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First Characterization of Sphingomyeline Phosphodiesterase Expression in the Bumblebee, *Bombus lantschouensis*

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Abstract

The bumblebee (*Bombus lantschouensis* Vogt) is an important pollinator of wild plants. Sphingomyelin phosphodiesterase (SMPD) is a hydrolase that plays a major role in sphingolipid metabolism reactions. We report the preparation and characterization of a polyclonal antibody for bumblebee SMPD. We then use the polyclonal antiserum to detect the SMPD protein at different development stages and in different tissues. Our results showed that a 1228bp fragment homologous with the *B. terrestris* SMPD gene was successfully amplified. The molecular weight of the fusion protein was about 70kDa by SDS-PAGE. An effective polyclonal antibody against SMPD was also obtained from mice and found to have a higher specificity for bumblebee SMPD. Western blotting detection showed that SMPD was expressed at a high level in queen ovaries, although expression was lower in the midgut and venom gland. SMPD expression decreased from the egg stage until the pbl pupal stage. We interpret our results as showing that the development of an effective polyclonal antiserum for the SMPD protein of a bumblebee, which provides a tool for exploring the function of the SMPD gene. In addition, the work has confirmed that SMPD should be considered as an important enzyme during bumblebee egg and larval stages.

Introduction

Sphingomyeline phosphodiesterase (SMase) is a hydrolytic enzyme that is involved in metabolic reactions of sphingolipids. SMase generates ceramide through the hydrolysis of sphingomyelin to phosphocholine and ceramide to regulate the ceramide-mediated cell (Ago et al., 2006; Hofmann et al., 2000; Kramer et al., 2015). Sphingomyeline phosphodiesterase gene (*SMPD*) was first cloned from *Bacillus cereus* (Yamada et al., 1988). SMases are classified into three categories based on its pH activity profiles. For the optimal activity pH value, the acid SMase (aSMases) was reported to be 4.5~5 (Brady et al., 1966; Jenkins et al., 2009), neutral SMase (nSMases) near pH 7 (Sabourdy et al., 2008), and the alkaline SMase is about pH 9 (Kraut, 2011). Among these sphingomyelinases, the acid SMase was the most explored in the past few years (Brady et al., 1966; Jenkins et al., 2009; Bartelsen et al., 1998).

Currently, it has been reported that SMase is correlated to many human diseases, such as Niemann-Pick, lung or liver diseases, neuronal disorders, sepsis organ failure, non-hemolytic enterotoxin, and regulation of immune response (Ago et al., 2006; Horinouchi et al., 1995; Smith & Schuchman 2008; Doll et al., 2013). It has been demonstrated that the function of SMase can inhibit colonic cancer and colitis (Herterving et al., 1997; Sjöqvist

et al., 2002). Moreover, increased secretion levels of SMase could also lead to accelerated at herogenesis (Truman et al., 2011). Although the function of SMase in human research has been explained clearly, little work has been done on insect SMase, except in flies. Based on the homology analysis of *Drosophila* (Diptera: Drosophiloidade) genome, five paralogues of *SMPD* gene were annotated and research explorations have demonstrated that they are highly expressed at flies embryonic development stages (Renault et al., 2002). Furthermore, based on microarray analysis, it was found that *SMPD* expression levels decreased with the sugar regulation (Zinke et al., 2002). Interestingly, it was suggested that the silence of *SMPD* gene by siRNA has strong influence on pregnancy and progeny development in *Glossina morsitans morsitans* Westwood (Diptera: Glossinidade) (Benoit et al., 2012). However, the function of SMPD in bees hasn't been explored.

Bombus lantschouensis Vogt (Hymenoptera: Apidae) is a native bumblebee species that play an important role for wild plant pollination in China (Peng et al., 2009; An et al., 2010). Furthermore, it has been a candidate species for year round rearing and greenhouse crop pollination in recent years. The investigations of basic biology of *B.lantschouensis* are very important for its successful year round rearing. According to a comparative proteomics analysis, *SMPD* gene was found to be significantly different in expressions between egg-laying and non-egg-laying queens (Unpublished data). Therefore, it was important to explore the basic biology of SMPD in bumblebee.

Since SMPD plays an important role in sphingolipid metabolic reactions, investigating SMPD protein profileswill help us to understand its functions in bumblebees. In this study, we described the clone and expression of the recombinant protein with a partial fragment of *SMPD* gene from *B. lantschouensis*. The fusion protein was purified as antigen for antiserum preparation against mice. Furthermore, the specificity of antiserum was validated on His-tag expression fusion protein and original bumblebee protein. Finally, the SMPD expression profile was detected at the different tissues and development stages of *B. lantschouensis* by western blotting. This antiserum can be used to explore the function of *SMPD* gene in further research.

Materials and Methods

Bumblebees

Colonies of *B. lantschouensis* were obtained from Department of Insect Pollination and Ecology laboratory of Institute of Apicultural Research, Chinese Academy of Agricultural Sciences. The colonies were supplied with pollen pellet and sugar syrup (1:1) every other day. The rearing boxes were kept at a temperature of 28 ± 2°C and 60 ± 10% relative humidity according to a method described by Kwon (Kwon et al., 2003). All samples were prepared and kept at -80°C until used.

SMPD amplification and fusion expression vector construction

The total RNA was isolated from the abdomen of *B. lantschouensis* workers through a technique that uses TRIzol (Invitrogen, USA), according to the manufacturer's instruction. The complementary DNA was synthesized from 1μg of RNA using a kit (Takara, Dalian, China) following manufacturer's manual. The forward primer 5'-CCGGAATTCAACAGTTTGTGGCGTTGTTCTA-3' and reverse primer 5'- AAATATGCGGCCGCGTGTTTCA TTCTTTGCTGCTG-3' with *Not*I and *Eco*RI restriction enzyme sites were designed and used to amplify SMPD coding gene sequence. At the same time, a pair of similar primers with *Eco*RI and *Sac*I restriction enzyme sites was synthesized for His-tag plasmid construction. PCR amplification reaction system contained1μl cDNA template,0.5 μM of each primer, and 10 μl of the PCR mix and finally topped to 20 μl volume with nuclease-free water. PCR was performed under the following conditions: an initial denaturation at 94°C for 3 min followed by 35 cycles of amplification (with 30 sec of denaturation at 94°C, 30 sec of annealing at 56°C, 1.5 min of extension at 72°C, and 10 min of further elongation at 72°C). The PCR products amplified were then sequenced (SinoGenoMax, Beijing) from both ends to validate the *SMPD* amplicon. PCR products were purified and digested with *Not*I and *Eco*RI restriction enzymes for $p^{GEX\text{-}6P\text{-}1}$ vector recombination and *Eco*RI and *Sac*I restriction enzymes for $p^{Cold\text{-}II}$ vector recombination. More specifically, the digested PCR products were gel purified and ligated into $p^{GEX\text{-}6P\text{-}1}$ and $p^{Cold\text{-}II}$ vectors using T4 DNA ligase at 16°C. The ligated constructs were transformed into *E. coli*BL21 (DE3) competent cells and cultured overnight at 37°C. We selected an ampicillin-resistant clone and sub-cultured in 5mL LB liquid medium to obtain optimum amount of the expression vector.

Expression and purification of the fusion protein

Positive clones of *E. coli* BL21 were cultured at 37°C in ampicillin containing LB liquid medium. Isopropy-D-thiogalactoside (IPTG) was added to the culture at a final concentration of 1 mmol/L when the optical density at 600 nm (OD600) reached a value of 0.6~0.8 to induce expression of fusion protein. After 5 hours incubation, the culture was centrifuged for 3 min at 10000g at 4°C to collect the cell. The precipitate was washed 3 times in 10 mL phosphate-buffered saline (PBS) for 10 min at 6000g and 4°C. Cells was re-suspended in 15 mL PBS and sonicated with 400W at 6s and 15s interval for 90 cycles. Cells were centrifuged again for 10 min at 6000g and 4°C. The supernatant and precipitate were collected separately. The precipitate was then diluted in PBS, and 2× loading buffer was added and boiled at 100°C for 5 min. SDS-PAGE electrophoresis was used to check the characteristics of protein expressions. The expressed recombinant protein band was cut from the gel and eluted in phosphate-buffered saline and kept at -80°C until used (Yu et al., 2007).

Production of polyclonal antibodies and western blotting validation

Fifty µg of purified recombinant protein was injected into BALB/c type female mice (6 weeks old) with an equal volume of Freund's complete adjuvant. This was immunized for the second time after two weeks. Negative serum was collected from the injected mice's tail before immunization. The tail of experimental mice was cut to check the antibody through western blotting two weeks after the final immunization. Blood was sampled from mice eyes and centrifuged at 5000 rpm and 4°C for 20 min. The serum was decanted and stored at -80°C for further use.

Polyclonal antibody specificity was then checked using western blotting. At this step, total protein from *B. lantschouensis* was extracted and separated on 12% SDS-PAGE, then the gel was transferred onto nitrocellulose membrane in a western blot system (BioRad, USA) for 60 min. Nitrocellulose (NC) membrane was blocked with a commercial blocking buffer for 1.5 hours (ComWin, Beijing). The polyclonal antibody was diluted at a ratio of 1:3000 and used for incubating with the transferred NC membrane. The membrane was then washed three times with 1*TBST containing 0.1% Tween 20 and the europium chelate coated mice IgG was used as secondary antibody to be incubated with NC membrane. Finally, the treated NC membrane was scanned and images were detected on microplate spectrophotometer (Molecular Devices, USA).

SMPD expression characteristics in bumblebee

In order to understand the SMPD expression status, total proteins from different tissues and development stages of *B. lantschouensis* queens and workers were extracted and protein concentration was determined using a BCA method. Twenty µg of total protein was then electrophoresed by SDS-PAGE and immunoblotting was carried out as described above. Protein expression levels in different tissues (antenna, head, muscle, leg, body wall, midgut, fat body, ovary, and poison gland) of three egg-laying queens were quantified. The different development stages including eggs, larvae (at day 5 and 10) and pupae (Pw, PP, Pb, Pb1, Pbdand Pha) were also detected for the protein expression levels (Duchateau & Velthuis, 1989; Dallacqua et al., 2007).

Results

SMPD cDNA cloning and construction of its expression vector

A part of SMPD cDNA was PCR amplified using a pair of specific primers designed from *B.terrestris* cDNA sequence and PCR products were electrophoresed on 1% Agarose gel. According to the result, a PCR product with *Not*I and *Eco*RI restriction enzyme sites has produced a clear band at about 1200 bp (Fig 1). Furthermore, the DNA sequence and homogenous analyses of this product has clearly elucidated that it is a segment of SMPD gene. It has been also predicted that its relative molecular mass and isoelectric point were 50.8 kDa and 6.17, respectively. The PCR product was subcloned into the $p^{GEX\text{-}6P\text{-}1}$ expression vector, and verified with double enzyme digestion and DNA sequencing (Fig 2). Moreover, the recombinant vector$p^{GEX\text{-}6P\text{-}1}$-SMPD was successfully transformed into *E. Coli* BL21 (DE3) cells.

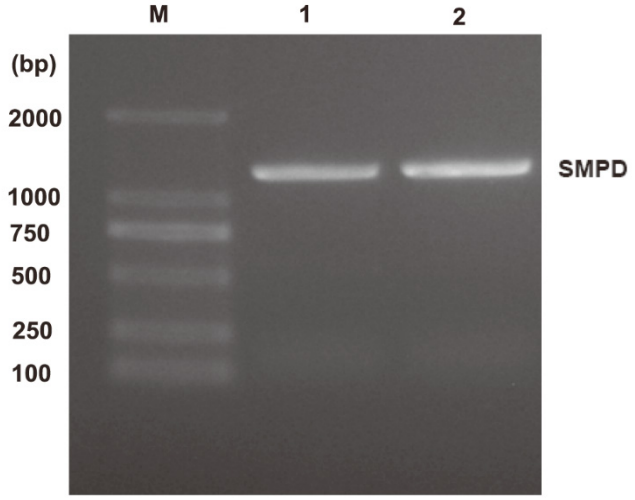

Fig 1. PCR amplification of SMPD gene sequence from *B. lantschouensis*. M, molecular mass marker D2000; lane 1 and 2, amplified product (about 1200bp).

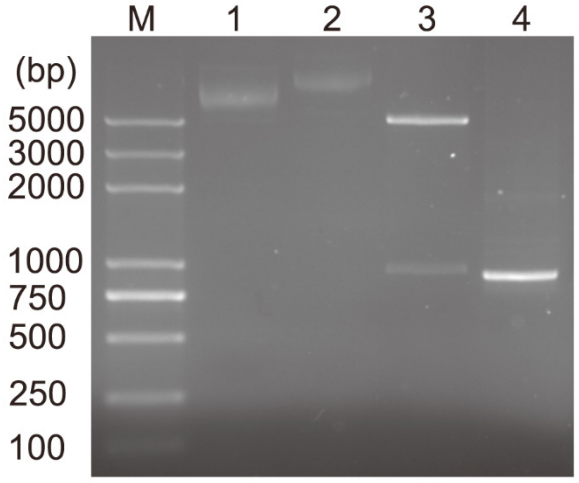

Fig 2. Recombinant plasmid $p^{GEX\text{-}6P\text{-}1}$-SMPD validated by restriction enzyme *Eco*RI and *Not*I at agarose gel electrophoresis. M, molecular mass marker D2000; lane 1,$p^{GEX\text{-}6P\text{-}1}$ empty plasmid; lane 2, $p^{GEX\text{-}6P\text{-}1}$-SMPD recombinant plasmid; lane 3,$p^{GEX\text{-}6P\text{-}1}$-SMPD recombinant plasmid product cut by enzyme *Eco*RIand *Not*I; lane 4: SMPDPCR amplification product.

Recombinant protein expression and purification

Small-scale culture of positive $p^{GEX\text{-}6P\text{-}1}$-SMPD clone was induced by IPTG to express the recombinant protein. Accordingly, polyacrylamide gel electrophoresis showed that the fusion protein has been successfully expressed at an expected molecular mass of about 70 kDa. Simultaneously, a similar protein band was not observed from the control treatment lanes (Fig 3). Large-scale expression of SMPD fusion protein was separated by SDS-PAGE and the predicted

SMPD band was then collected. Furthermore, electrophoresis has confirmed that the fusion protein was the major band on the SDS-PAGE indicating its purity (Fig 4) which further indicated that we have successfully obtained the fusion GST-SMPD protein from *E. coli*.

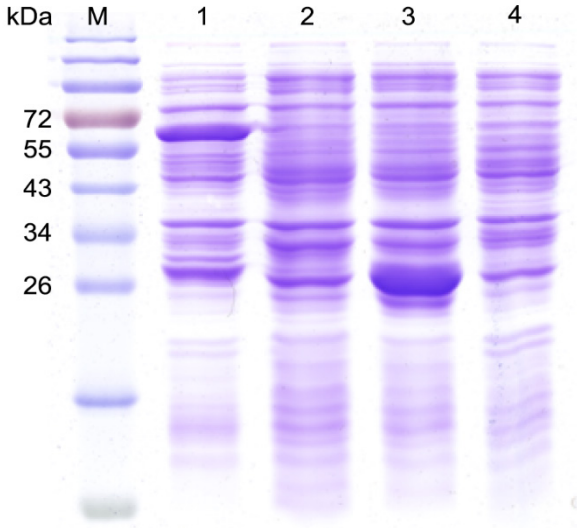

Fig 3. SDS-PAGE profile of SMPD expressed in $p^{GEX-6P-1}$/BL21 system. M, protein molecular weight standards; lane 1, protein expressed in BL21 cell with $p^{GEX-6P-1}$-SMPD plasmid; lane 2, protein expressed in BL21 cell with un-induced $p^{GEX-6P-1}$-SMPD plasmid; lanes 3 and 4, protein expressed in BL21 cell with induced and un-induced $p^{GEX-6P-1}$ plasmid respectively.

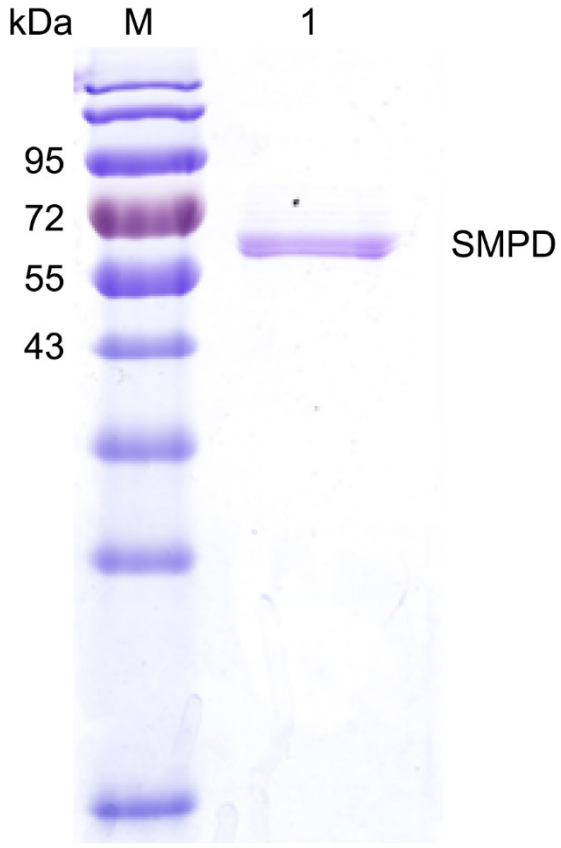

Fig 4. Purified GST-SMPD protein separated in SDS-PAGE electrophoresis. M, protein molecular weight standards; lane1, recombinant protein was purified by gel isolation.

Polyclonal antibody preparation and western blot identification

In this experiment, 500 μL of blood samples were collected from each of the experimental mouse at the end of two mice immunizations. Furthermore, the antiserum was then divided into aliquot 200 μL tubes and stored at -80°C until use. Accordingly, Western blot analysis of the SMPD polyclonal and His-tag monoclonal antibodies against His-SMPD fusion protein demonstrated that both antibodies produced a similar band size at about 40 kDa (Fig 5). The SMPD polyclonal antibody which was hybridized with the bumblebee protein produced a 70 kDa protein band as was expected. Simultaneously, the pre-immunized mice serum, the negative control, didn't produce a detectable band signal (Fig 6). Based on the results from western blotting, it has been confirmed that we have successfully developed the SMPD polyclonal antibody for the first time which could be used to characterize SMPD protein in the future.

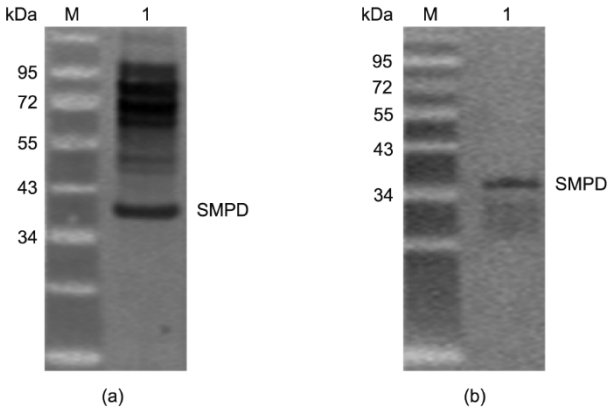

Fig 5. SMPD polyclonalantibody (a) and His-tag monoclonal antibody (b) against the expressed His-SMPD fusion protein.

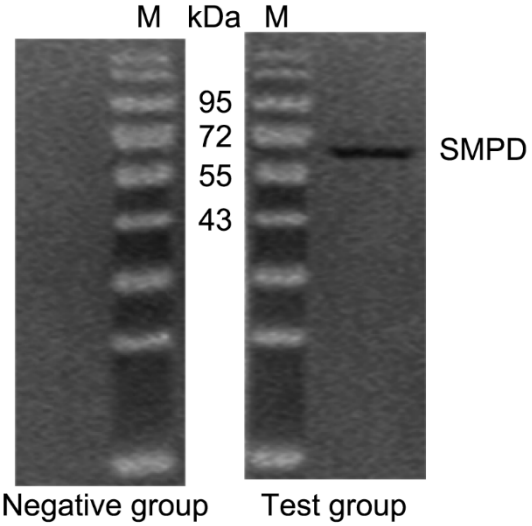

Fig 6. Polyclonal antibody and negative antiserum hybrid with bumblebee total protein using western blotting.

Expression characteristics of SMPD in bumblebee

In order to understand the expression profile of SMPD in bumblebee, the developed polyclonal antibody was used to detect SMPD expression levels at different tissues and developmental stages of *B. lantschouensis*. Accordingly, western blotting analysis showed significantly varied in SMPD expression levels base on the same loading volume of total protein electrophoresis. Among the nine majorly considered tissues, seven (antenna, head, muscle, leg, body wall, fat body and ovary) of them were detected with signals on western blotting. More specifically, SMPD was highly expressed in the muscle, ovary and head. However, we didn't detect SMPD expression in the midgut and venom gland (Fig 7(a)). Furthermore, it has been confirmed that SMPD expression decreased with increasing developmental stages. Specifically, its expression levels were higher at egg and 5 days old larvae whereas SMPD expression levels were lower at the pbd and Pha developmental stages (Fig 7(b)).

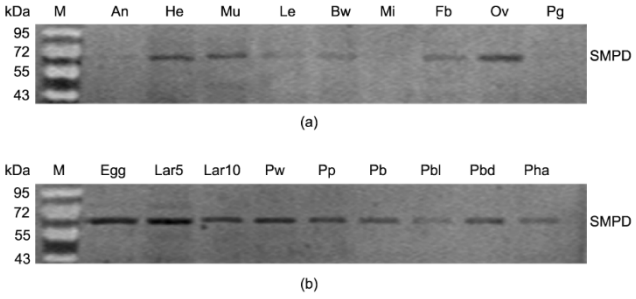

Fig 7. Expression levels of SMPD proteinat different tissues(a) and different development stages(b) determinedusing prepared polyclonal antibody. The different tissues include: antenna (An), head (He), muscle (Mu), leg (Le), body wall (Bw), midgut (Mi), fat body (Fb), ovary (Ov) and poison gland (Pg) of the queen. Likewise, the differentstages include: egg, larvae atday 5, larvae atday 10, Pw, Pp, Pb, Pbl, Pbd and Pha stage.

Discussion

Recently, the development of next generation sequencing technologies made the predictions about biological features in the genome of non-model organisms easier. Furthermore, such technologies also facilitated and supported the possible characterization of more and more novel genes and proteins. Based on *Bombus terrestris* Vogt (Hymenoptera: Apidae) genome annotation and previously conducted experiment in our laboratory, SMPD was identified from different expressed proteins by comparing hemolymph proteome of egg-laying and non-egg-laying queens. In this current study, SMPD gene was cloned from *B. lantschouensis* for the first time. Concurrently, it was implicated that SMPD is involved in the mammalian oocyte apoptosis (Zhang et al., 2009). Thus, this could be explained that SMPD is possibly involved in regulating egg-laying in bumblebee.

According to our experiment, SMPD gene recombinant plasmid was successfully constructed and expressed in a prokaryotic expression system which was a fast and inexpensive way to obtain plenty of interesting proteins. Despite the high solubility of GST fusion protein, we didn't get solubilized proteins during the expression conditions of our experiment. It was, thus, confirmed that there was no fusion protein found in the supernatant of cell disruption which may be associated with the high efficiency expression of $p^{GEX\text{-}6P\text{-}1}$ system (Zhao et al., 2000; Ding et al., 2006; Liu et al., 2011). Therefore, it needs to change the expression to acquire the soluble protein for studying the activity of SMPD.

As western blot results of this work suggested, fusion protein from prokaryotic expression had immunogenicity characteristics and successfully produced the SMPD polyclonal antibody in mice. Both the His-tag antigen and bumblebee total protein western Blot demonstrated that the polyclonal antibody was effective and specific to SMPD protein. Generally, the specificity of this kind of polyclonal antibody has been found to be lower than the monoclonal antibody (Chu & Englund, 2013). However, our Western blots suggested that the specificity of this kind was higher than expected which may require using a Eu-labeled goat anti-mouse kit which also could improve sensitivity and reduce background to guaranty higher blotting quality (Park et al., 2015; Lynch et al., 2016).

SMPD protein has been described to play an important role in sphingolipid metabolism reactions. In line with this, consequently, we have found that SMPD expression was higher at the early ages (egg and larval stages) of a bumblebee. This is because SMPD might be produced at those stages in higher quantity to involve in sphingolipids metabolism. In this case, similar results were reported from *Drosophila* embryos (Renault et al., 2002). Furthermore, higher expression of SMPD observed at ovary and head could imply that these regions might be the functional sites of SMPD in bumblebees.

In conclusion, this study has displayed successful amplification of SMPD gene cDNA fragment from bumblebee for the first time. AGST-SMPD recombinant protein was successfully expressed in *E. coli* and a highly specific polyclonal antibody against SMPD was produced. The antibody has also demonstrated that it can serve as a good tool in the analysis of SMPD protein profiles in future studies. Moreover, SMPD expression has indicated that its role might be associated with egg and larvae development. Finally, we believe that this work could provide an immense importance in further investigation of SMPD functions in bumblebees.

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