

Molecular cloning and characterization of presenilin gene in *Bombyx mori*

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Abstract. Presenilin (PS), the catalytic core of the γ -secretase complex, is considered to be a causative protein of the early-onset familial form of Alzheimer's disease. Aging is a risk factor for Alzheimer's disease and a number of genetic studies have utilized *Bombyx mori* (*B. mori*) as a model, making it possible to use *B. mori* to investigate Alzheimer's disease. However, the homologous gene of human *PS* in *B. mori* has remained to be elucidated. In the present study, the *PS* homologue gene in *B. mori* was identified and characterized, and six *B. mori* presenilin (*BmPS*) mRNA transcripts were generated by selecting multiple transcription start sites and/or alternative splice sites. The longest mRNA of *BmPS* (termed *BmPS1*) contains a 153 nt 5' untranslated region (UTR), a 1,440 nt open reading frame and a 1,063 nt 3' UTR. The predicted protein of *BmPS1* consists of 479 amino acid residues and has two highly-conserved aspartate residues, which form the catalytic core of aspartic proteases. It exhibits a sequence identity of ~44 and 51% with homologues in *Homo sapiens* and *Drosophila melanogaster*, respectively. However, the amino acid sequence of the *BmPS* loop region does not completely match between the two *B. mori* strains R13Q and Dazao. Genomic analysis revealed that *B. mori* had a single copy of the *BmPS* gene, which was composed of 14 exons. A total of four isoforms of *BmPS* (*BmPS*-A, -B, -C and -D) owing to multiple transcriptional start sites and alternative splice sites were identified. The alternative splicing events occurring in the loop region improved the diversity of the *BmPS* protein and were detectable in all tissues, as determined using reverse transcription quantitative polymerase chain reaction (RT-qPCR). Furthermore, the expression levels of *BmPS* in the brain at different developmental stages were detected using RT-qPCR, and significantly higher expression levels of *BmPS* were found in the adult stage compared with those in the larval and pupal stages. The present study on *BmPS* provided insight

into the pathogenesis of Alzheimer's disease and mechanisms of silkworm developmental regulation.

Introduction

Alzheimer's disease (AD) is a prototypical age-associated neurodegenerative disorder and is the most common progressive form of dementia. Although the pathogenesis of AD remains to be clarified, missense mutations in three associated genes coding for the proteins amyloid precursor protein (APP) and the presenilins (PSs) have been identified as a causative factor in early-onset familial AD (EOFAD) (1-3). Of note, mutations within the PS gene are the most common cause of EOFAD. PS1 mutations account for 18-50% of EOFAD cases (4), while mutations in PS2 are rare (5). PS1 and PS2 are integral membrane proteins with nine transmembrane domains (TMDs) similar in amino acid composition (65%) and intracellular location (6). Of note, PS1 and PS2 exhibit a different tissue-specific expression pattern in transcription level. *PS1* is uniformly expressed (7), while *PS2* is restricted to the heart, skeletal muscle and pancreas (8).

PS is the catalytic core of the γ -secretase complex, containing nicastrin, PS enhancer 2 and anterior pharynx defective 1 (2,9,10). γ -Secretases cleave various type I transmembrane proteins, primarily APP, the Notch receptor, N-cadherin and E-cadherin, which are associated with AD in vertebrates (11). APP, the most well-known substrate of γ -secretase, generates a 37-49 amino acid peptide termed amyloid- β ($A\beta$) following cleavage. These peptides, particularly $A\beta_{42}$, are abundantly released and aggregate into oligomeric structures in the brain of patients with an APP or PS missense mutation, and are considered to be the initiating factors of AD (12). In addition, PS has also been reported to regulate the kinase activity and control tau phosphorylation, which is the causative factor of neurofibrillary tangles in AD and other neurodegenerative disorders (13).

A number of invertebrate model organisms, including *Caenorhabditis* (*C. elegans*) and *Drosophila* (*D. melanogaster*), have been employed to investigate AD, and have provided insight into AD progression and the function of genes involved (14-17). In *C. elegans* and *D. melanogaster*, homologues of PSs have been identified. *Sel-12*, the homologous gene of PS in *C. elegans*, has been confirmed to be involved in the lin-12-mediated cell signaling pathway, which is homologous to the Notch pathway in *D. melanogaster* and vertebrates (18). Accumulating evidence has demonstrated that the Notch receptor is the hydrolytic

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substrate of *Drosophila* presenilin (DPS) in *D. melanogaster*, and overexpression of wild-type DPS or DPS mutations in *Drosophila* resulted in several phenotypes due to loss-of-function of Notch, including wing margin loss, wing vein thickening and rough eye (19-23), which are considered to be the results of an apoptotic process of the developmental wing and eye discs (24). Although Notch is present in the adult brain and is required for long-term memory in *Drosophila* (25), it remains to be elucidated whether DPS regulates long-term memory via Notch signaling. A DPS-null mutation has been reported to impair learning and memory, which, however, cannot be exclusively attributed to loss-of-function in the Notch pathway (26). In addition, DPS is also required for calcium homeostasis, the disruption of which may lead to cognitive deficits (27-29). Similarly to investigations in invertebrates, Notch has also been confirmed to interact with PSs and APP in mammals (30,31) and the expression of Notch 1 is increased in the brains of patients with AD (32). Thus, the understanding of PS and Notch signaling in AD may aid in the development of novel strategies for the treatment of AD. Molecular and genetic studies of PS homologues in invertebrates may also provide important insight into the pathogenic mechanism of this gene family in mammals.

The silkworm, *Bombyx mori* (*B. mori*), is widely used in genetics and molecular biology and has numerous beneficial traits as a model compared with mammals or other insects. In particular, the whole genome of the silkworm has been sequenced, which allows for genetic manipulation, and there are already a number of ongoing research projects that utilize transgenic and genetically modified silkworms (33,34). Of note, a number of previous studies have demonstrated the possibility of applying the results obtained in silkworms to mammals (33,34).

To gain insight into the function of *B. mori* presenilin (*BmPS*), the present study cloned and characterized the *BmPS* gene and assessed its homology with PS from other species. Furthermore, the expression of *BmPS* in various *B. mori* tissues at the adult and larval/pupal stages was assessed. The present study provided a basis for the understanding of the function of PS, particularly with regards to the development of AD.

Materials and methods

Reagents. The reagents and detection kits used in the present study were as follows: LATAq, ExTaq, T4 Ligase, RNase-free DNase I, pMD18 T-Vector, DL2000 DNA marker, DL5000 DNA marker, 5'-Full RACE kit and 3'-Full RACE kit were purchased from Takara Bio, Inc. (Otsu, Japan); ReverTra Ace[®] kit, KOD Plus ver.2, 100 bp DNA Marker and SYBR[®] Green real-time PCR master mix were purchased from Toyobo Co., Ltd. (Osaka, Japan); Agarose gel extraction kit, Plasmid mini preparation kit and PCR clean-up kit were purchased from Axygen (Union City, CA, USA); T7 polymerase and PolyATtract[®] mRNA isolation system III were purchased from Promega Corporation (Madison, WI, USA); TRIzol reagent was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animal model. The two *B. mori* strains, Dazao and R13Q, were kindly provided by Professor Mu-wang Li and Professor An-yin Xu (Department of Biotechnology, Jiangsu

University of Science and Technology, Zhenjiang, China). The larvae were reared on fresh mulberries at 25°C under a 12 h-light/dark cycle. Eggs, pupae and adults were maintained under the same light and temperature conditions.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR). Total RNA from various tissues of *B. mori* was extracted with TRIzol reagent. A total of 1 µg total RNA was denatured at 65°C for 5 min and immediately chilled on ice. First-strand cDNA was synthesized using ReverTra Ace[®] according to the manufacturer's instructions, with Oligo (dT)₂₀ primers. Two specific primers F1 and R1 (as shown in Table I) designed from the flanks of the predicted *BmPS* coding sequence (CDS) present in the expressed sequence tag (EST) sequences (BJ983375, BY936068, CK563766, CK490866, CK488524, CK488055 and CK485294) from the SilkBase (<http://silkworm.genomics.org.cn/>) were used for amplification of the actual CDS. All PCR procedures were performed using LA Taq with GC buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and consisted of 3 min of denaturation at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 58°C and 80 sec at 72°C, and a final extension step at 72°C for 5 min.

The expression of *BmPS* alternative splice sites was investigated by RT-PCR using total RNA extracted from R13Q 3rd- and 5th instar larval tissues with the primers of F4 and R5 (as shown in Table I). All PCRs were performed using KOD Plus with 5% dimethyl sulfoxide and consisted of 2 min of denaturation at 94°C followed by 28 cycles of 10 sec at 98°C, 30 sec at 58°C and 20 sec at 68°C.

The PCR products were purified by 1% agarose gel electrophoresis, sub-cloned into the pMD18-T vector and sequenced by Shanghai Invitrogen Biotechnology Co., Ltd. (Shanghai, China).

Rapid amplification of cDNA ends (RACE). To clone the full-length cDNA of *BmPS*, 5'- and 3'-RACE was performed using the 5'- and 3'-Full RACE kit according to the manufacturer's instructions. For the 5'-RACE, first-strand cDNA was generated by RT of poly(A)⁺ RNA from the pupal brains of the R13Q strain using the 5'-cDNA synthesis (CDS) primer provided in the kits. The cDNA was amplified by PCR using gene-specific primers (GSPs) and R2/R3 (as shown in Table I), which were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). To determine the *BmPS* transcripts with multiple transcription start sites and alternative splice sites, 5'-RACE PCR was performed again with the downstream primer R4 and the nest primer R5. In 3'-RACE, first-strand cDNA was generated using the 3'-CDS primer. The cDNA was amplified by PCR using GSPs and F2/F3 (as shown in Table I). The primers F1-1 and R1-1 were used to assess whether the PCR products of 5' and 3' RACE belonged to a single transcript.

RT-quantitative (q)PCR analysis. The brains of 4th instar larvae, 5th instar larvae, pupae and adults were collected from the R13Q strain. The total extracted RNA was treated with DNase to eliminate potential genomic DNA contamination. The RT was performed using the ReverTra Ace kit according to the manufacturer's instructions and was used in the RT-qPCR (iQ5; Bio-Rad Laboratories, Inc.) assays. The negative controls without the RT enzyme were prepared and analyzed in parallel with the unknown samples during the

RT-qPCR assay. RT-qPCR was performed in a 20- μ l volume using SYBR Green real-time PCR master mix and the CFX96 system (Bio-Rad Laboratories, Inc.) with template cDNA equivalent to 200 ng RNA and 0.4-mM primers. The primer sequences for *BmPS* are shown in Table I, and the primers for the *BmActin3* gene, as the endogenous control, were as follows: Forward, 5'-GCGCGGCTACTCGTTCCTACC-3' and reverse, 5'-GGATGTCCACGTCGCACTTCA-3'. The two-step amplification protocol consisted of 3 min at 95°C followed by target amplification via 40 cycles at 95°C for 15 sec, 63.5°C for 30 sec and 72°C for 30 sec. The standard curves were generated by a 10-fold serial dilution of template cDNA, and the reaction efficiency (*E*) was determined by the equation $E=10^{(-1/\text{slope})}$. Quantification analyses of *BmPS* transcript expression relative to *BmActin3* were calculated according to the $2^{-\Delta\Delta C_t}$ method using the CFX Manager software (version 1.6; Bio-Rad Laboratories, Inc.). Data from triplicate experiments are expressed as the mean \pm standard deviation.

Sequence analysis. To analyze the intron/exon of the *BmPS*, the *BmPS* sequences were compared with silkworm genomic sequences from the National Center for Biotechnology Information (NCBI) GenBank database (<http://www.ncbi.nlm.nih.gov/>). The amino acid sequences of *PS* homologues from various species were also retrieved from the GenBank database using the basic local alignment search tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple sequence alignments were performed using the DNAMAN, and phylogenetic analysis was performed with MEGA software, version 5 (<http://www.megasoftware.net/>).

Statistical analysis of data. Student's one-tailed unpaired t-test was used to perform statistical analysis. A minimum of three independent replicates were used for each treatment and the error bars represent the mean \pm standard error.

Results

Cloning of the *BmPS* gene and its variants. To characterize the *B. mori* presenilin gene (*BmPS*), *BmPS* was cloned from 5th instar larval brains of the R13Q strain. A 1,460-bp fragment (designated BmC-1) was PCR-amplified using the specific primers F1 and R1 (as shown in Table I), which were designed from a conserved amino acid fragment of PSs from the silkworm EST database. Based on the sequence of the original BmC-1 clone, primers were designed for 5'- and 3'-RACE. A total of three fragments (394 bp, 345 bp and 195 bp) were amplified by 5'-RACE with the primer R2 and the nest primer R3 (as shown in Table I). Analysis of the sequence of three fragments indicated that the 5'-region of the *BmPS* transcripts contained at least three transcription start sites and upstream open reading frames (uORFs; Figs. 1 and 2). Only a 1,113-bp fragment was determined by 3'-RACE using the F2 and F3 primers (as shown in Table I and Fig. 2), which exhibited a putative polyadenylation signal (AATAAA) starting 15 nucleotides upstream of the polyA tail (Fig. 1).

To confirm that 5'-RACE, BmC-1 and 3'-RACE belong to the same transcript, the PCR amplification with primers F1-1 and R1-1 (as shown in Table I and Fig. 2) designed from the

Table I. Primer sequences designed for *Bombyx mori* presenilin cloning.

Primer	Primer sequence (5'-3')	Position ^a
F1	AGTGACTTAGACGCAACCGAGCA	Exon1
R1	GACTTCTATCTACCTTACGCACA	Exon10
F1-1	TTTGACTTTTATGGTTTTACC	Exon1
R1-1	TTAGTTACGATTTCGAATTTG	Exon10
R2	GCTGGAACTCGAGGTTGCTGGG	Exon2
R3	CCCCGATGGGCCACCTTCA	Exon2
R4	CACCTAGACCAAGTTTTACGCCT	Exon9
R5	ACCTTCGCCCTCGACAGTTC	Exon8
F2	ATTTGCGGACGCTTTGGC	Exon10
F3	GAACAGGTGTTTCATTTAGC	Exon10
F4	AACGCAATGAGCCTATATTTCCA	Exon5
F5	ATGGACGGCCACATCGCAGAA	Exon1

^aPositions correspond to Fig. 2. F, forward; R, reverse.

5'-UTR and 3'-UTR of *BmPS*s was performed. Only one fragment was obtained and sequence analysis indicated that the PCR products of 5'-RACE, BmC-1 and 3'-RACE belonged to the same transcript. In addition, a novel transcript was identified which had a 33 bp-deletion due to the alternative splicing (Fig. 1).

To determine the *BmPS* transcript combinations with multiple transcription start sites and the 33 bp of alternative splicing, 5'-RACE PCR was performed again with primers R4/R5, which are downstream of the 33-bp deletion. The two bands were amplified and sequence analysis of the 30 clones revealed that the *BmPS* gene had six transcripts termed *BmPS1* (2,656 bp), *BmPS2* (2,646 bp), *BmPS3* (2,630 bp), *BmPS4* (2,597 bp), *BmPS5* (2,480 bp) and *BmPS6* (2,447 bp), respectively (Fig. 2). The nucleotide sequences of *BmPS1* and *BmPS2* have been submitted to the GenBank/NCBI database with the accession numbers JQ993471.1 and JQ993472.1, respectively.

Compared with the silkworm genome, *BmPS* was a single gene in *B. mori* and there were at least six splice variants, which were composed of 14 exons (Fig. 2A). In all the alternative splice variants, the introns were in accordance with the GT-AG boundaries rule (data not shown). *BmPS1* (2,656 bp) was the longest transcript, which comprised 13 exons (exon 2-14) and contained a 153 nt 5'-UTR, a 1,440 nt ORF encoding a putative protein of 479 amino acids and a 1,063 nt 3'-UTR (Fig. 1). In addition, three uORFs were identified in the 5'UTRs of BmPS1-6. Among them, BmPS1 possessed uORF1 and uORF3, BmPS2/3/4 possessed uORF2 and uORF3 and BmPS5/6 did not possess any uORFs (Figs. 1 and 2B). The alternative splicing forms of BmPS2/4/6 has a 33 bp deletion (Fig. 2C).

Sequence alignment and phylogenetic analysis. The six transcripts encoded four *BmPS* isoforms, termed *BmPS-A* (*BmPS1/3*), *BmPS-B* (*BmPS2/4*), *BmPS-C* (*BmPS5*) and *BmPS-D* (*BmPS6*), which consisted of 479, 468, 463 and 452 amino acid residues, respectively. Alignment of the amino

AAAGTCTGACAGGTACCAGTACTTTAATGGTTTTACCTCATACTACTTTGTCGTGAGTTTATCTTTGTG 72
TTTTTTAAGATACTACGCCTACGCCTAAATTTAAACCTTGCCTTTTAAACAATGGGAGCATTATTCCAAAGATATTGA 153
ATGAGTGACTCAGGCAGTACTTAGACCGCAACCGAGCACACTGCACATCATGGACGGCCACATCGCAGAAGCTCGTCCCTGAC 234
1 M S E S G S D L D A T E H T A L M D G H I A E A R P D

AGGGAGTTAGCTGAAGGATAGCTAGGAAACGAAAGACTAAATCCGATAATCAGCGCTCCTATGGCAGTGTAGAACAGATT 315
28 R E L A E R I A R K R K T K S D N Q R S Y G S V E Q I

GAAGGTGGCCCATCAGGGGCTTCTATTGCCAGCAACCTCGAGTTCCAGCAGCACCAGTGGAAACCACAGATTGAGGAGCTG 396
55 E G G P S G A S I A Q Q P R V P A A P V E P Q I E E L

GAGTTGAAGTATGGGGCTCGCCATGTCATCAAACCTGTTTGTGCCTGTCACATTGTGCATGCTCGTAGTTGTTGCAACAATA 477
82 E L K Y G A R H V I K L F V P V T L C M L V V V A T I

TCATCTATAAATTTTTACTCCGTTAAGATGTATATTTGCCATATACACCGTTTCATGAAGAGAGTCCATACCCATCAACA 558
109 S S I N F Y S V K D V Y L A Y T P P H E E S P Y A S T

AAAGTGTGGAATGCATTGGCCAAATCGATGATCTTGTGTGTGTGATTGCATTAAATGACGGTGTACTTATTGTCTTTAC 639
136 K V W N A L A N S M I L L C V I A L M T V L L I V L Y

AAGAAAAATGCATAAGACTATTTCATGGATGGTTGATTCTCTCATCATTAACTTGTCTGTTTCTATTCTCATACTTGTAT 720
163 K K K C Y K T I H G W L I L S S L I L L F L F S Y L Y

ATTGAGGAAGCACTCATTGCCTATAATATACCAATGGATTATATAACTTTAGGCTTTGTGTGTGGAATTTTGGAGTCATG 801
190 I E E A L I A Y N I P M D Y I T L G F V L W N P G V M

GGCATGATTGTAATACATTGGAAGGGTCCGCTGAGGCTACAACAAGCTTACTTGATCTTCATTGCAGCCCTTATGGCACTT 882
217 G M I V I H W K G P L R L Q Q A Y L I F I A A L M A L

GTGTTTCATCAAGTATTTCGGGAATGGACAACATGGGCCGCTCCTTGCAGTATCTATTTGGGATTTGGTGGCTGTATTA 963
244 V F I K Y L P E W T T W A V L A V I S I W D L V A V L

ACACCGAAGGGACCTTTGAGAATATTAGTTGAAAACAGCACAGGAACGCAATGAGCCTATATTTCCAGCTCTCATTATTTC 1044
271 T P K G P L R I L V E T A Q E R N E P I F P A L I Y S

TCGACAGTGTACTGCATGGCGGAGCGAGCCCCCGCGCCCGCGCGAGGTGAGCGCGAGCTGGCCCGCTGCCAACCG 1125
298 S T V M Y C M A A A S P A P G A A G E R E L R P L Q P

CAAGGAATCTCGAGGGTGAAGGTCCGACGGCAGGAGCGCGGGGGGGGGAGGGCCGGTTTCGACGGCGCTGGAGGGAG 1206
325 H G T V E G E G R D G T E A G G G E A G P D A A W R E

CGGCGGATGAGGGTGGCGCGCGCGCGGCTACGGGTGCACTGGCGCGCGCGCTACGTCACGGCCCTCGACCCGCGG 1287
352 R R D E G A A P R R L R V D S A P P R Y V T R L D P P

CACGCGCCGCCACACGCGCCGACCTCGACGAGGAGAAGGGCGTAAACCTTGGTCTGGGTGATTCATATTCTATAGTGTG 1368
379 H A P P H A P D L D E E K G V K L G L G D F I F Y S V

CTCGTAGGAAAAGCAAGTTCATACGGCGATTGGAATACAACATTAGCCTGTTTTGTAGCTATACTTATAGGTTTATGTCTA 1449
406 L V G K A S S Y G D W N T T L A C F V A I L I G L C L

ACACTCCTGCTGCTCGCCATATTGAAGAAGGCTCTCCGGCGCTCCCTATATCGATAACATTCCGACTTATATTCTATTTTC 1530
433 T L L L L A I L K K A L P A L P I S I T P G L I F Y F

GCAACGGATCGGTCGTGAAGCCATTTCCGGACGCTTTGGCGGGGAACAGGTGTTCATTAG 1593
460 A T R S V V K P F A D A L A A E Q V F I *

CAAATACTCGTGTGCGTAAGGTAGATAGAAGTCGACAAACATTCTGTAAGAAGCAGATCACATTTTATGTTAAAATCAA 1674
TTTGCAATTTTTATTTAATTATTTTCGATTAATTAATAATGTGTTTCGATATGTGTATAAAGGAATTTATTTGTTGCA 1755
AAAAAACTCCAATATTTTCATTGAACGATGGGAAAAATAACTTATTTTGAATAATATCGGTAATTTCTTAAATCCGGA 1836
ACTAACGGATTTGGAAATAGTCAGGGTATCCCCGTAACCTAAGTTTCGCTAACCATACCGAATTTGATTTTTATTGCTAC 1917
CAGAGTCAATTTGATAGAGGGCCCGCTAGTGGTAATTACCATAGCCCGTGGACATCTGTGAAGATGAGCGCGGTGAGCTC 1998
ACTGTTAGCCTTTTTCTGTAATATCGCTCAATTTACCTTCAAATACAAAATTTGTTTACCATATAGACTTGTCTTAATA 2079
TCGGTCTGCATTAATGACAAATGTAATAATTAATTTGATTCCCTATTCATCGGCTTTGACTCCGCCCAATCGACGCAT 2160
AGGAGTGAAGGAACCTATTTTGGCGGACTAATGCTTCGCTTTTGTCTCAAATTTCCCATGCTGATCGTAGCAAGTGTAG 2241
TTAGCTGCGGCGTTTCCGTCGAAACGACGTTCCCTCATTAGTGAAGAAAGACTTTGAGTAGCGCCTCTAGTCCACGCTTAT 2322
AATGAAATACCCAGTCTAAAAGAACACTGAAAATACTGCAGACAAAATAAAAACACTATGTATTACAAAGATAACTCAA 2403
TTCGAATCGTAACATAAAATAAAATATTTCGTTTTTCATTTAGGATTTTCATACAAATTCACAACAATACAAACAAAAA 2484
CATTTTCAAAGTAAATGAACATTCCACGCAATTTGACTTATGGTAATTTAAATTAATTTATGTTAGTTAATATTATTAT 2565
AATTATCTGTTTGAAGTAACGTTTCGCAATGTAATAAAATTTAATACATAAGTTTAACTTTTATTTTAAATAAAATTTGA 2646
AACACCATAC polyA 2656

Figure 1. Full-length nucleotide and deduced amino acid sequences of the *BmPS* gene. Numbers on the right refer to the last nucleotide in each line in the corresponding line. The start codon (ATG), stop codon (TAG), and the polyadenylation signal are indicated by a broken underline, double underline and bold underline, respectively. The deduced amino acid sequence is stated below the nucleotide sequence and is numbered to the left. The upstream open reading frames are indicated by a hollow box. The alternative splicing area is shaded. *BmPS*, *Bombyx mori* presenilin.

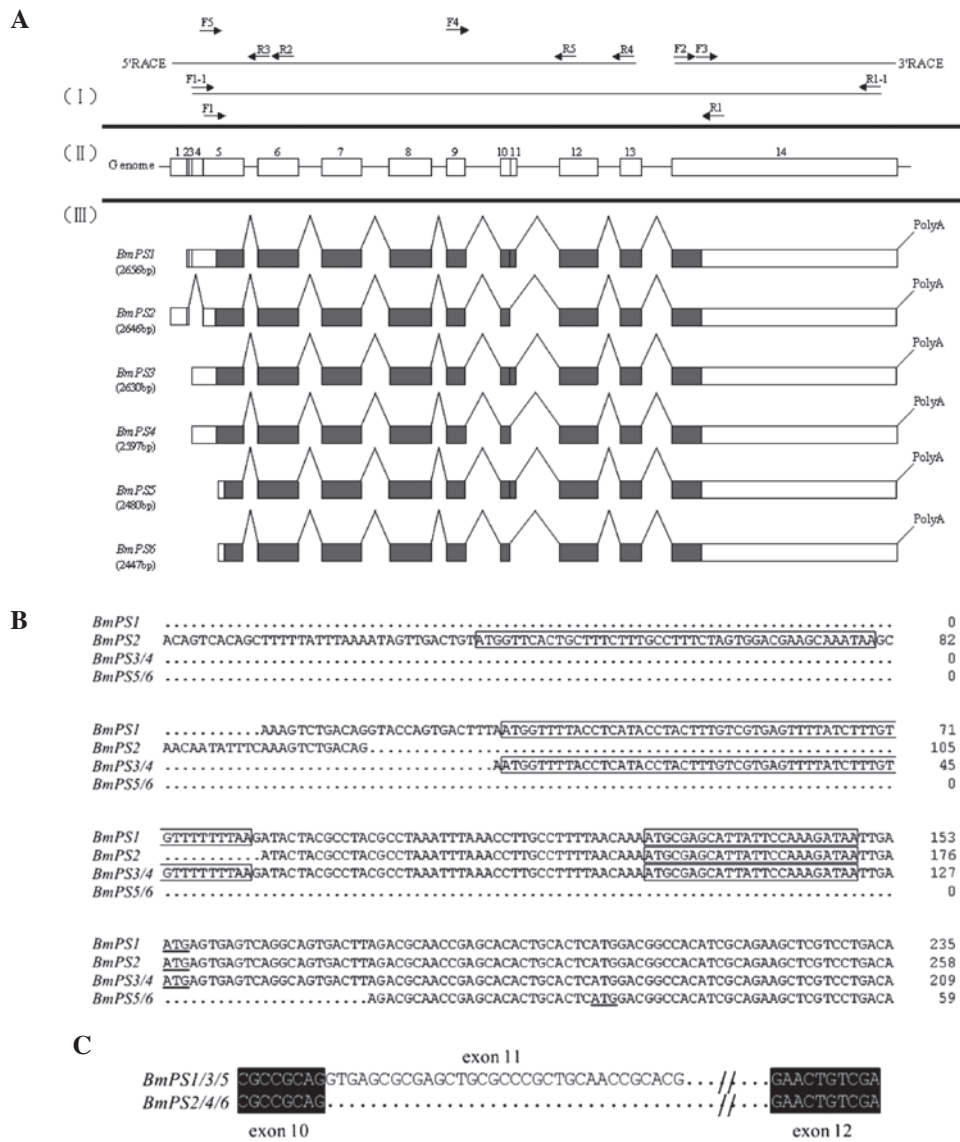


Figure 2. *BmPS* gene structure and splice variants. (A) (I): Primers for the polymerase chain reaction for *BmPS* cloning. Arrows indicate the position and direction of primers. A (II): Genomic organization of the *BmPS* gene. Exons and introns are indicated by white boxes and lines, respectively. A (III): Diagram of the *BmPS* splice variants. Coding regions are indicated by black boxes, non-coding regions by white boxes. Introns are indicated by disconnected lines. ATG: translation initiation; TAG: translation termination. (B) Multiple transcription start sites of *BmPS* transcripts. The upstream open reading frames and translation initiation sites are indicated by hollow boxes and underlines, respectively. (C) The nucleotide sequence spliced region of the *BmPS* transcripts in exon 11. Identical nucleotide residues of exon 10 and exon 12 are highlighted in black. *BmPS*, *Bombix mori* presentinlin; RACE, rapid amplification of cDNA ends; F, forward; R, reverse.

acid sequences of the BmPSs with those from mammals and insects indicated a high level of conservation (Fig. 3A). BmPS-A exhibited ~44% sequence identity to hPS1 and hPS2, ~51% to DPS and ~40% to sel-12. In the TMDs, BmPS shared sequence identities of 70% with the human PSs, including the two catalytic aspartate residues (Fig. 3A). Due to the sequence identities in the functional regions of the PS proteins, it was hypothesized that the BmPSs belong to the PS family. In addition, similarly to DPS, one of the BmPS alternative splicing events occurs in the loop region, which is located between TMD6 and TMD7 and had a markedly low level of conservation between species (Fig. 3A). The primers BmPS F1 and BmPS R1 were used to amplify the BmPS in the brains of the Dazao strain, and it was found that there was a 3-amino acid residue insertion in the loop region compared with the R13Q strain (Fig. 3A).

A phylogenetic tree was constructed to analyze the evolutionary associations of BmPS with PSs found in other insects, nematodes, amphibians, reptiles, avian and mammalian/primate species (Fig. 3B). The clustering pattern confirmed that BmPS was grouped in the lineage of other insects (red flour beetle, honey bee, fruit fly and mosquito), with a high bootstrap value, the highest similarity with the PS of *Tribolium castaneum*, whereas avian species (chicken), amphibians (frog), teleosts (zebrafish) and mammals (human and rat) grouped into distinct lineages.

Expression patterns of the B. mori PS gene. In order to determine whether the alternative splicing event in the loop region exhibited tissue-specific expression, the RT-PCR experiments with primers F4 and R5, detecting exon 11 in the ORF, were examined in different tissues of 5th instar silkworm larvae.

The two bands corresponding to the alternative splicing of *BmPS1/3/5* and *BmPS2/4/6* were detected in the silkworm tissues. The expression of *BmPS2/4/6* was more abundant than that of *BmPS1/3/5*; however, the ratio of *BmPS1/3/5* to *BmPS2/4/6* was 2.2-2.4 in the brain, fat body, silk gland and testes, and 2.7-3.0 in the prothoracic gland, the midgut, malpighian tubules and the ovaries (Fig. 4). This result indicated that *BmPS* and its alternative splicing are expressed in all of the tissues assessed in the present study.

As PS is important in neurodegenerative disorders in mammals and the developmental processes in *Drosophila*, the temporal expression of *BmPS* in the brain was investigated in the present study using RT-qPCR from the larval, pupal and adult stages (Fig. 5). The expression levels of *BmPS* were constant in the 4th instar and the initial stage of the 5th instar, and slowly declined between day 0 of the 5th instar and day 0 of the pupal stage. However, during the development of the pupal stage, the *BmPS* expression levels persistently increased and suddenly markedly improved in the adult stage. This dynamic expression pattern in the brain suggested that *BmPS* may have roles during *B. mori* development, particularly in the adult stages.

Discussion

PSs are highly conserved transmembrane proteins, which are aspartyl proteases with catalytic residues localized inside the lipid bilayer on TMD6 and TMD7 (2). There are several motifs conserved evolutionarily across numerous species in the two amino acid sequences. The most important motifs include YD and GxGD motifs, which contain catalytic aspartyl residues or an endoplasmic reticulum-retention sequence (35-37). The topology of PSs has established a view of PS as containing nine TMDs (38-40). In metazoans, PS is synthesized as a holoprotein and subsequently undergoes autocatalytic endoproteolysis between TMD6 and TMD7. The conserved two catalytic aspartate residues (Asp²⁵⁷ in TM6 and Asp³⁸⁵ in TM7 of hPS1) are essential for the released amino- and carboxy-terminal fragments (NTF and CTF, respectively), which are associated with conformational changes in the complex and function together as a heterodimer (41-43). In the present study, a *B. mori* homologue of the PS gene was cloned and characterized. The deduced amino acid sequence of BmPS is equally homologous to DPS (~51% sequence identity), hPS1/hPS2 (~44% sequence identity), the highest similarity with insect PS, particularly *T. castaneum* PS. A total of nine TMDs and the hydrophilic domains at the beginning of the TM6-TM7 loop domain exhibited >70% identity to DPS and hPSs. Two highly-conserved aspartate residues of PS, Asp²⁵⁷ in TM6 and Asp³⁸⁵ in TM7 of hPS1, corresponded to Asp²⁶⁵ and Asp³⁹⁷ in BmPS-A. Furthermore, the mutations in hPS1/hPS2, present in human familial AD, have been reported (for a list of the mutations, see molgen.ua.ac.be/ADMutations) (4,44,45). Of note, among the 20 amino acid residues of hPS1/hPS2 mutated in patients with familial AD, 17 are identical and three represent conserved substitutions in BmPS, indicating that the conserved mutations may be important for functions and/or structures of PS. This result suggested that the BmPS cloned in the present study is a member of the PS family and may be relevant to the pathogenesis of AD.

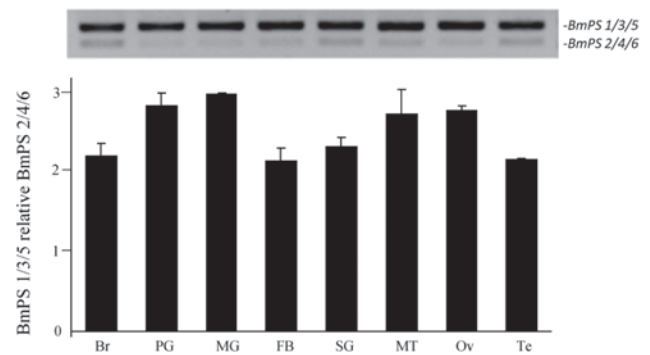


Figure 4. RT-PCR analysis of *BmPS* splice variants in tissues of 3rd- and 5th-day instar larvae of *Bombyx mori* (R13Q strain). *BmPS1/3/5* and *BmPS2/4/6* were detected in the Br, PG, MG, FB, SG, MT, Ov and Te, and the quantification of the RT-PCR results produced by gel imaging system is shown. Data are presented as the mean \pm standard error; 3 independent experiments. L, larval; P, pupal; A, adult; *BmPS*, *Bombyx mori* presenilin; RT-PCR, reverse transcription quantitative polymerase chain reaction; Br, brain; PG, prothoracic gland; MG, midgut; FB, fat body; SG, silk gland; MT, malpighian tubules; Ov, ovaries; TE, testis.

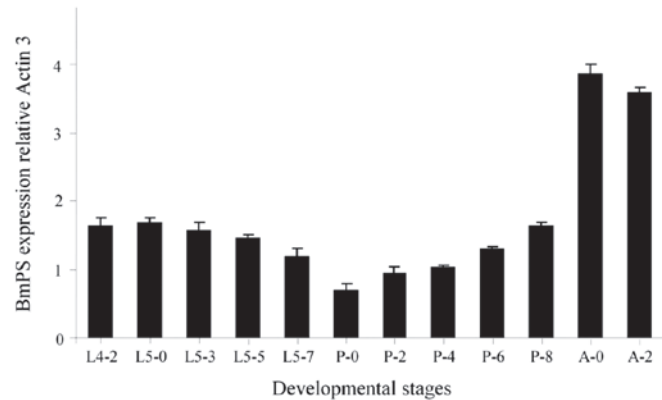


Figure 5. Temporal expression pattern of *BmPS* investigated by reverse transcription-quantitative polymerase chain reaction in the brains of *Bombyx mori* (Dazao strain) developmental stages. L4, L5, P and A present fourth larval instar, fifth larval instar, pupal and adult development, respectively. Numbers in each stage refer to the day in the corresponding developmental stage. Total RNA was extracted from the brain. Expression levels were normalized to the expression of the silkworm Actin 3 gene. Each point represents the mean values of three experiments and error bars indicate the standard error. L, larval; P, pupal; A, adult; *BmPS*, *Bombyx mori* presenilin.

Human PSs have two homologues, hPS1 and hPS2, encoded by two genes on chromosome 14 and chromosome 1, respectively. However, invertebrate PSs, such as *Drosophila* PS, derived from a single gene, exhibit several splice variants (46,47). In *B. mori*, PS has at least six alternative splice sites corresponding to four isoforms, which belong to a single gene. Of note, three uORFs were identified in the 5'-UTR of *BmPS*. uORFs in the 5'-UTR of an mRNA transcript are able to modulate the translational efficiency of the major ORF (mORF), and disruptions of a functional uORF are associated with human genetic diseases (48-51). In *Drosophila*, ~40% of transcripts contain uORFs (52). *DPS* genes also have three uORFs in the 5'-UTR of mRNA (data from FlyBase <http://flybase.org/>). An upstream ORF has been observed to be involved in Sex lethal to inhibit *msl-2* translation during *Drosophila* gender determination (53). Although

uORF-mediated translational control has been rarely reported in *Bombyx mori*, the translational efficiency of the mORF of *BmPS* may be modulated by the uORFs, highlighting the potential physiological implications of *BmPS*.

Although PSs share extensive homology throughout their length, the N-terminal domain and the large hydrophilic loop facing the cytosolic compartment between TM6 and TM7 are highly divergent between PS1 and PS2, and are poorly conserved across species. However, numerous PS-linked mutations have been mapped to specific amino acid residues within the loop region, which are highly conserved between species (54,55), the filamin (an actin-binding protein) and the methyltransferases (an evolutionary conserved protein in a variety of species) in *Drosophila* interact with the loop region of either human or *Drosophila* PSs (56,57), suggesting the functional importance of the loop. Analyzing the *BmPS* splice variants, the alternative splicing occurs only in the large hydrophilic loop region between TMD6 and TMD7, as exon 11. Similarly, the alternative splice variants of DPS predominantly occur in this region (58). Of note, comparison of *BmPS* between two silkworm strains R13Q and Dazao showed that the three amino acid residues were inserted in this region in Dazao. It has been established that the strains R13Q and Dazao exhibit morphological and physiological differences, including body stripe, size, voltinism and life span. Although it remains to be elucidated whether the alternative splicing in the TM6-TM7 loop of *BmPS* has an important function, there remains reason to hypothesize that *BmPS* may be associated with certain physiological processes in *B. mori*.

PS has been implicated in numerous molecular processes, including Notch signaling, the metabolism of β -catenin and calcium homeostasis (2,19,59-62). mRNAs of PSs are ubiquitously detected in a number of human and mouse tissues, including the brain, heart, kidney and muscle (63). In *Drosophila*, DPS is expressed at different developmental stages, exhibiting higher levels of expression in adults than larvae, and is predominantly expressed in the central nervous system (46,47,58). In the present study, *BmPS* and its splice variants were observed to be expressed in the silkworm tissues, including the brain, fat body, silk gland, testis, prothoracic gland, midgut, malpighian tubules and ovaries, and the expression of *BmPS* in the developmental brain was higher in the adult stage. This dynamic expression pattern of *BmPS* suggested that it may have multiple roles during *Bombyx* development.

While vertebrate models provide a closer match to humans from an evolutionary perspective, invertebrates, including the nematode worm *C. elegans* and the fruit fly *D. melanogaster*, have been used extensively in transgenic experiments and classical genetic analyses to investigate the function of AD-relevant genes and to analyze the underlying mechanisms of AD (16,17,64). The Notch signaling pathway was initially linked to AD in *C. elegans* and *D. melanogaster*, contributing greatly to the current understanding of the PS-mediated pathogenesis of AD. Although the silkworm *B. mori*, as a model organism, has not been previously utilized in the study of AD, the present study on *B. mori* PSs may provide valuable insight into the mechanisms of silkworm development regulation and the pathogenesis of AD.

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