The pro-phenoloxidase of coleopteran insect, *Tenebrio molitor*, larvae was activated during cell clump/cell adhesion of insect cellular defense reactions

Hyun Seong Lee[a], Mi Young Cho[a], Kwang Moon Lee[a], Tae Hyuk Kwon[a], Ko-ichi Homma[b], Shunji Natori[b], Bok Luel Lee[a,*]

[a] *College of Pharmacy, Pusan National University, Jangjeon Dong, Kumjeong Ku, Pusan 609-735, South Korea*
[b] *Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan*

Received 15 December 1998

Abstract To characterize the proteins involved in cell clump/cell adhesion of insect cellular defense reactions, we induced the cell clump/cell adhesion reaction in vitro with the hemolymph of larvae of the coleopteran insect, *Tenebrio molitor*. The 72 kDa protein was specifically enriched in the residues of cell clump/cell adhesion and was purified to homogeneity. A cDNA clone for the 72 kDa protein was isolated. We found that the 72 kDa protein was an activated phenoloxidase from *Tenebrio* pro-phenoloxidase. We suggest that activated phenoloxidase is involved in the cell clump/cell adhesion reaction as well as in the synthesis of melanin.

Key words: Insect immunity; Pro-phenoloxidase; Cell clump; Phenoloxidase; Defense protein

1. Introduction

The endogenous defense of insects is based on cellular and humoral immune responses. The latter include the synthesis of a broad spectrum of potent antimicrobial proteins and the activation of the pro-phenoloxidase (pro-PO) cascade. Previously, we examined the humoral responses of larvae of the coleopteran insects, *Tenebrio molitor* and *Holotrichia diomphalia* and reported the primary structures of antimicrobial proteins in their hemolymph [1–5]. Recently, we have isolated a cDNA clone for a pro-PO-activating factor (PPAF-I) and determined the complete amino acid sequence of PPAF-I [6,7]. PPAF-I is highly homologous to *Drosophila* easter serine protease (42.9% identity), an essential serine protease zymogen for pattern formation in normal embryonic development.

The cellular defense reactions in the presence of foreign substances in insects are cell clump/cell adhesion and encapsulation. Recently, the component that interferes with cellular encapsulation was purified and characterized [8]. Cell clump/cell adhesion has been reported to occur in insects in response to a variety of foreign substances including bacteria, insert materials, and foreign tissue implants [9]. The development of the clumps/cell adhesion is always preceded by the initial attachment of hemocytes to the foreign material [10]. The first stage of recognition is followed by the adherence of hemocytes. However, the biochemical processes of cell clump/cell adhesion in insects are still obscure at the molecular level.

This paper presents evidence that activated phenoloxidase (PO) from pro-PO is engaged in the cell clump/cell adhesion reaction.

2. Materials and methods

2.1. Animals and in vitro cell clump/cell adhesion

T. molitor larvae (mealworm) were maintained on a laboratory bench in terraria containing wheat bran. Vegetables were placed on top of the bran to provide water. Hemolymph and hemocyte were collected as previously described [1]. To induce cell clump/cell adhesion in vitro, 80 μl of *T. molitor* hemolymph was suspended in 100 μl of insect saline (130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, pH 6.0) in a petri dish and incubated for 20 min at room temperature. After incubation, the supernatant was carefully withdrawn with a micropipette and the morphology of cell clump/cell adhesion was observed at 400× magnification under phase contrast optics with a light microscope (Zeiss Axiovert). As a control, 80 μl of hemolymph was suspended in a decoagulation buffer (30 mM trisodium citrate, 26 mM citric acid, 15 mM sodium chloride, 20 mM EDTA, pH 4.6) under the conditions described above. The clumped materials on the surface of the petri dish were regained by 6 M urea in 50 mM Tris-HCl (pH 6.5) containing 2% SDS and 5 mM dithiothreitol (DTT). The proteins were precipitated with trichloroacetic acid (TCA) and analyzed on 12% SDS-PAGE under reducing conditions. The protein concentrations were determined by the method of Lowry et al. [11] using bovine serum albumin as the standard.

2.2. Purification and molecular cloning of 72 kDa cell clump/cell adhesion related protein

The 72 kDa protein was purified to homogeneity from polyacrylamide gel using the Micro-Electroeluter (Centrilutor, Amicon) according to the manufacturer's instructions. The purified 72 kDa protein was reduced and *S*-pyridylated according to a previously published method [6]. The *S*-pyridylated 72 kDa protein was digested with trypsin, and the resulting peptides were separated by HPLC on a C_{18} reverse phase column (Gilson). To determine the amino-terminal sequences of the 72 kDa protein, the purified 72 kDa protein was subjected to SDS-PAGE under reducing conditions. The bands of the 72 kDa protein were blotted onto a polyvinylidene difluoride filter (PVDF filter, Millipore), cut out from the filter, and subjected to automated amino acid sequence analysis [12]. A cDNA library of *T. molitor* larvae was constructed by a previously published method [1]. To screen the 72 kDa protein cDNA clones, we performed immunoscreening using an affinity-purified antibody against the 72 kDa protein. Following isopropyl-β-D-thiogalactoside (IPTG) induction, 5×10^4 plaques were screened with an affinity-purified antibody against the purified 72 kDa protein. The secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG, Bio-Rad) was used at a dilution of 1:1000. Phage DNA was isolated from phage lysates by using the lambda DNA preparation kit according to the manufactur-

*Corresponding author. Fax: (82) (51) 513-6754.
E-mail: brlee@hyowon.pusan.ac.kr

Abbreviations: pro-PO, pro-phenoloxidase; PO, phenoloxidase; PPAF, pro-PO-activating factors; TCA, trichloroacetic acid; PVDF, polyvinylidene difluoride

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with accession number AB020738.

PII: S0014-5793(99)00067-8

er's instructions. We analyzed all of the plaques showing positive signals on immunoscreening. We sequenced the clones by the dideoxy chain-termination method of Sanger et al. [13]. The amino acid sequences of the 72 kDa protein were compared with the protein sequence database of the National Cancer for Biotechnology Information (NCBI) using the Genetyx system (Software Development Co., Ltd., Tokyo).

2.3. Antibody and immunoblotting

Antibody against the 72 kDa protein was raised by injecting 20 μg of the purified protein into a male albino rabbit with complete Freund's adjuvant and giving a booster injection of the same amount of protein 14 days later [14]. The resulting antibody was affinity-purified as described before [7]. For immunoblotting, the proteins separated on the gel by electrophoresis were transferred electrophoretically to a PVDF filter and the filters were immersed in 5% skim milk solution containing 1% horse serum for 12 h, transferred to rinse solution I (20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.1% Tween 20 and 2.5% skim milk) containing the affinity-purified antibody against 72 kDa (50 ng/ml), and kept at 4°C for 2 h. The bound antibodies were detected using the ECL Western blotting reagent kit (Amersham Life Science).

3. Results

3.1. Identification and purification of 72 kDa protein

To isolate the protein involved in the cell clump/cell adhesion reaction, we first examined the morphology of induced cell clump/cell adhesion with the hemolymph of *T. molitor* larvae. As shown in Fig. 1A, *T. molitor* hemocytes clumped in the presence of insect saline on the surface of the petri dish after 20 min incubation. However, hemocytes were not clumped in the presence of the decoagulation buffer as shown in Fig. 1B. To examine the difference between clumped cells and non-clumped cells, we recovered proteins from the cell-clumped residues on the surface of the petri dish. As shown in Fig. 2, one band (72 kDa band shown by the arrow) was specifically enriched from the cell-clumped residues (lane 2 in Fig. 2). This band was not detected in the supernatant (lane 3), the residues of hemolymph incubated with decoagulation buffer (lane 4), and its supernatant (lane 5). These results suggest that the 72 kDa protein is related to cell clump/cell adhesion of *T. molitor* hemocytes. To characterize this 72 kDa protein, we first purified 72 kDa protein to homogeneity from cell-clumped residues (lane 6 in Fig. 2). Then, we determined the amino-terminal sequence of the 72 kDa protein from the blotted filter as FGEDADERIDVKKIS. We also determined three partial amino acid sequences as follows: EQATVVPEGS, YTENQLNFPGVTVS and PQGGEELAQFNF.

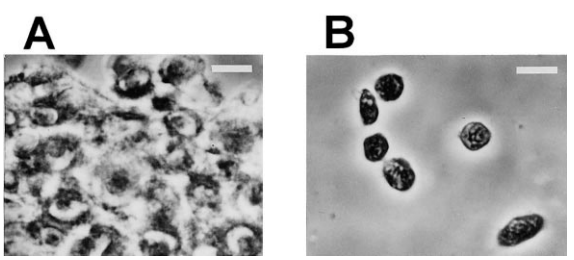

Fig. 1. Morphology of in vitro induced cell clump/cell adhesion with the hemolymph of *T. molitor* larvae. Photographs of *T. molitor* hemocytes in the presence of insect saline (A) and decoagulation buffer (B). A: Cell clump/cell adhesion was induced at room temperature for 20 min. B: Cell clump/cell adhesion was not observed in the decoagulation buffer at the same conditions. Bar = 10 μm.

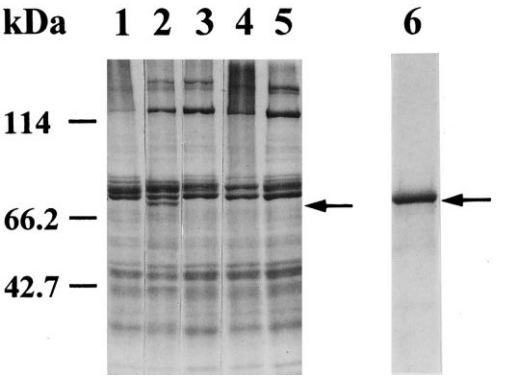

Fig. 2. 12% SDS-PAGE analysis of cell clump/cell adhesion related protein. The proteins were retrieved as described in Section 2. The arrow indicates the 72 kDa protein. Lane 1, fresh hemolymph (10 μg) of *T. molitor* larvae was immediately precipitated with TCA and loaded on the gel. The retrieved proteins (10 μg) from the cell-clumped residues were incubated in the presence of insect saline (lane 2), supernatant solution (10 μg) in the presence of insect saline (lane 3), hemocyte residues (10 μg) incubated in the presence of decoagulation buffer (lane 4), supernatant solution (20 μg) in the presence of decoagulation buffer (lane 5). The purified 72 kDa protein (2 μg) was loaded on lane 6. Molecular mass markers from the top: 114, 66.2 and 42.7 kDa.

3.2. cDNA cloning of 72 kDa protein

To determine the whole amino acid sequence of the 72 kDa protein, we screened the cDNA library of *T. molitor* larvae with an affinity-purified antibody against 72 kDa protein by an immunoscreening method. We obtained 10 positive clones for 72 kDa protein. The nucleotide sequence and the deduced amino acid sequence of one of these clones, named clump-2, are shown in Fig. 3A. This cDNA contained an open reading frame of 2052 nucleotides corresponding to 684 amino acid residues. The chemically determined 15 amino acid residues of the amino-terminus of the 72 kDa protein were present in this sequence starting from Phe at position 51 (indicated by the triangle in Fig. 3A). Moreover, three chemically determined partial amino acid sequences of 72 kDa protein coincided with the deduced amino acid sequences in this open reading frame (indicated by underlines). Therefore, we concluded that this is a cDNA for the 72 kDa protein. There were six potential *N*-glycosylation sites (Asn-Xaa-Set/Thr), indicated by filled diamonds. We searched the sequence homology between the sequence of the 72 kDa protein and those in the NCBI database and found that the deduced amino acid sequence of the 72 kDa protein coincided well with known pro-POs. As shown in Fig. 3B, the 72 kDa protein was found to contain copper binding sites with significant similarity to the known pro-POs, suggesting that the 72 kDa protein is a pro-PO, but lacking the amino-terminal 50 amino acid residues which are assumed to be a pro-sequence of *Tenebrio* pro-PO. We and other groups have suggested that pro-PO was activated by pro-PO-activating serine proteinase with a limited proteolysis at the sequence motif Asn-Arg-Phe-Gly [6,16]. The amino-terminal two residues of the 72 kDa protein were Phe and Gly, whereas the carboxy-terminal two residues of the pro-segment were Asn and Arg. These results clearly indicate that the serine proteinase cleaves *Tenebrio* pro-PO between Arg and Phe in Asn-Arg-Phe-Gly, resulting in its activation. Thus, the 72 kDa is an activated PO from *Tenebrio* pro-PO.

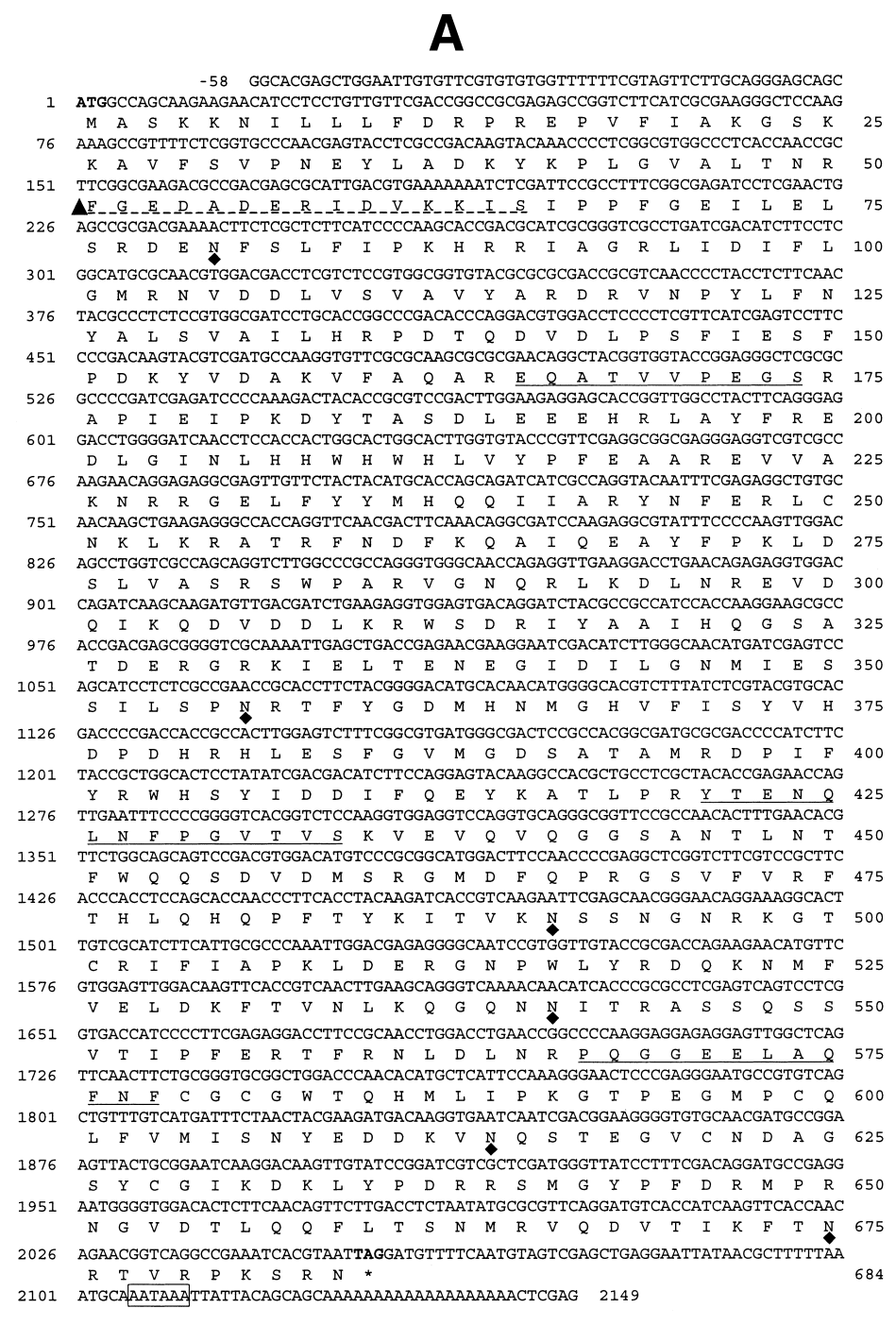

Fig. 3. Nucleotide and deduced amino acid sequence of cloned cDNA encoding the 72 kDa protein (A) and comparison of putative copper binding sites in *Tenebrio* pro-PO with those of other known pro-POs (B). A: Numbers of nucleotides starting from the first base at the 5′ end are shown on the left of each line; the deduced amino acid sequence is numbered from the initiating Met residue on the right of each line. The chemically determined amino-terminal sequence of the 72 kDa protein is dot-lined. The chemically determined partial amino acid sequences of the 72 kDa protein are underlined. The potential attachment sites for the *N*-linked carbohydrate chain is indicated by filled diamonds. The putative cleavage site of pro-PO is shown by the triangle. A poly(A) additional signal is boxed. B: Numbers at the left of each lane represent the amino acid residues of proteins. Gaps (-) are introduced to optimize alignment. Solid lines enclose the highly conservative amino acid residues. Asterisks show the suggested positions of histidine residues that are identified as ligands for copper atoms in arthropod hemocyanin. TMproPO, *Tenebrio molitor* pro-PO; BMproPO1, *Bombyx mori* pro-PO1 [15]; MSproPO, *Manduca sexta* pro-PO [19]; DMproPO, *Drosophila melanogaster* pro-PO [20]; PLproPO, *Pacifastacus leniusculus* (crayfish) pro-PO [17].

This is the first time that PO has been shown to be involved in cell clump/cell adhesion in insects.

3.3. Immunoblotting analysis

To prove that the purified 72 kDa protein was a component of cell clump/adhesion, we performed immunoblotting experiments. As is evident from Fig. 4, the recovered solution from cell-clumped proteins on the surface of the petri dish was found to contain the 72 kDa protein (shown by the arrow, lane 2 in Fig. 4). The position of this signal coincided with

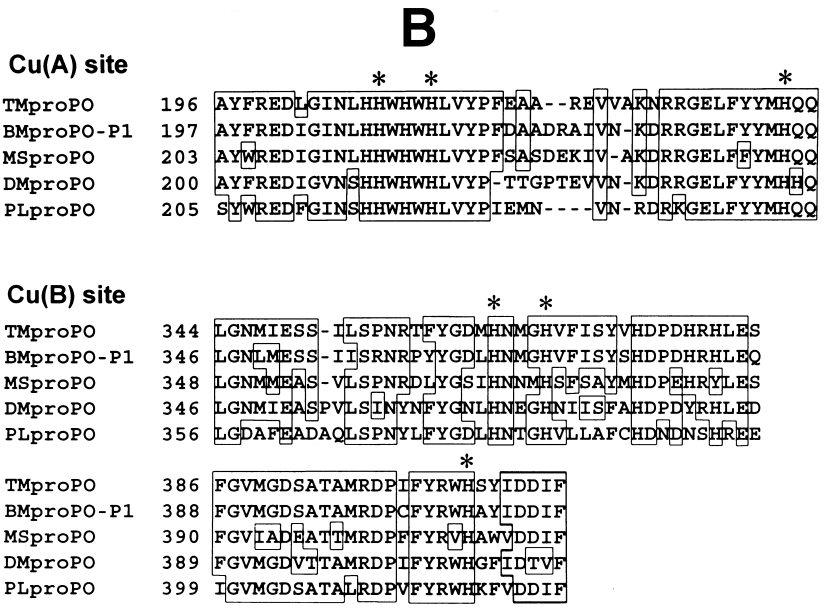

Fig. 3 (*continued*).

that of the purified 72 kDa protein. The residues and supernatant incubated with decoagulation buffer did not contain the 72 kDa protein (lane 4 and 5, respectively). However, 76 kDa bands of pro-PO (shown by the arrowhead) were shown in both the decoagulation-treated solution and the supernatant of the insect saline-treated solution (lanes 3 and 4, respectively).

4. Discussion

PO in arthropods catalyzes the oxidation of phenols to quinones, which are then polymerized non-enzymatically to melanin. It has long been recognized that defense reactions in many insects are often accompanied by melanization; the synthesized melanin encapsulates invading foreign organisms and immobilizes them. In vitro studies have shown that PO exists as an inactive precursor, pro-PO, which is activated by a stepwise process involving serine proteases activated by microbial cell wall components, such as 1,3-β-glucan, peptidoglycan or lipopolysaccharide [15,16]. Recently, pro-POs from several invertebrate species have been cloned and sequenced as pro-PO [15,17–22]. There is as yet no report about the molecular structure of the activated PO from arthropods. It was suggested that it was difficult to purify the activated PO to homogeneity from insect hemolymph because of its stickiness and non-specific crosslinking properties with hemolymph proteins [23].

In view of the biological functions of activated PO in insect defense reactions, it has been proposed that PO will have a positive correlation between non-self-recognition and a subsequent stimulation of cellular responses, such as cell adhesion, degranulation, and hemocyte locomotion, in vitro or in vivo [24]. However, the biochemical evidence concerning the role of such a correlation response has not yet been determined, and little is known about the cell adhesion mechanism at the molecular level. Here, we have provided evidence that activated PO is localized in cell clump/cell adhesion.

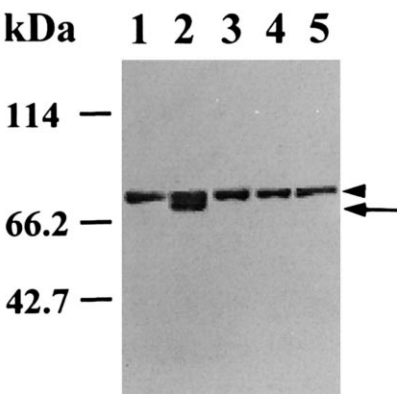

Fig. 4. Immunoblotting of 72 kDa protein antibody against the retrieved cell clump/cell adhesion proteins. Lane 1, fresh hemolymph (2 μg) of *T. molitor* larvae was immediately precipitated with TCA and loaded on the gel. The retrieved proteins (2 μg) from the cell clump/cell adhesion residues were incubated in the presence of insect saline (lane 2), supernatant solution (2 μg) in the presence of insect saline (lane 3), the residues (2 μg) incubated in the presence of decoagulation buffer (lane 4), supernatant solution (2 μg) in the presence of decoagulation buffer (lane 5). The arrowhead and arrow indicate 76 kDa pro-PO and 72 kDa activated PO, respectively.

Acknowledgements: This study was supported by the Academic Research Fund (GE 97) of the Ministry of Education of South Korea.

References

[1] Moon, H.J., Lee, S.Y., Kurata, S., Natori, S. and Lee, B.L. (1994) J. Biochem. (Tokyo) 116, 53–58.
[2] Lee, H.Y., Hong, S.U., Oh, J.E., Kwon, M.Y., Yoon, J.H., Lee, J.H., Lee, B.L. and Moon, H.M. (1998) Biochem. J. 334, 99–105.
[3] Lee, S.Y., Moon, H.J., Kurata, S., Kurama, T., Natori, S. and Lee, B.L. (1994) J. Biochem. (Tokyo) 115, 82–86.
[4] Lee, S.Y., Moon, H.J., Kawabata, S., Kurata, S., Natori, S. and Lee, B.L. (1995) Biol. Pharm. Bull. 18, 457–459.
[5] Lee, S.Y., Moon, H.J., Kurata, S., Natori, S. and Lee, B.L. (1995) Biol. Pharm. Bull. 18, 1049–1052.

[6] Lee, S.Y., Kwon, T.H., Hyun, J.H., Choi, J.S., Kawabata, S., Iwanaga, S. and Lee, B.L. (1998) Eur. J. Biochem. 254, 90–97.
[7] Lee, S.Y., Cho, M.Y., Hyun, J.H., Lee, K.M., Homma, K., Natori, S., Kawabata, S., Iwanaga, S. and Lee, B.L. (1998) Eur. J. Biochem. 257, 615–621.
[8] Asgari, S., Theopold, U., Wellby, C. and Schmidt, O. (1998) Proc. Natl. Acad. Sci. USA 95, 3690–3695.
[9] Ratcliffe, N.A. and Rowley, A.F. (1979) in: Insect Hemocytes (Gupta, A.P., Ed.), pp. 331–414, Cambridge University Press, Cambridge.
[10] Gagen, S.J. and Ratcliffe, N.A. (1976) J. Invertebr. Pathol. 28, 17–22.
[11] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[12] LeGendre, N. and Matsudaira, P. (1988) BioTechniques 6, 154–159.
[13] Sanger, F.S., Nickel, S. and Cousin, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[14] McCauley, R. and Racker, E. (1973) Mol. Cell. Biol. 1, 73–81.
[15] Kawabata, T., Yasuhara, Y., Ochiai, M., Matsuura, S. and Ashida, M. (1995) Proc. Natl. Acad. Sci. USA 92, 7774–7778.
[16] Ashida, M. and Brey, P (1998) in: Molecular Mechanisms of Immune Responses in Insects (Brey, P.E. and Hultmark, D., Eds.), pp. 135–172, Chapman and Hall, London.
[17] Aspan, A., Huang, T.S., Cerénius, L. and Söderhäll, K. (1995) Proc. Natl. Acad. Sci. USA 92, 939–943.
[18] Cho, W.L., Liu, H.S., Lee, C.H., Kuo, C.C., Chang, T.Y., Liu, C.T. and Chen, C.C. (1998) Insect Mol. Biol. 7, 31–40.
[19] Hall, M., Scott, T., Sugumaran, M., Söderhäll, K. and Law, J.H. (1995) Proc. Natl. Acad. Sci. USA 92, 7764–7768.
[20] Fujimoto, K., Okino, N., Kawabata, S., Iwanaga, S. and Ohnishi, E. (1995) Proc. Natl. Acad. Sci. USA 92, 7769–7773.
[21] Park, D.S., Shin, S.W., Kim, M.G., Park, S.S., Lee, W.J., Brey, P.T. and Park, H.Y. (1998) Insect Biochem. Mol. Biol. 27, 983–992.
[22] Lee, W.J., Ahmed, A., della Torre, A., Kobayashi, A., Ashida, M. and Brey, P.T. (1997) Insect Mol. Biol. 7, 41–50.
[23] Ashida, M. and Yamazaki, I.H. (1990) in: Molting and Metamorphosis (Ohnishi, E. and Ishizaki, H., Eds.), pp. 239–265, Japan Science Societies Press, Tokyo.
[24] Söderhäll, K., Cerenius, L. and Johansson, M.W. (1996) in: New Directions in Invertebrate Immunolgy (Söderhäll, K., Iwanaga, S. and Vasta, G., Eds.), pp. 229–253, SOS, Fair Haven, NJ.