 31, 633–644.

Mori, H., Ohyane, M., Ito, M., Iwamoto, S., Matsumoto, T., Sumida, M., Matsubara, F., 1989. Induction of a hemagglutinating activity in the hemolymph of the silkworm, *Bombyx mori*, infected with cytoplasmic polyhedrosis virus. J. Invertebr. Pathol. 54, 112–116.

Morimoto, M., Mori, H., Otake, T., Ueba, N., Kunita, N., Niwa, M., Murakami, T., Iwanaga, S., 1991. Inhibitory effect of tachyplesin I on the proliferation of human immunodeficiency virus in vitro. Chemotherapy 37, 206–211.

Mukai, J., Inoue, J., Akune, S., 1969. A red, fluorescent protein from silkworm. Agric. Biol. Chem. 33, 125–127.

Murakami, T., Niwa, M., Tokunaga, F., Miyata, T., 1991. Direct virus inactivation of tachyplesin I and its isopeptides from horseshoe crab hemocytes. Chemotherapy 37, 327–334.

Murphy, M.E., Kariwa, H., Mizutani, T., Arikawa, J., Takashima, I., 2000. In vitro antiviral activity of lactoferrin and ribavirin upon hantavirus. Arch. Virol. 145, 1571–1582.

Nagaraja-Sethuraman, B., Nagaraju, N., Datta, R.K., 1993. Purification and partial characterization of antiviral protein in silkworm, *Bombyx mori*. Indian J. Seric. 32, 63–67.

Nozaki, A., Ikeda, M., Naganuma, A., Nakamura, T., Inudoh, M., Tanaka, K., Kato, N., 2003. Identification of a lactoferrin-derived peptide possessing binding activity to hepatitis C virus E2 envelope protein. J. Biol. Chem. 278, 10162–10173.

Peterson, A.M., Fernando, G.J.P., Wells, M.A., 1995. Purification, characterization and cDNA sequence of an alkaline chymotrypsin from the midgut of *Manduca sexta*. Insect Biochem. Mol. Biol. 25, 765–774.

Ponnuvel, K.M., Nakazawa, H., Furukawa, S., Asaoka, A., Ishibashi, J., Tanaka, M., Yamakawa, M., 2003. A lipase isolated from the silkworm *Bombyx mori* shows antiviral activity against nucleopolyhedrovirus. J. Virol. 77, 10725–10729.

Reading, P.C., Holmskov, U., Anders, E.M., 1998. Antiviral activity of bovine collectins against rotaviruses. J. Gen. Virol. 79, 2255–2263.

Sambrook, J., Fritisch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Siciliano, R., Rega, B., Marchetti, M., Seganti, L., Antonini, G., Valenti, P., 1999. Bovine lactoferrin peptidic fragments involved in inhibition of herpes simplex virus type I infection. Biochem. Biophys. Res. Commun. 264, 19–23.

Singh, I.P., Chopra, A.K., Coppenhaver, D.H., Ananatharamaiah, G.M., Baron, S., 1999. Lipoproteins account for part of the broad non-specific antiviral activity of human serum. Antiviral Res. 42, 211–218.

Summers, M.D., Smith, G.E., 1978. Baculovirus structural polypeptides. Virology 84, 390–402.

Tomita, S., Kanaya, T., Kobayashi, J., Imanishi, S., 1995. Isolation of p10 gene from *Bombyx mori* nuclear polyhedrosis virus and study of its promoter activity in recombinant baculovirus vector system. Cytotechnology 17, 65–70.

Trudeau, D., Washburn, J.O., Volkman, L.E., 2001. Central role of hemocytes in *Autographa californica* M nucleopolyhedrovirus pathogenesis in *Heliothis virescens* and *Helicoverpa zea*. J. Virol. 75, 996–1003.

Wachinger, M., Kleinschmidt, A., Winder, D., von Pechmann, N., Ludvigsen, A., Neumann, M., Holle, R., Salmons, B., Erfle, V., Brack-Werner, R., 1998. Antimicrobial peptides melittin and cecropin inhibit replication of human immunodeficiency virus 1 by suppressing viral gene expression. J. Gen. Virol. 79, 731–740.