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REVIEW

Hemocytes and hematopoiesis in the silkworm, *Bombyx mori*

F Liu[1,2], Q Xu[2], Q Zhang[2], A Lu[2], BT Beerntsen[3], E Ling[2]

[1] *Department of Biological Science and Technology, Shaanxi Xueqian Normal University, Xi'an, Shaanxi 710100, China*
[2]*Key Laboratory of Insect Developmental and Evolutionary Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China*
[3]*Department of Veterinary Pathobiology, University of Missouri, Columbia, MO 65211, USA*

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Abstract

The silkworm, *Bombyx mori*, is a typical Lepidopteran insect. In the silkworm hemolymph, there are 5 types of circulating hemocytes that are classified as prohemocytes, granulocytes, plasmatocytes, spherulocytes and oenocytoids. All of them are involved in humoral and cellular immunity either directly or indirectly. Insect hematopoietic organs can produce hemocytes that are continuously released into the circulation. Recent studies indicate that in the hematopoietic organs of silkworm larvae, there are mainly prohemocytes and oenocytoids. Based on *in vitro* observations, silkworm prohemocytes can differentiate into plasmatocytes and granulocytes, and granulocytes can differentiate into spherulocytes. The silkworm also has a novel type of hematopoiesis. When its hematopoietic organs are extirpated through a surgical operation, circulating hemocytes can still remain at a high level through the wandering stage due to an increase in the level of cell division. Previously, oenocytoids have been considered as the only source of prophenoloxidase (PPO) which is an important immunity protein in insects. However, recent studies in different insect species, as well as in the silkworm, show that additional hemocyte types contain PPO. Furthermore, PPO can be produced by epidermal cells in the hindgut of the silkworm. Consequently, the silkworm is a valuable model to study hemocyte development and cellular and humoral immune responses.

Key Words: hemocytes; hematopoiesis; *Bombyx mori*

Introduction

In most insects, there are several types of circulating hemocytes (Gillespie *et al*., 1997; Lavine and Strand, 2002; Strand, 2008). In typical Lepidopterans, like *Bombyx mori* and *Manduca sexta*, there are 5 types of hemocytes (Akai and Sato, 1973; Beaulaton, 1979; Han *et al*., 1998; Ling *et al*., 2003b; Tan *et al*., 2013). All circulating hemocytes are produced by either hematopoietic organs or through cell division while in circulation (Gillespie *et al*., 1997; Lavine and Strand, 2002; Ling *et al*., 2003c; Strand, 2008; Tan *et al*., 2013). Most insect circulating hemocytes can be easily identified using light or fluorescent microscopy following different staining methods (Gillespie *et al*., 1997; Lavine and Strand, 2002; Ling *et al*., 2003b; Ling and Yu, 2006b). Investigations on the development and differentiation of circulating hemocytes are a very popular research focus in the field of developmental biology (Gillespie *et al*., 1997).

Insect hemocytes are a key component of immunity (Gillespie *et al*., 1997; Lavine and Strand, 2002; Strand, 2008). Insects have no adaptive immune system, and they have to defend against foreign bodies via innate immune responses (Gillespie *et al*., 1997; Lavine and Strand, 2002; Kanost *et al*., 2004; Strand, 2008; Jiang *et al*., 2010; Tanaka and Yamakawa, 2011). Insect innate immunity is composed of both humoral and cellular responses (Gillespie *et al*., 1997; Lavine and Strand, 2002; Kanost *et al*., 2004; Strand, 2008; Liu *et al*., 2009; Jiang *et al*., 2010). Humoral immune responses include the production of proteins like antibacterial peptides and prophenoloxidase (PPO) (Gillespie *et al*., 1997; Lavine and Strand, 2002; Kanost *et al*., 2004; Strand, 2008; Liu *et al*., 2009; Jiang *et al*., 2010; Tanaka and Yamakawa, 2011). Antibacterial peptides are produced primarily by hemocytes and fat bodies (Gillespie *et al*., 1997; Kanost *et al*., 2004; Jiang *et al*., 2010), while insect

Corresponding author:
Erjun Ling
Key Laboratory of Insect Developmental and Evolutionary Biology, Institute of Plant Physiology and Ecology
Shanghai Institutes for Biological Sciences
Chinese Academy of Sciences
Shanghai, 200032, People's Republic of China
E-mail: erjunling@sippe.ac.cn

PPO is considered to be produced within hemocytes (Ashida and Brey, 1998). In Lepidopteran insects, cellular immunity is primarily via granulocytes and plasmatocytes (Lavine and Strand, 2002; Ling and Yu, 2006b). However, in *M. sexta*, five types of circulating hemocytes all take part in the cellular response of encapsulation (Ling and Yu, 2006a). In *Drosophila melanogaster*, plasmatocytes and lamellocytes are responsible for cellular immunity (Lavine and Strand, 2002).

Circulating hemocytes

In *D. melanogaster* there are three terminally differentiated types of hemocytes, which are plasmatocytes, crystal cells and lamellocytes (Lanot *et al*., 2001; Evans and Banerjee, 2003; Wertheim *et al*., 2005; Strand, 2008). All three types of hemocytes are differentiated from prohemocytes (Strand, 2008), and they are involved in larval immune functions like phagocytosis, melanization and encapsulation (Honti *et al*., 2013). Approximately 90%-95% of circulating hemocytes are plasmatocytes that are responsible for phagocytosis of bacteria and dead host cells (Lanot *et al*., 2001; Wertheim *et al*., 2005; Strand, 2008). Crystal cells are not adhesive *in vitro*, occupy ~5% of the total hemocytes, and can produce PPO in *Drosophila* (Strand, 2008; Tang, 2009). Lamellocytes are a type of large, flat, adhesive hemocyte (Strand, 2008). Prohemocytes quickly differentiate into lamellocytes when *Drosophila* is infected by parasites or during the process of metamorphosis. In *Drosophil*a, lamellocytes are responsible for encapsulating large parasites and other foreign bodies (Lanot *et al*., 2001).

In larvae of *B. mori*, the 5 types of circulating hemocytes are prohemocytes, granulocytes, plasmatocytes, spherulocytes and oenocytoids (Akai and Sato, 1973; Han *et al*., 1998; Ling *et al*., 2003b; Tan *et al*., 2013). Prohemocytes are progenitor stem cells which can differentiate into other types of hemocytes according to light and electron microscopy observations (Beaulaton, 1979; Yamashita and Iwabuchi, 2001). In the silkworm, most circulating hemocytes are granulocytes that have many granules inside the cytoplasm (Ling *et al*., 2003b). This type of hemocyte can recognize invading pathogens, which are then subjected to phagocytosis or encapsulation, and these cells are also important for wound healing (Akai and Sato, 1973; Gillespie *et al*., 1997; Strand, 2008; Tanaka and Yamakawa, 2011). In the silkworm, oenocytoids are the largest type of hemocyte in the silkworm (Akai and Sato, 1973; Ling *et al*., 2003b). They are easily classified using light microscopy because of their large size and opaque appearance when compared to other hemocytes (Wago, 1991). Plasmatocytes are smaller than oenocytoids but larger than the other hemocytes (Akai and Sato, 1973; Ling *et al*., 2003b). This type of hemocyte can adhere to the surfaces of foreign entities very quickly upon contact by asymmetrically extending pseudopods (Ling *et al*., 2003b). Plasmatocytes are the main type of hemocytes that are responsible for encapsulation of foreign bodies (Strand, 2008). Spherulocytes in the silkworm have no ability to adhere. Thus, they must be observed immediately after spreading on a glass slide (Ling *et al*., 2003b). Just like granulocytes, spherulocytes have many obvious large granules in the cytoplasm (Akai and Sato, 1973; Ling *et al*., 2003b). Consequently, most of the 5 types of circulating hemocytes in silkworm larvae can be readily identified if a researcher is familiar with their morphological properties.

Although *D. melanogaster* and *B. mori* are two typical insect models for studying hemocyte differentiation and development, there are a number of differences in hemocyte nomenclature between the two species. Based on the phagocytosis function, *Drosophila* plasmatocytes, which are phagocytotic, appear to be more similar to silkworm granulocytes than to silkworm plasmatocytes (Ribeiro and Brehelin, 2006). In the silkworm, plasmatocytes are normally larger than granulocytes and plasmatocytes are the major hemocyte type that takes part in encapsulation (Strand *et al*., 2008). Thus, plasmatocytes in the silkworm are not exactly the same as plasmatocytes in *D. melanogaster* and these two types of hemocytes have different cellular functions even though they have the same name (Ribeiro and Brehelin, 2006). Lamellocytes are probably the equivalent of the silkworm plasmatocytes due to their capacity to adhere to foreign surfaces (Ribeiro and Brehelin, 2006). Oenocytoids and spherulocytes are both non-adhesive hemocyte types. It is thought that spherulocytes may function to transfer necessary materials to the cuticle via epidermal cells (Sass *et al*., 1994). The silkworm spherulocytes have no equivalent in *D. melanogaster* (Ribeiro and Brehelin, 2006). Oenocytoids are considered to be the main type of hemocyte that can produce PPO in Lepidopteran insects (Ashida and Dohke, 1980; Iwama and Ashida, 1986; Jiang *et al*., 1997; Ling *et al*., 2005a). Crystal cells in *Drosophila* show strong similarities both in structure and function with silkworm oenocytoids (Ribeiro and Brehelin, 2006). However, recent studies indicate that other types of hemocytes and even insect hindgut cells have PPO. Therefore, it is necessary to be cognizant of these differences when investigating insect hemocytes.

The primary method to identify and classify insect hemocytes is according to hemocyte morphological differences determined via histochemical observations and specific physiological functions (Gupta, 1985; Brehelin and Zachary, 1986). Cell morphology is the basic method which is performed using a variety of microscopy formats, including light, electron, fluorescence, confocal and differential interference contrast (DIC) microscopy.

Fluorescent staining with chemicals like acridine orange (AO) and propidium iodide (PI) has been used to stain silkworm hemocytes for classification (Ling *et al*., 2003b). After staining, each type of hemocyte has a specific fluorescence, which makes it easier to identify them using fluorescent microscopy (Ling *et al*., 2003b; Ling *et al*., 2005a). Granulocytes have many granules inside their cytoplasm which exhibit strong green fluorescence (Ling *et al*., 2003b; Ling *et al*., 2005a). Spherulocytes also have many green granules after

staining with AO and PI. However, the green granules inside spherulocytes are much larger than those inside granulocytes (Ling *et al*., 2003b; Ling *et al*., 2005a). Prohemocytes and plasmatocytes cannot be positively stained by AO, but their long and irregular shapes make them easily identifiable without such staining. Oenocytoids can be stained by PI to show red nuclei (Ling *et al*., 2003b). Neutral red is also a good staining solution for the differentiation of hemocytes in *M. sexta* because these hemocytes exhibit different properties after staining (Ling and Yu, 2006b).

Monoclonal antibodies against specific types of hemocytes in *M. sexta* and *Pseudoplusia includens* have been created and used for hemocyte identification, which is a novel way to study hemocyte development, differentiation and function (Gillespie *et al*., 1997; Gardiner and Strand, 1999; Levin *et al*., 2005). Using monoclonal antibodies against *Manduca* granulocytes and plasmatocytes separately, granulocytes were found to specifically phagocytose apoptotic cells while plasmatocytes phagocytosed injected fluorescent beads (Ling and Yu, 2006b). In *Drosophila*, cell markers like antibody against P1 antigen (an uncharacterized surface marker) to differentiated plasmatocytes was also very useful (Strand, 2008). Thus, for future studies, it is important to find additional specific markers to label insect hemocytes and aid in identification.

Although there are diffferent methods that can be used to identify insect hemocytes as described above, most of them are not very convenient. For example, the use of monoclonal antibodies is inconvenient to identify large numbers of hemocytes within a short time frame, since this method requires fixation, washing and staining. There also are a number of hemocytes that are difficult to differentiate from each other using light microscopy (Akai and Sato, 1973; Gillespie *et al*., 1997). For example, the younger granulocytes that contain very few granules are difficult to distinguish from prohemocytes, making it hard to classify them as a granulocyte or prohemocyte. Also, the younger plasmatocytes that have no obvious pseudopods are similar to young granulocytes and/or prohemocytes (Ling *et al*., personal observations). All in all, it is necessary to develop additional methods that provide convenience and precision in identifying hemocytes.

Just like other cells, hemocytes also undergo cell death, like necrosis and apoptosis, and insect hemocytes have been observed undergoing apoptosis (Liu and Ling, personal observation). However, hemocytes also can phagocytose apoptotic cells, making it difficult to distinguish them from apoptotic hemocytes. For example, in the silkworm and *Manduca*, many hemocytes can phagocytose apoptotic bodies (Ling *et al*., 2003b; Ling and Yu, 2006b). Those hemocytes containing phagocytosed apoptotic bodies might be misleadingly considered as undergoing apoptosis if their nuclei are not counterstained by DAPI (4',6-diamidino-2-phenylindole) when the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) method is used for identifying apoptosis.

Hematopoietic organs and hematopoiesis

In insects, hematopoietic organs are the primary source of hemocytes (Akai and Sato, 1971; Gillespie *et al*., 1997; Strand, 2008). However, in different species of insects, there are likely many differences in hematopoietic organs and even hematopoiesis.

In *D. melanogaster*, hemocytes are produced in two different ways with each occurring during different developmental stages (Strand, 2008). When *Drosophila* is still an embryo, some cells in the head or dorsal mesoderm differentiate into blood progenitors, and this process is under the control of transcription factors (Grigorian *et al*., 2011). During the larval stage, hemocytes are produced by lymph glands, which are the *Drosophila* hematopoietic organ. These lymph glands are located bilaterally along the anterior part of the dorsal vessel (Jung *et al*., 2005).

In Lepidopteran larvae, hematopoietic organs and hematopoiesis are very different from *Drosophila*. In the silkworm, there are four hematopoietic organs that are located in the meso- and meta-thorax (Akai and Sato, 1971; Ling *et al*., 2003a). Each hematopoietic organ is tightly attached to a wing disc, and the two anterior hematopoietic organs are larger than the two posterior ones (Akai and Sato, 1971; Ling *et al*., 2003a; Ling *et al*., 2006). These hematopoietic organs are circled by a layer of an acellular sheath, which is broken during the larval wandering stage to release hemocytes (Akai and Sato, 1971; Han *et al*., 1998). Within the organs many new hemocytes are produced inside the islets (Akai and Sato, 1971; Ling *et al*., 2003a). Hemocytes produced inside the hematopoietic organs are continuously released into the hemolymph during the larval stages. At the same time, circulating hemocytes, derived from the embryo, can still proliferate and differentiate into other types of hemocytes (Akai and Sato, 1971; Gillespie *et al*., 1997; Gardiner and Strand, 2000; Nardi, 2004; Strand, 2008), which contributes to hematopoiesis. Recently, an *in vitro* assay has demonstrated that the neighboring wing discs and fat body tissue are also important to hematopoiesis (Wang *et al*., 2010), which has not been reported with *Drosophila*. Therefore, as the typical Lepidopteran insect, the silkworm has a different type of hematopoiesis than does *Drosophila*.

As mentioned above, although insect hematopoietic organs are very important to hematopoiesis, hemocytes can also be produced through self cell division. In the silkworm, when the four larval hematopoietic organs were destroyed by pinpointed heavy ion beams or were physically extirpated via a surgical operation, the silkworm larvae did not die as predicted (Ling *et al*., 2003c). Very surprisingly, the number of circulating hemocytes in larvae with the four hematopoietic organs destroyed still increased during the wandering stage (Ling *et al*., 2003c). When cell division of circulating hemocytes was studied, increased hemocyte division was observed in larvae with hematopoietic organs destroyed or extirpated as compared to naive larvae (Ling *et al*., 2003c). These results indicate that cell division of circulating

hemocytes can contribute considerably to the total amount of hematopoiesis (Ling *et al*., 2003c; Tan *et al*., 2013). Also, when the silkworm hematopoietic organs were targeted by heavy ion beams and were seriously damaged, the organs regenerated later via the assistance of circulating hemocytes (Ling *et al*., 2003a). Based upon the hypothesis that circulating hemocytes might enter the damaged hematopoietic organs to remove dead cells, silkworm larvae were injected with fluorescent beads, which served as a tracer to distinguish circulating hemocytes that phagocytosed the beads from hemocytes present in the hematopoietic organs. After an initial injection of fluorescent beads into larvae to label circulating hemocytes, the hematopoietic organs of these larvae were heavy ion beam irradiated. It then was demonstrated that circulating hemocytes containing phagocytosed fluorescent beads had entered the targeted hematopoietic organs in order to phagocytose and clear the dead cells (Ling *et al*., 2006). Subsequently, the invading hemocytes remained in the irradiated hematopoietic organs to induce the targeted organs to regenerate (Ling *et al*., 2006).

Hormones may also affect insect hematopoiesis (Ling *et al*., 2003c). Silkworm hemocyte density increases abruptly during the wandering stage and then decreases during the spinning and prepupal stages (Ling *et al*., 2003c). When silkworm larvae were injected with 20-ecdysone (20-E), hemocyte density significantly increased at approximately 12-18 h post injection (Ling *et al*., 2003c). However, the application of a juvenile hormone analogue (methoprene) to the injected silkworm larvae kept the hemocyte level stable without an obvious change (Ling *et al*., 2003c).

In summary, hemocytes produced by hematopoietic organs are the main source of circulating hemocytes in the silkworm. However, hemocyte division in the circulation may also contribute considerably to hematopoiesis if the hematopoietic organs are destroyed, which is unheard of for mammalian hematopoiesis. During the hematopoietic process, hormones like 20-E and JH, also may take part in the regulation of hematopoiesis (Ling *et al*., 2003c).

Hemocyte differentiation

In the silkworm hematopoietic organs, a previous study demonstrated that there are prohemocytes, plasmatocytes, granulocytes and oenocytoids based upon electron microscopy observations (Akai and Sato, 1973). However, in the hematopoietic organs of *Spodoptera frugiperda* and *M. sexta* for example, there are primarily prohemocytes and plasmatocytes (Gardiner and Strand, 2000; Nardi *et al*., 2003; Strand, 2008). In the silkworm, prohemocytes appear to differentiate into plasmatocytes in the hematopoietic organs before release (Beaulaton, 1979). Subsequently, in the circulation of the silkworm, these plasmatocytes then differentiate into granulocytes, spherulocytes and oenocytoids (Beaulaton, 1979). Obviously, there are still disagreements in regards to hemocyte differentiation even within one insect species of insect (like the silkworm) if the studies were performed in different labs. Therefore, it is necessary to discover new methods and techniques to study hemocyte differentiation in order to minimize discrepancies in hemocyte descriptions.

One such method to study hemocyte proliferation and differentiation is the use of tissue culture. When silkworm hematopoietic organs were cultured in Grace's medium supplemented with 10% heated silkworm plasma, large numbers of hemocytes were released during the process (Nakahara *et al*., 2003; Wang *et al*., 2010). After several days of tissue culture, most newly released hemocytes were plasmatocytes and prohemocytes (Nakahara *et al*., 2003). But when using AO and PI to stain hemocytes immediately after the silkworm hematopoietic organs were physically split open to release hemocytes, we found there were mainly prohemocytes (60%-70%) and oenocytoids (30%-40%) (Ling *et al*., 2005b). During the process of transient cell culture, prohemocytes newly released from silkworm hematopoietic organs could transform into granulocytes and plasmatocytes (Ling *et al*., 2005b). Yamashita and Iwabuchi (2001) separated silkworm prohemocytes from other circulating hemocytes in a single cell culture and observed that prohemocytes can differentiate into plasmatocytes and granulocytes, and granulocytes can differentiate into spherulocytes. However, circulating prohemocytes did not differentiate into oenocytoids *in vitro* (Yamashita and Iwabuchi, 2001). In another study, circulating oenocytoids from *P. includens* and *S. frugiperda* were not labeled by 5-bromo-2-deoxyuridine (BrdU), which indicated that circulating oenocytoids cannot proliferate via cell division (Gardiner and Strand, 2000). Therefore, oenocytoids in Lepidopeteran insects are likely derived only from hematopoietic organs.

PPO is produced not only by hemocytes

In insects, hemocytes have been considered as the only source of PPO (Ashida and Brey, 1998). However, recent studies have shown that there is also PPO in the hindgut and wing discs of the silkworm (Diao *et al*., 2012; Shao *et al*., 2012). Therefore, it is necessary to address this topic and briefly summarize this research because it is important to know whether the PPO that is present in those tissues has a close relationship with circulating hemocytes, and whether PPO in the wing discs and/or hindgut can induce melanization as occurs in the hemocoel and that involves the participation of hemocytes (Ashida and Brey, 1998).

PPO-positive hemocyte identification

In most insects, oenocytoids can produce PPO (Ashida and Brey, 1998; Hillyer and Christensen, 2002; Hillyer *et al*., 2003). Since PPO has no signal peptide, it is thought to be released after cell lysis (Ashida and Brey, 1998). As described above, PPO is a good protein marker for identifying hemocytes that can produce PPO. The traditional methods to identity PPO-positive hemocytes are to use immuno-staining or *in situ* methods to detect PPO protein or transcription within hemocytes (Jiang *et al*., 1997; Ashida and Brey, 1998). However, due to the absence of antibodies against insect PPO and other

limitations, PPO-positive hemocyte identification has not been extensively tried in most insects. Yet there could be some simple methods to identify PPO-positive hemocytes, if hemocytes containing PPO can first be activated followed by staining using a substrate like L-DOPA or dopamine.

In insects, PPO is activated by the regulation of a cascade composed of several serine proteases and serpins (Ashida and Brey, 1998; Kanost *et al*., 2004; Jiang *et al*., 2010). However, many chemicals like the detergent sodium dodecyl sulfate (SDS), cetylpyridinium chloride (CPC) and ethanol also can activate PPO by an unknown mechanism (Ashida and Brey, 1998). Researchers have utilized this property to identify PPO-positive hemocytes in insects. Since ethanol can activate insect PPO, a mixture containing 35% ethanol and L-DOPA was used to activate PPO inside silkworm hemocytes for staining, by which many more types of hemocytes were found to contain PPO (Ling *et al*., 2005a). Using the same method, Ling and Yu (2005) found that most PPO-positive hemocytes were oenocytoids and a few spherulocytes and granulocytes also contained PPO in *M. sexta*. Further studies indicated that 10% of hemocytes exhibited surface PPO (Ling and Yu, 2005), but oenocytoids showed no surface PPO (Ling and Yu, 2005). In *M. sexta*, oenocytoids were also found to encapsulate immulectin-2 coated agarose beads when a mixture containing ethanol and dopamine was used to identify PPO-positive hemocytes (Ling and Yu, 2006a). This was the first study to show that oenocytoids may take part in encapsulation.

Using the same methods that were performed to identify PPO-positive hemocytes in the silkworm and *Manduca*, hemocytes that have PPO were also identified in mosquitoes. Hillyer and colleagues (Hillyer *et al*., 2003) used a formaldehyde solution (4%) to fix hemocytes followed by staining with a 2 mg/ml L-DOPA solution, which resulted in the identification of PPO-positive hemocytes in the mosquitoes, *Aedes aegypti* and *Armigeres subalbatus*. In the mosquito *Culex pipiens quinquefasciatus*, PPO-positive hemocytes were identified in larvae and pupae, and many more types of hemocytes were found to have PPO (Wang *et al*., 2011). Very interestingly, PPO-positive plasmatocytes were found only in larvae, and blood-feeding could specifically induce different types of PPO-positive hemocytes in adult female mosquitoes (Wang *et al*., 2011). We are unsure as to why PPO-positive hemocytes in mosquitoes are so complicated and whether those PPO-positive hemocytes may be involved some specific innate immunity responses, like phagocytosis or encapsulation. These methods of activating PPO within cells should be tried with other insects in order to obtain a more complete picture of PPO-positive hemocytes in different insect species.

PPO in the wing disc: a source of plasma prophenoloxidase in the silkworm

In the silkworm, each hematopoietic organ is attached to a wing disc (Akai and Sato, 1971; Han *et al*., 1998; Ling *et al*., 2006). When wing discs were separated from hematopoietic organs for tissue staining and Western blot assays, all results indicated that the silkworm wing discs contain PPO that can be released into the culture medium after a period of tissue culture (Diao *et al*., 2012). This result was also verified by LC-MS/MS after the band containing PPO was excised for protein identification (Diao *et al*., 2012).

PPO protein was also found in the hindwing of *Tribolium castaneum* (Dittmer *et al*., 2011) and an *in situ* assay showed that a few free cells in the cavity of the wing disc contained PPO1 and PPO2 mRNA (Diao *et al*., 2012). Silkworm wing discs are connected with the hematopoietic organs through many tube-like materials (Ling *et al*., 2006). Those cells in the cavity of the silkworm wing disc are likely hemocytes that were released from the hematopoietic organs and made their way to the disc via the tube-like connections. When PPO-positive hemocytes were broken within the wing disc, PPO was released into the wing discs. Therefore, PPO released via the wing disc may contribute to the hemolymph PPO (Diao *et al*., 2012).

PPO is produced by epidermal cells in the hindgut

A very familiar phenomenon is that silkworm larvae feed on green mulberry leaves but excrete black feces. Various methods, including PPO activity staining, immuno-staining, Western blot and *in situ* assays, were performed to show that the large epidermal cells and many small cells in the hindgut can produce PPO and release it into the hindgut lumen (Shao *et al*., 2012). When PPO activity was inhibited by giving larvae phenylthiourea (PTU), the silkworm larvae excreted green feces. However, large amounts of bacteria then easily reproduced in the green feces. On the contrary, bacteria in the black feces were found to be dead (Shao *et al*., 2012). This study demonstrated that, besides hemocytes, silkworm hindgut cells can also produce PPO in order to remove microorganisms in the feces through melanization, thereby avoiding contamination of the mulberry leaves and the surrounding living environment.

Utilization of Drosophila macrophage-like cell line S2 cells to study PPO structure and activity

To date, we know very little about the relationship of PPO structure and enzyme activitiy. *Drosophila* has three PPO genes and when all three *Drosophila* PPO genes were over-expressed in S2 cells, different biochemical properties of the three PPOs were found (Liu *et al*., 2012). PPO1 and PPO2 need additional Cu^{2+} in order to subsequently become activated by ethanol and this was demonstrated by native gel separation of samples with or without Cu^{2+} added during cell transfection. For PPO3, it has enzyme activity directly (i.e., auto-activation), without the need for activation by either ethanol or any other method, after over-expression in S2 cells. PPO3 also is able to induce auto-melanization if additional Cu^{2+} is added (Liu *et al*., 2012). When the predicted PPO3 structure was compared with those of PPO1 and PPO2, results showed that two key amino acids, around the active site pocket of PPO3, occupy less space than the comparable amino acids in PPO1 and PPO2. These two key amino acids in PPO3 make the entrance pocket larger such that there is

gap between the placeholder and the entrance, thereby permitting auto-activation since substrate can enter at any time (Chen *et al*., 2012). When the two key amino acids in PPO3 were mutated to make the active site pocket smaller so that the placeholder occupies the entrance of the active site pocket without a gap, the mutant PPO3 acts like PPO1 and PPO2 and needs to be activated either by ethanol or serine protease (Chen *et al*., 2012).

Conclusions

In most insects, there are several types of circulating hemocytes (Gillespie *et al*., 1997; Lavine and Strand, 2002; Strand, 2008) and these hemocytes have been the focus of research on cell development and differentiation. Insect hemocytes are important factors in innate immunity (Gillespie *et al*., 1997; Lavine and Strand, 2002; Strand, 2008). They can produce many immune proteins like antibacterial peptides and PPO (Gillespie *et al*., 1997; Ashida and Brey, 1998; Kanost *et al*., 2004; Jiang *et al*., 2010). In addition, hemocytes are directly involved in cellular immunity like phagocytosis, encapsulation and nodule formation (Gillespie *et al*., 1997). In the field of entomology, hemocytes are an attractive research arena for many scientists. Using the silkworm as a model, scientists have been extensively studying hemocyte identification, development, differentiation and cellular immune responses. Silkworm larvae have five types of circulating hemocytes that are produced by hematopoietic organs and released into the hemolymph. However, several types of circulating hemocytes also can proliferate through cell division, which contributed significantly to hematopoiesis after the larval hematopoietic organs were extirpated via a surgical procedure. Novel types of hematopoiesis in the silkworm make this insect species a valuable model to study hemocyte-related activities in the future. Now with the silkworm genome available (Xia *et al*., 2004), we anticipate being able to explore insect hemocyte immune activities at the molecular level.

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