Molecular Cloning, Expression and Characterization of *BmIDGF* Gene from *Bombyx mori*

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Imaginal disc growth factors (IDGF) play a key role in insect development, but their mechanism remains unclear. In this study, we cloned a novel IDGF gene in *Bombyx mori* and designated it as *BmIDGF*. We found that the *BmIDGF* gene contains eight exons and seven introns, encoding a peptide of 434 amino-acid residues. The protein was predicted to contain one conserved motif of the glycosyl hydrolases family 18 and fall into group V chitinases. Sequence alignment showed that BmIDGF shares extensive homology with other invertebrate IDGF. RT-PCR analysis showed that BmIDGF is expressed in all developmental stages of silkworm larvae and various larvae tissues, which was further confirmed by Western blot analysis. Subcellular localization analysis indicated that BmIDGF is located in the extracellular space. We also successfully expressed it in *E. coli* and further characterized it by SDS-PAGE and mass spectrometry. Taken together, our data suggests that BmIDGF is a chitinase-like extracellular protein, and provides an excellent platform for subsequent studies on its enzyme activity and role in *B. mori* development.

Key words: IDGF, *Bombyx mori*, Subcellular Localization

Introduction

In multicellular animals, cell size and cell number are tightly regulated by different growth factors. Some growth factors act externally through cell surface receptors such as epidermal, platelet-derived, and insulin-like factors, whereas some have binding proteins associated with them (Hayakawa and Ohnishi, 1998). Some growth factors have been found in insects, such as the imaginal disc growth factor (IDGF) (Kawamura *et al.*, 1999), adenosine deaminase-related growth factor (ADGF) (Homma *et al.*, 1996; Zurovec *et al.*, 2002), bombyxin (Nagata *et al.*, 1995), growth-blocking peptide (Hayakawa and Ohnishi, 1998), and tsetse salivary growth factor from *Glossina morsitans* (Li and Aksoy, 2000).

Chitinases play an important role in the development of insects, some insect chitinases are involved in degradation of chitin from the exoskeleton cuticle or from the midgut peritrophic membrane during molts. The IDGF shows high similarity to chitinase enzymes and belongs to group V of the chitinase-like family (Zhu *et al.*, 2008a). The IDGF is a soluble polypeptide growth factor that was firstly identified from the conditioned medium of *Drosophila* imaginal disc C1.8+ cells, but it is also expressed in larval glands and fat body. It is secreted and transported to target tissues *via* the hemolymph (Kawamura *et al.*, 1999). However, the *Drosophila melanogaster* IDGF, *Drosophila melanogaster* DS47, *Tribolium castaneum* IDGF2, and *Tribolium castaneum* IDGF4 were demonstrated to be devoid of chitinolytic activity, although they could act as carbohydrate-binding proteins and bind very tightly to an insoluble ligand, colloidal chitin. Therefore, it will be very interesting to further investigate their physiological roles. In *Bombyx mori*, multiple chitinase genes were also identified (Abdel-Banat and Koga, 2001; Kanost *et al.*, 1994; Mikitani *et al.*, 2000), but, to our knowledge, there is no report on the characterization of the *B. mori* IDGF (*BmIDGF*) gene. In the present study, we carried out the cloning

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and characterization of the *BmIDGF* gene, and found that BmIDGF is expressed in all developmental stages of silkworm larvae and various larvae tissues. We also demonstrated that BmIDGF is located in the extracellular space. This cloned gene has been registered in GenBank under the accession number AB183872.

Experimental

Insect and cell line

B. mori strain C108 (standard strain of silkworm) was maintained in our laboratory. All larvae were reared with fresh mulberry leaves at 27 ºC under a 12 h light/12 h dark photoperiod.

The BmN cell line was cultured at 27 ºC in TC-100 insect medium supplemented with 10% (v/v) fetal bovine serum (Gibco-BRL, Carlsbad, USA) using standard techniques.

Bioinformatic analysis

Sequence analysis was carried out online at http://www.ncbi.nlm.nih.gov and http://cn.expasy.org. In order to obtain the genomic organization, the cDNA sequence was blasted to the contigs of *B. mori* genome in GenBank, and SIM4 (http://pbil.univlyon1.fr/sim4.php) (Florea *et al.*, 1998) was used to align the cDNA sequence with the genomic sequences to search potential introns. The sequence comparison was conducted through database search using the BLAST program (National Center for Biotechnology Services, http://www.ncbi.nlm.nih.gov), and similarity analysis was performed by GENEDOC. A phylogenetic tree was constructed using MEGA version 4.1 (Tamura *et al.*, 2007) from CLUSTAL W alignments. The neighbour-joining method (Saitou and Nei, 1987) was used to construct the tree. Determination of the location of the gene on *B. mori* chromosomes was carried out online at http://silkworm.swu.edu.cn/silksoft/silkmap.html. PLOC (http://www.genome.jp/SIT/plocdir/) (Park and Kanehisa, 2003) was used to predict the subcellular location.

RNA extraction and RT-PCR

The eggs from 0 h to 10 d and 1st to 5th instar larvae were collected and the various tissues (testis, midgut, hemocytes, silk gland, Malpighian tubule, fat body, and ovary) from C108 larvae at day 5 of the 5th instar were dissected, then immediately frozen in liquid N_2, and stored at −80 ºC for later use. Total RNA was extracted from frozen samples with RNeasy mini kit (Qiagen, Hilden, Germany), treated for 20 min at 37 ºC with RNase-free DNaseI (TaKaRa, Dalian, China), and finally dissolved in diethylpyrocarbonate (DEPC)-treated water. cDNAs were generated from these RNA using M-MLV RTase (Promega, Madison, WI, USA) and an oligo-DT primer following the manufacturer's instructions.

To determine the tissue distribution of *BmIDGF* transcripts, the 5th strain C108 larvae tissues of testis, midgut, hemocytes, silk gland, Malpighian tubule, fat body, and ovary were analyzed by RT-PCR. PCR was performed using the following primers for *BmIDGF*: BmIDGF-F, 5' cg GAATTC (EcoR I) ATGAAGCTATTTATCGCTCTAGTCG 3'; BmIDGF-R, 5' ccg CTCGAG (Xho I) GAGACGGTATTTAGCGGCC 3', where the underlined characters indicate the restriction enzyme sites. A 284-bp fragment of *B. mori* actin A3 (*Bombyx mori* cytoplasmic actin gene A3) was amplified in parallel, as an internal control for quantification, using the following primers: Bm-actin A3-F, 5'-GCGCGGCTACTCGTTCACTACC-3'; Bm-actin A3-R, 5'-GGATGTCCACGTCGCACTTCA-3'. The PCR reaction was carried out for 35 amplification cycles (94 ºC/60 s, 58 ºC/45 s, and 72 ºC/90 s) in a Gene Amp 2400 System thermocycler (Applied Biosystem, Foster City, CA, USA). Agarose gels (1%) were used for electrophoreses.

To compare the expression levels of the *BmIDGF* gene during the different developing stages, similar PCR analysis was also performed with egg samples from 0 h to 10 d and 1st to 5th instar larvae.

Protein expression in E. coli

The above PCR product was ligated into the pMD18-T vector (TaKaRa) using T4 DNA ligase (TaKaRa) and then transformed into *E. coli* (TG1 strain) for amplification. The obtained plasmid pMD18-T/BmIDGF was digested with EcoR I and Xho I, and then the purified fragment was subcloned into the pET30a (+) expression vector (Novagen, La Jolla, USA) in frame with the *N*-terminal 6×His tag and transformed into *E. coli* (BL21 strain) for protein expression.

For expression of recombinant protein, a positive clone was cultured in LB medium supple-

mented with kanamycin (50 μg/ml) overnight at 37 ºC with shaking. This culture was added into fresh LB medium and cultured at 37 ºC with vigorous shaking to an A_{600} value of 0.6. The culture was then induced with IPTG (final concentration of 0.2 mmol/l) and further cultured for another 5 h at 37 ºC. 12% SDS-PAGE was used to analyze the recombinant protein. SDS-PAGE was performed in the Mini-Protein system (Bio-Rad, Richmond, CA, USA). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R250 to visualize the protein bands.

Mass spectrometric analysis

According to the protocol for mass spectrometry as described by instruction, briefly, the specific bands were excised manually from the gel with a sterile scalpel and digested with trypsin after a series of processing. The digested samples were analyzed using an ultraflex II MALDI-TOF mass spectrometer (Bruker, Bremen, Germany). Peptide mass fingerprinting was performed using MASCOT search engine (http://www.matrixscience.com) against the NCBI protein database.

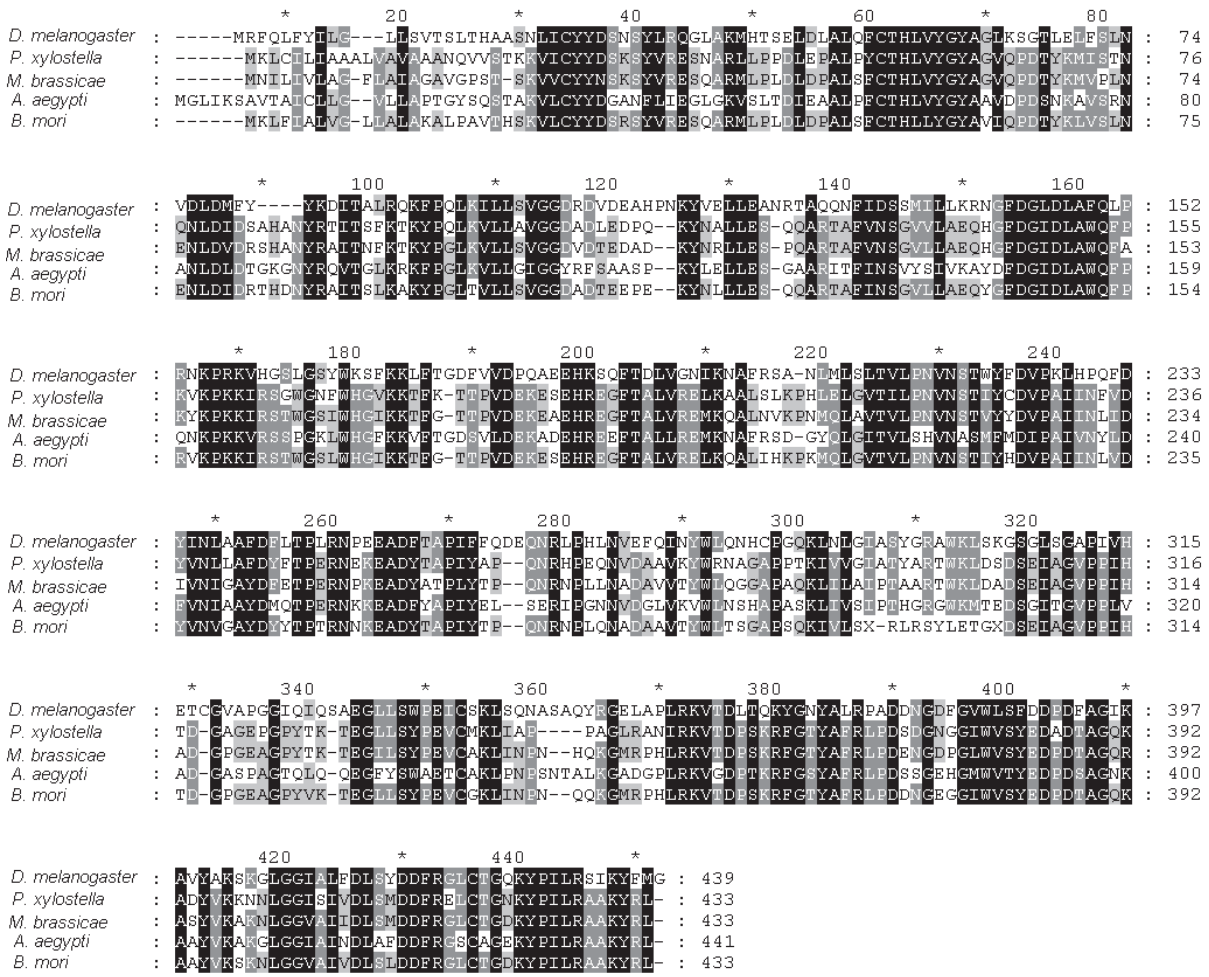

Fig. 1. Sequence alignment of *B. mori* IDGF protein and group V chitinases. The alignment was performed by BLAST and edited with GENEDOC. The identical amino acids are shown in white with a black background, and the conserved amino acids are shown in white with a grey background. The sources of sequences are: *Bombyx mori* (accession no. AB183872), *Drosophila melanogaster* (accession no. AAM69636), *Plutella xylostella* (accession no. AB282642), *Mamestra brassicae* (accession no. DQ355162), and *Aedes aegypti* (accession no. XP_001660748).

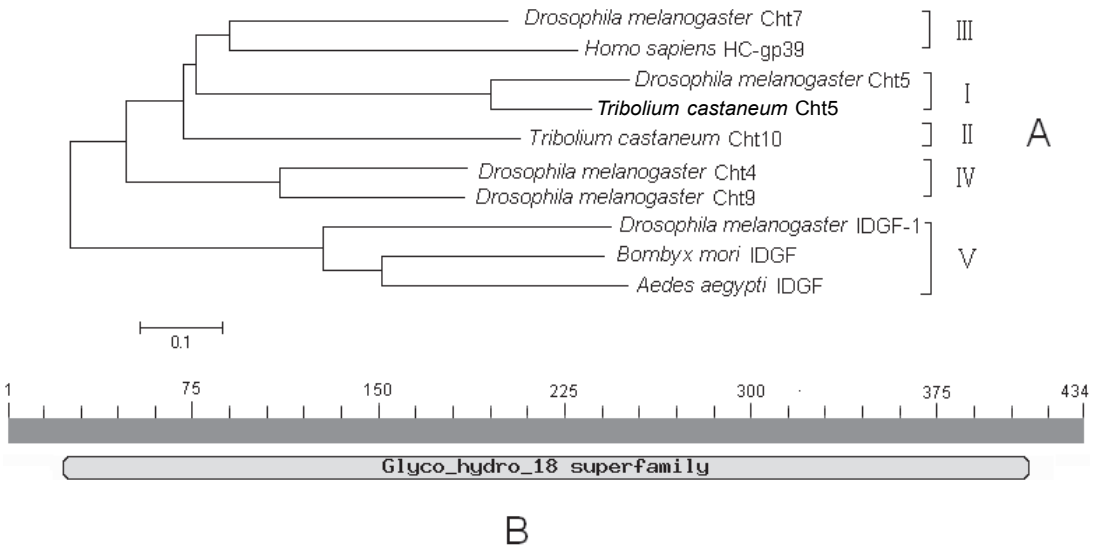

Fig. 2. (A) A neighbour-joining tree showing the phylogenetic relationship of BmIDGF with other chitinases family members. The distance scale indicates the number of amino acid substitutions per site. The deduced amino acid sequences in this figure are from GenBank with the following accession numbers: *Bombyx mori* IDGF (AB183872), *Drosophila melanogaster* Cht4 (NP_524962), *Drosophila melanogaster* Cht5 (NP_650314), *Drosophila melanogaster* Cht7 (NP_647768), *Drosophila melanogaster* Cht9 (NP_611543), *Drosophila melanogaster* IDGF-1 (AAM69636), *Tribolium castaneum* Cht5 (NP_001034517), *Tribolium castaneum* Cht10 (NP_001036067), *Homo sapiens* HC-gp39 (NP_001267), *Aedes aegypti* IDGF (XP_001660748). (B) Prediction of conserved domain of BmIDGF protein.

Antibody production and immunoblot analysis

The 6×His-tagged recombinant BmIDGF protein was purified using the Ni resin (Novagen) and used to raise polyclonal antibodies in rabbits. The antibody was prepared by standard techniques. Briefly, purified BmIDGF protein (about 2 mg) was injected subcutaneously to immunize New Zealand white rabbits in complete Freund's adjuvant, followed by two booster injections in incomplete Freund's adjuvant within a gap of 2 weeks before exsanguinations. Then, the polyclonal rabbit antibody against 6×His-BmIDGF was obtained and used for the immunoassay.

After SDS-PAGE (Bio-Rad Mini-Protean II) was finished, the proteins were transferred to a PVDF membrane with a Bio-Rad liquid transfer apparatus for Western blot. The rabbit anti-BmIDGF polyclonal antibodies (1:1,000 dilution) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (1:2,000 dilution) were used, and signals were detected by diaminobenzidine (DAB) (Sigma, St. Louis, MO, USA).

Immunofluorescence microscopy

BmN cells seeded onto coverslips were washed with PBS, and fixed with 2 ml of 4% paraformaldehyde for 15 min. Then cells were washed three times with PBS and permeabilized with 0.1% Triton X-100 in PBS for 15 min. After washing three times with PBS, cells were incubated with anti-BmIDGF antibody (1:1,000) as primary antibody and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody as secondary antibody (1:3,000) (Sigma). Then nuclei were stained with DAPI (Roche, Basel, Switzerland) and examined with a confocal laser scanning microscope (Zeiss lsm 5 live, Jena, Germany).

Results

Bioinformatic analysis of BmIDGF gene

The *BmIDGF* cDNA contains an open reading frame of 1,305 bp encoding a protein of 434 amino-acid residues with a calculated molecular mass of 48.2 kDa. The protein consists of only one conserved signature motif of the glycosyl hydrolases family 18. The BmIDGF protein was predicted to

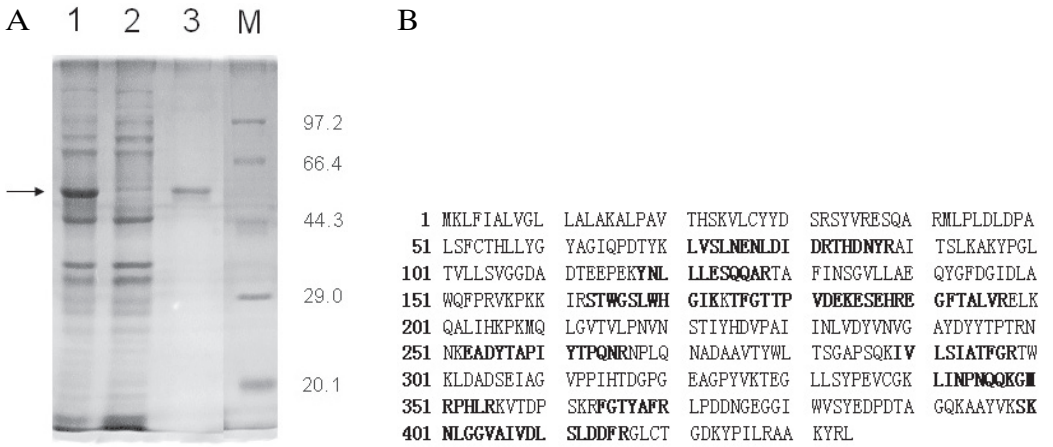

Fig. 3. (A) Expression of BmIDGF protein in *E. coli*. Lane 1, total cell lysate for pET30a (+)-BmIDGF transformants; lane 2, total cell lysate for pET30a (+) transformants as control; lane 3, the purified recombinant BmIDGF protein; M: molecular mass marker. (B) Identification of the BmIDGF protein by MALDI-TOF analysis. The amino-acid sequence of BmIDGF peptide sequences identified by mass spectrometry is shown as bold characters.

be an extracellular protein. Using SIM4 to align the cDNA sequence with Ctg000293, eight exons and seven introns were found in the genome DNA sequence.

By BLAST the amino-acid sequence of the BmIDGF protein showed an identity of 48%, 77%, 81%, and 55% to the corresponding genes from *Drosophila melanogaster* (accession no. AAM69636), *Plutella xylostella* (accession no. AB282642), *Mamestra brassicae* (accession no. DQ355162), and *Aedes aegypti* (accession no. XP_001660748). Analyzed by GENEDOC (Fig. 1), the BmIDGF protein has high homology with the IDGF from other insect species, and according to the phylogenetic tree analysis (Fig. 2A) and conserved motif prediction (Fig. 2B), the BmIDGF protein belongs to the group V chitinases.

Protein expression and characterization by mass spectrometry

The plasmid pET30a (+)-BmIDGF was transformed into *E. coli* (BL21); then IPTG was used to induce protein expression (Fig. 3A). The expressed protein was mainly in form of inclusion body. The protein was purified by a Ni resin, and the purified protein was used as antigen to immunize New Zealand white rabbits to prepare polyclonal antibodies.

The peptide mass fingerprint has a high signal-to-noise ratio (Fig. 3B). Using mascot to analyze the mass spectrometric data, the protein was proved to be the BmIDGF protein with a high score of 113, and the sequence coverage was 29%, which demonstrated the credibility of our result.

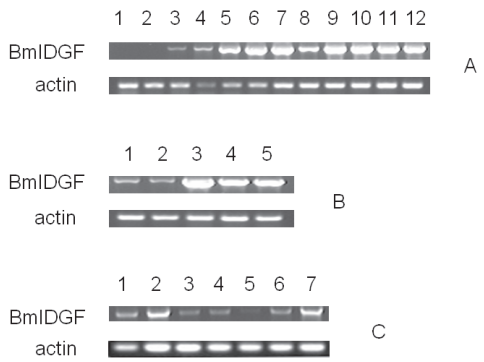

Fig. 4. (A) RT-PCR analysis of *BmIDGF* gene in developing *B. mori* eggs. Lanes 1–12 represent 0 h, 5 h, 1st day, 2nd day, 3rd day, 4th day, 5th day, 6th day, 7th day, 8th day, 9th day, and 10th day. (B) RT-PCR analysis of *BmIDGF* gene in developing *B. mori* larvae. Lanes 1–5 represent 1st instar, 2nd instar, 3rd instar, 4th instar, and 5th instar. (C) Expression profile of *BmIDGF* gene in different tissues of *B. mori*. Lanes 1–7 represent fat body, testis, hemocytes, silk gland, midgut, Malpighian tubule, and ovary. *B. mori* actin A3 was used as control to normalize the PCR reactions.

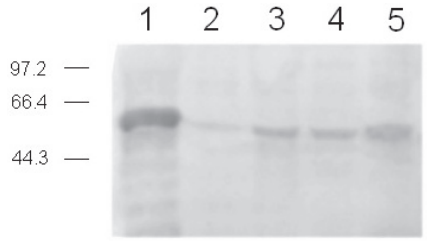

Fig. 5. Western blot analysis of BmIDGF protein in different *B. mori* tissues. Molecular mass standards are shown on the left lane. Lanes 1–5 indicate hemocyte, gut, ovary, silk gland, and fat body.

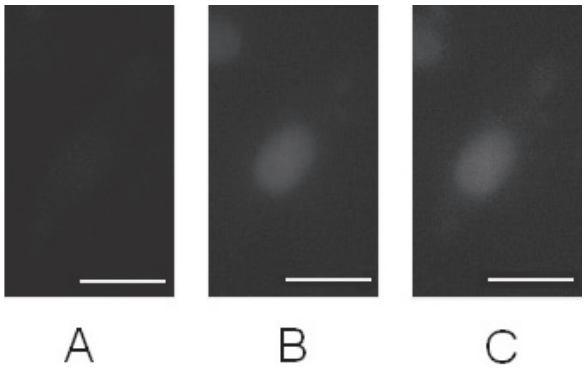

Fig. 6. Subcellular localization of BmIDGF protein in BmN cells. BmN cells were treated with anti-BmIDGF antibody, followed by treatment with FITC-conjugated goat anti-rabbit IgG and DAPI. (A) Fluorescence (green) for BmIDGF protein. (B) Nuclei were stained blue by DAPI. (C) The merged images. Bars = 10 μm.

Developmental and tissue expression pattern of BmIDGF gene

The levels of *BmIDGF* gene transcripts in various developmental stages of silkworm were examined by RT-PCR (Figs. 4A and B). The results indicated that mRNA was detectable from egg to 5th instar larvae. To investigate the expression profile of the *BmIDGF* gene, the total RNA from 5th instar larvae was isolated from testis, ovary, hemocytes, fat body, midgut, silk gland, and Malpighian tubules and subjected to RT-PCR. The results showed that *BmIDGF* gene expression could be detected in all tissues (Fig. 4C). Western blot analysis of different tissues further confirmed the presence of the BmIDGF protein in gut, ovary, silk gland, hemocyte, and fat body (Fig. 5).

Subcellular localization of BmIDGF protein

The intracellular localization of the BmIDGF protein was determined by immunofluorescence using prepared anti-BmIDGF serum. The BmIDGF protein was not detected in the cytoplasm and nucleus, suggesting that the BmIDGF protein is a secreted extracellular protein (Fig. 6).

Discussion

Insect chitinase-like proteins (glycosyl hydrolase family 18) are encoded by a rather large and diverse group of genes, and classified into five groups based on phylogenetic analyses. Little is known about the functions of most of these groups of chitinase-like proteins or their properties with the exception of the group I enzymes and one group IV enzyme (Genta *et al.*, 2006). The imaginal disc growth factors (IDGF) belong to the group V chitinases, which are chitinase-like in amino-acid sequence but are devoid of catalytic activity, most likely, these proteins bind to chitin or other carbohydrates containing *N*-acetylglucosamine (GlcNAc) (Zhu *et al.*, 2008a). Included in this group is another protein, HAIP from the hemolymph of *M. sexta*, which inhibits hemocyte aggregation (Kanost *et al.*, 1994). Thus, our work presented here represents the first report about cloning and characterization of a *B. mori* IDGF, designated as *BmIDGF*.

Through nucleotide sequence analysis we found that this gene contains an 1305-bp ORF, encoding a peptide of 343 amino-acid residues, and has eight exons. The BmIDGF protein has high homology with IDGF of other species, indicating a highly conserved and similar role for these IDGF. RT-PCR analysis revealed that this transcript widely exist in different *B. mori* tissues and different stages of development, suggesting the essentiality of its activity. We further successfully expressed the BmIDGF protein in *E. coli* and characterized it by SDS-PAGE and mass spectrometry.

Finally, our subcellular localization analysis showed that the BmIDGF protein is a extracellular protein. The extracellular IDGF may interact with membrane-bound glycoprotein receptors and modulate their signal transduction cascades.

Alternatively, they may affect the adhesive properties of the target cells (Zhu *et al.*, 2008b). Therefore, BmIDGF is believed to play an important role in the development of *B. mori*. The identification and analysis of the BmIDGF should help to understand the functions of the group V chitinases and their relationships to disease states.

Acknowledgements

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