Arch Pharm Res Vol 30, No 3, 361-365, 2007

Archives of Pharmacal Research
http://apr.psk.or.kr

Isolation of Fatty Acids with Anticancer Activity from *Protaetia brevitarsis* Larva

Yung-Choon Yoo[1,*], Byung-Hoon Shin*, Jang-Hee Hong, Junglim Lee[1], Hee-Youn Chee[2], Kyung-Sik Song[2], and Kyung-Bok Lee

Department of Biochemistry, College of Medicine, Konyang University, Daejeon 302-801, Korea, [1]Department of Microbiology, College of Medicine, Konyang University, Daejeon 302-801, Korea, [2]Department of Cell Biology, College of Medicine, Konyang University, Daejeon 302-801, Korea, and [3]Division of Applied Biology and Chemistry, College of Agriculture and Life Sciences, Kyungpook National University, Daegu 702-701, Korea

(Received November 1, 2006)

In this study, biologically active compounds were isolated from *Protaetia brevitarsis* larva (PBL) by dichloromethane extraction. The dichloromethane extract from PBL was highly cytotoxic to various cancer cells. From a silica gel column chromatograpy of this extract, we obtained four fractions (F-2, F-4, F-5 and F-7) having apoptosis-inducing activity. These fractions induced DNA ladder and caspase-3 activation during apoptosis in colon 26 tumor cells. In ^{1}H and ^{13}C NMR and mass spectral analysis of the fraction F-2 showing the highest apoptosis-inducing activity, we found that the fraction was composed of three free fatty acids such as palmitic acid, (Z)-9-octadecenoic acid and octadecenoic acid. These results indicate that the dichloromethane extract of PBL includes anticancer components composed of at least three fatty acids, and apoptosis-inducing activity of the extract was mediated by caspase-3 activation in tumor cells.

Key words: *Protaetia brevitarsis*, Apoptosis, Caspase-3, Fatty acid

INTRODUCTION

It has been well known that many natural source-derived constituents can suppress the proliferation of cancer cells (Yang *et al.*, 2003; Abdullaev and Espinosa-Aguirre, 2004; Cozzi *et al.*, 2004; Farkya *et al.*, 2004; Kostova, 2005). In the traditional medicine, many insects and their larva such as *Mylabris sidae, Bombyx mori, Protaetia brevitarsis, Pheretima asiatica, Hirudo nipponia, Scolopeudra subspinipes multilas and Buthus martensis* were broadly applied to the therapy of cancer and various types of adult diseases (Xu *et al.*, 2004; Goo *et al.*, 2004; Park *et al.*, 2004; Tanaka *et al.*, 1999; Tettamanti *et al.*, 2004; You *et al.*, 2004; Fu *et al.*, 2004). During of metamorphosis, the insects produce a variety of bio-active materials to fulfill tissue remodeling and development of the host. Especially the amount of the bio-active materials is increased in the larva stage. For these reasons, many investigators have tried to find new biologically active compounds from insects and their larva (Cai *et al.*, 2004; Vergote *et al.*, 2004; Xu *et al.*, 2004).

P. brevitarsis larva (PBL), called Jejo in Korea, has been often used for treatment of hepatic cancer, liver cirrhosis, hepatitis, breast cancer and inflammatory disease in a folk remedy (Boman, 1995; Lee *et al.*, 1997; Tossi *et al.*, 1997; Yoon *et al.*, 2003). However no scientific evidence for anticancer activity of PBL has been reported yet. It prompted us to investigate anticancer activity of the extract of PBL and to isolate components related to its anticancer activity. In this study we investigated anticancer activity of PBL to induce apoptosis in tumor cells, and isolated three fatty acids responsible for its apoptosis-inducing activity.

MATERIALS AND METHODS

General

PBL from Jeju island was purchased from the market place located in Seoul, Korea. ^{1}H- and ^{13}C-NMR were recorded on a Bruker Avance Digital 400 Spectrometer

*These two authors equally contributed to this work as the first author.
Correspondence to: Kyung-Bok Lee, Department of Biochemistry, College of Medicine, Konyang University, Seo-ku, Daejeon 302-801, Korea
Tel: 82-42-600-6495, Fax: 82-42-600-6495
E-mail: yc_yoo@konyang.ac.kr

(Karlsruhe, Germany) at 400 and 100 MHz, respectively. HPLC was a Jasco system (Japan) equipped with a PU2080 pump, AS2055 auto sampler, and MD2010 PDA. GC-MS was analyzed with a JMS 700 (JEOL, Japan) with an HP-1 column (0.25 mm×30 m) at 70 eV. TLC was performed on a pre-coated silica gel plate (Kieselgel 60 F_{254}, Merck, NJ, U.S.A.). Silica gel chromatography was carried out with Kieselgel 60 (Art.7734, 70-230 mesh, Merck, NJ, U.S.A.). Authentic fatty acids were the products of Sigma (U.S.A.).

Extraction and isolation

As preliminary test, 10 g of PBL powder was extracted with 500 mL of dichloromethane twice. The filtrate was combined and evaporated to dryness (PBL-CH_2Cl_2). The residue was refluxed in ethyl acetate (EtOAc) to be evaporated (PBL-EtOAc) and the residue was extracted again with n-butanol to obtain PBL-BuOH. Five hundred g of PBL powder was refluxed thrice with 6 L of dichloromethane in a water bath for 3 h. The solution was filtered through filter paper and the filtrate was concentrated by rotary evaporator to give 37.7 g of dichloromethane soluble fraction. Silica gel column chromatography (12 × 45 cm, Merck Art. 7734, benzene-EtOAc = 100:1 100% EtOAc) of this fraction afforded Fr. 1 to 8.

Cell culture and cytotoxicity assay

Colon 26 murine carcinoma cells were maintained as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum, vitamin solution, sodium pyruvate, nonessential amino acids and L-glutamine. Colon 26 cells (5×10^4/well) in 96-well plates were incubated with various doses of the indicated samples at 37°C for 24 h. The cultures were added by 10 μL/well of Cell Counting Kit (Allexis, MA, U.S.A.) solution, and incubated for 2 h before termination. Cytotoxic activity was determined using absorbance value of each well at 450 nm.

DNA fragmentation

Fragmentation of genomic DNA of the cells was analyzed as described previously (Yoo *et al.*, 1997). Colon 26 cells (1×10^6) that had been treated with each fraction (100 μg/mL) of PBL-CH_2Cl_2 for 24 h were digested in in 50 μL of a lysis buffer, 0.5% sodium-N-lauroyl-sarcosinate, 10 mM EDTA and 50 mM Tris-HCl (pH 7.8) containing RNAse-A (20 μg/mL), at 50°C for 30 min. Digestion was continued for 60 min after addition of proteinase K (20 μg/mL). After centrifugation at 10000 rpm for 1 min, DNA samples were loaded on 1% agarose gel containing 0.1 μg/mL ethidium bromide and visualized under UV light. As the positive control, the cells were treated with 0.5 μg/mL of adriamycin (ADR).

Assay of caspase-3 enzyme activity

Enzyme activity of the intracellular caspase-3 in colon 26 cells undergoing apoptosis induced by the fractions of PBL extract was determined using a caspase-3 activity determination kit (Allexis) according to the manufacturer's recommendation.

RESULTS AND DISCUSSION

The PBL powder was consecutively extracted with dichloromethane (PBL-CH_2Cl_2), ethyl acetate (PBL-EtOAc), and butanol (PBL-BuOH). In the assay for cytotoxic activity of the PBL extracts using colon 26 murine tumor cells, PBL-CH_2Cl_2, but not PBL-EtOAc and PBL-BuOH, showed high cytotoxic activity to colon 26 tumor cells in a dose-dependent manner (Fig. 1). It was also likely that PBL-

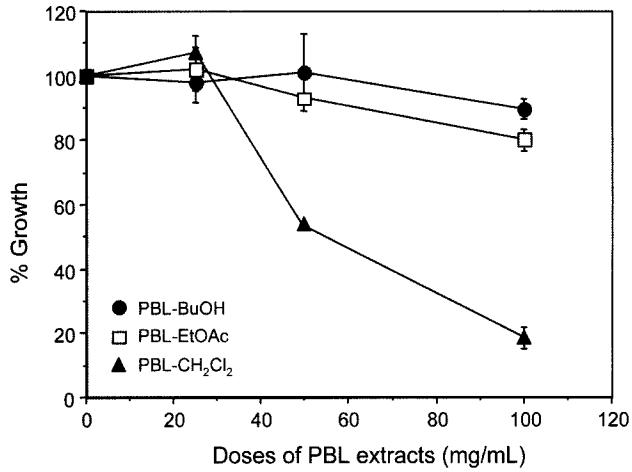

Fig. 1. Cytotoxic effect of PBL extracts on colon 26 tumor cells. Colon 26 tumor cells (5×10^4/well) were treated with the indicated doses of PBL-CH_2Cl_2, PBL-EtOAc or PBL-BuOH for 24 h. Cytotoxicity was evaluated by Cell Counting Kit.

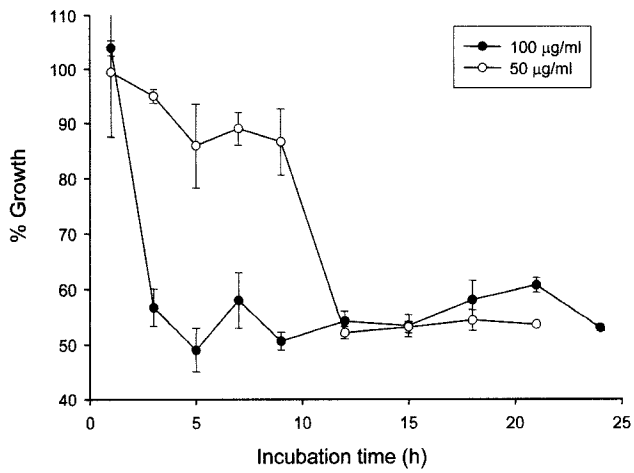

Fig. 2. Time-course analysis of PBL-CH_2Cl_2 on colon 26 tumor cells. Colon 26 cells were treated with 50 or 100 μg/mL of PBL-CH_2Cl_2 for the indicated times..

CH_2Cl_2 completely killed colon 26 tumor cells within 5 h at 100 μg/mL and within 12 h at 50 μg/mL (Fig. 2).

In order to investigate the active fractions responsible for PBL-CH_2Cl_2-induced cell death, the extract was applied to a silica gel column, and 8 fractions were obtained. Out of 8 fractions from the silica gel chromatography, 4 fractions (F-2, F-4, F-5 and F-7) showed high cytotoxic activity (Fig. 3). Among the 4 fractions, F-2 was the most effective, and this fraction appeared to be a single spot on the TLC plate. The cells undergoing cell death by these fractions exhibited apparent typical apoptotic characteristic like fragmented genomic DNA (Fig. 4). Furthermore, in an enzyme activity analysis, it was shown that treatment of each of the active fractions (100 mg/mL) for 2 h enhanced the intracellular level of caspase-3 enzyme activity (Fig. 5).

Next we tried to identify active compounds associated

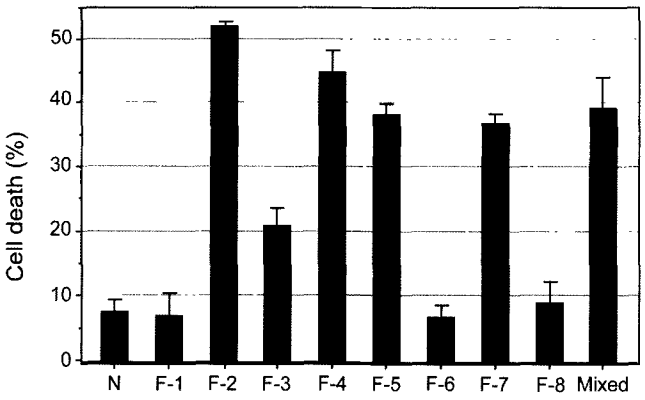

Fig. 3. Cytotoxic activity of various fractions obtained from a silica gel chromatography of PBL-CH_2Cl_2. Colon 26 cells were treated with each fraction (50 μg/mL) of PBL-CH_2Cl_2 for 24 h, and its cytotoxic activity was calculated by Cell Counting Kit.

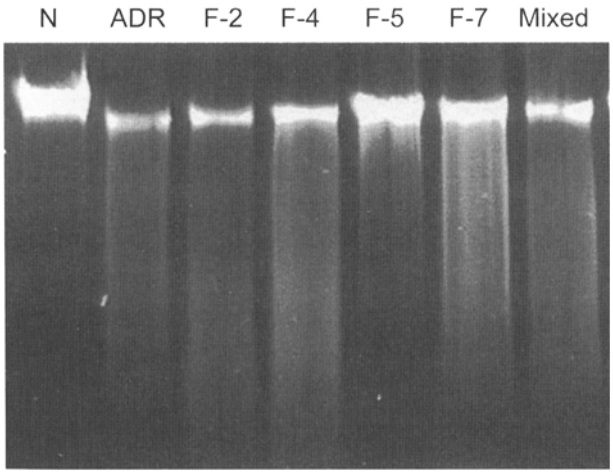

Fig. 4. DNA ladder induction by various fractions of PBL-CH_2Cl_2. Colon 26 cells (1×10^6/well) were treated with 50 μg/mL of the indicated fractions of PBL-CH_2Cl_2 for 24 h. Fragmented DNA was visualized by the method described in Materials and Methods.

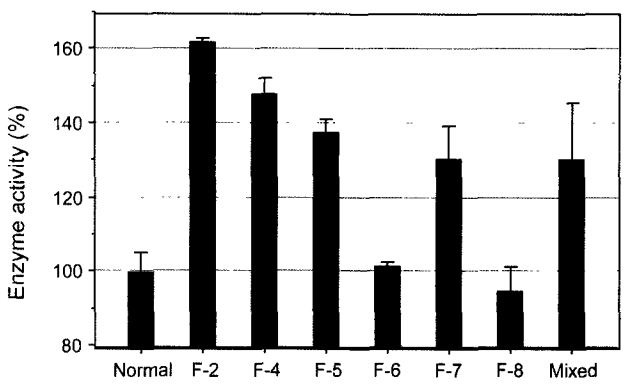

Fig.5. Activation of caspase-3 enzyme during apoptosis induced by PBL-CH_2Cl_2-derived fractions. Colon 26 cells (1×10^6/well) were treated with the indicated fractions (50 μg/mL) for 2 h. Caspase-3 activity of the cells was determined by a caspase-3 activity detection kit.

with apoptosis-inducing activity of F-2 fraction of PBL-CH_2Cl_2. In the ^{1}H-NMR (chloroform-*d*) (Palik, 1985), an olefinic proton at δ5.34 (*m*) was detected with the characteristic signals of fatty acids at δ2.37 (*t*, *J* =6.40), δ2.02 (*t*. *J* =7.56), δ1.64 (*m*), δ1.25 (*m*), 0.87 (*t*). In the ^{13}C-NMR (chloroform-*d*), two sp^2 carbon signals were detected at δ129.74 and 130.02, suggesting that F-2 was an unsaturated fatty acid. Overlapped carbonyl carbons at δ180.29 and 180.32 indicated that F-2 was a mixture of at least two fatty acids. In the GC-MS analysis, 3 peaks [t_R's (retention times); 18.04, 19.63, and 19.80 min] were found (Fig. 6). The peak at t_R 18.04 min was identified as palmitic acid by its molecular ion peak (*m/z* 256) and co-chromatography with an authentic sample. By means of similar methods, the peaks at t_R 19.63 and 19.80 min were identified as (*Z*)-9-octadecenoic acid and octadecanoic acid (stearic acid, *m/z* 282), respectively. The chemical structures of identified compounds are presented in Fig. 7. The peak ratio of palmitic acid, (*Z*)-9-octadecenoic acid, and octadecanoic acid was 37.4-40.5-22.1.

It is known that high concentration of certain fatty acids can cause cell death via apoptosis or necrosis (Andrade *et al.*, 2005). Palmitic acid has been also observed to induce apoptosis in tumor cells although the mechanism of this cytotoxicity is unsolved (Kong *et al.*, 2002). Since the dichloromethane extract of PBL contained palmitic acid, and the standard palmitic acid induced apoptosis effectively in colon 26 cells (data not shown), this fatty acid is thought to be a major molecule responsible for PBL-CH_2Cl_2-induced apoptosis. However, whether or not (*Z*)-9-octadecenoic acid and octadecanoic acid participate to apoptosis induction by PBL-CH_2Cl_2 is unclear.

This suggests that PBL contains cytotoxic compounds to induce apoptosis in tumor cells and its apoptosis-inducing activity is associated with caspase-3 enzyme activation. Further studies to isolate additional cytotoxic

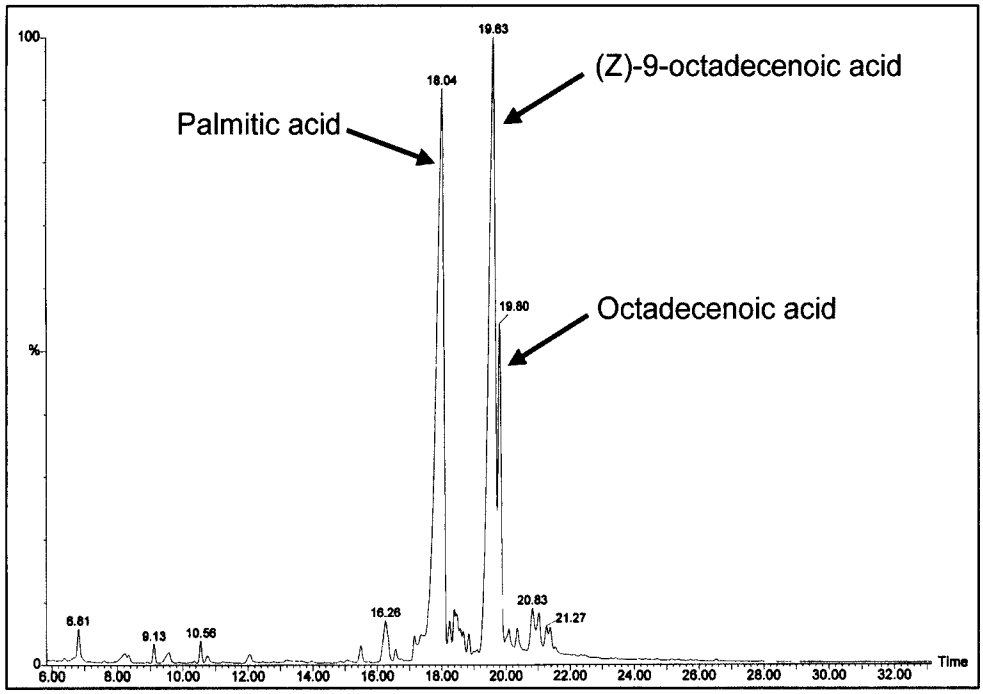

Fig. 6. Total ion chromatogram of F-2 of PBL-CH_2Cl_2

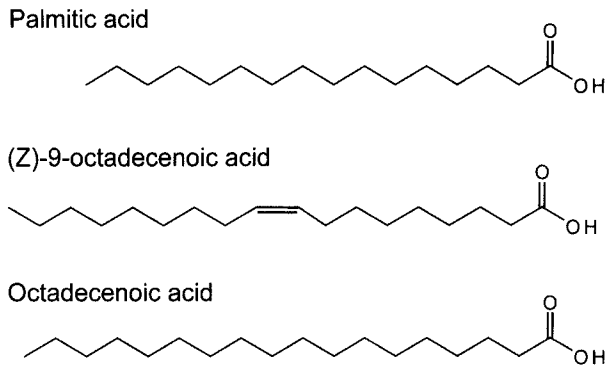

Fig. 7. The structure of palmitic acid, (Z)-9-octadecenoic acid and octadecenoic acid

molecules from PBL and to analyze the mechanisms of PBL-induced apoptosis are currently underway.

ACKNOWLEDGEMENTS

This study was performed as a link of the program for development of neutraceutical foods for the aged of RIS (2004-2005), Konyang University.

REFERRENCES

Abdullaev, F. I. and Espinosa-Aguirre, J. J., Biomedical properties of saffron and its potential use in cancer therapy and chemoprevention trials. *Cancer Detect Prev.*, 28, 426-432 (2004).

Andrade, L. N., de Lima, T. M., Curi, R., and Castrucci, A. M., Toxicity of fatty acids on murine and human melanoma cell lines. *Toxicol. In vitro*, 19, 553-660 (2005).

Boman, H. C., Peptide antibiotics and their role in innate immunity. *Annu. Rev. Immunol.*, 13, 61-92 (1995).

Cai, Z., Xu, C., Xu, Y., Lu, W., Chi, C. W., Shi, Y., and Wu, J., Solution structure of BmBKTx1, a new BKCa1 channel blocker from the Chinese scorpion *Buthus martensi* Karsch. *Biochem.*, 43, 3764-3771 (2004).

Cozzi, P., Mongelli, N., and Suarato, A., Recent anticancer cytotoxic agents. *Curr Med Chem Anti-cancer Agents*, 4, 93-121 (2004).

Farkya, S., Bisaria, V. S., and Srivastava, A. K., Biotechnological aspects of the production of the anticancer drug podophyllotoxin. *Appl Microbiol Biotechnol.*, 65, 504-519 (2004).

Fu, Y. J., Chai, B. F., Wang, W., Zhi, H., Yin, L.T., and Liang, A. H., Expression and purification of the BmK Mm2 neurotoxin from the scorpion *Buthus martensii* Karsch and its biological activity test. *Protein. Expr. Purif.*, 38, 45-50 (2004).

Goo, T. W., Yun, E. Y., Choi, K. H., Kim, S. H., Nho, S. K., Kang, S. W., and Kwon, O. Y., ATFC is a novel transducer for the unfolded protein response in *Bombyx mori* BM5 cells. *Biochem. Biophys. Res. Commun.*, 325, 626-631 (2004).

Kong, J. Y. and Rabkin, S. W., Lovastatin does not accentuate but is rather additive to palmitate-induced apoptosis in cardiomyocytes. *Prostaglandins Leukot. Essent. Fatty Acids*, 67, 293-302 (2002).

Kostova, I., Synthetic and natural coumarins as cytotoxic agents. Curr *Med Chem Anti-Canc Agents*, 5, 29-46 (2005).

Lee, D. G., Park, J. H., Lee, S. G., Kim, M. K., and Hahm, K. S., Design of novel analogue peptides with potent fungicidal but low hemolytic activity based on the cecropin A-mellitin hybrid structure. *Biochem. Mol. Biol. Int.,* 43, 489-498 (1997).

Palik, E. D., Handbook of proton-NMR spectra and data. Asahi Research Center, Japan (1985).

Park, C. W., Kim, J. H., Kim, K. M., Hwang, J. S., Kang, S. W., Kang, H. S., Cho, B. P., Yu, C. H., Kim, H. R., and Lee, B. H., Evidence for brain-derived neurotrophic factor-like neuropeptide in brain of the silk moth *Bombyx mori* during postembryonic periods. *Peptides,* 25, 1891-1897 (2004).

Tanaka, R., Noda, N., and Miyahara, K., Glycerophosphocholines of the earthworm, Pheretima asiatica. *Chem. Pharm. Bull. (Tokyo),* 47, 1167-1169 (1999).

Tettamanti, G., Grimaldi, A., Rinaldi, L., Arnaboldi, F., Congiu, T., Valvassori, R., and de Eguileor, M., The multifunctional role of fibroblasts during wound healing in Hirudo medicinalis (Annelida, Hirudinea). *Biol. Cell.,* 96, 443-455 (2004).

Tossi, A., Tarantino, C., and Romeo, D., Design of synthetic antimicrobial peptides based on sequence analogy and amphipathicity. *Eur. J. Biochem.,* 250, 549-558 (1997).

Yang, L. L., Wang, M. C., Chen, L. G., and Wang, C. C., Cytotoxic activity of coumarins from the fruits of *Cuidium monnieri* on leukemia cell lines. *Planta Med.*, 69, 1091-1095 (2003).

Yoo, Y. C., Watanabe, R., Koike, Y., Mitobe, M., Shimazaki, K., Watanabe, S., and Azuma, I., Apoptosis in human leukemic cells induced by lactoferricin, a bovine milk protein-derived peptide: involvement of reactive oxygen species. *Biochem. Biophys. Res. Commun.*, 237, 624-628 (1997).

Yoon, H. S., Lee, C. S., Lee, S. Y., Choi, C. S., Lee, I. H., Yeo, S. M., and Kim, H. R., Purification and cDNA cloning of inducible antibacterial peptides from *Protaetia brevitarsis*. *Arch. Insect. Biochem. Physiol.*, 52, 92-103 (2003).

You, W. K., Sohn, Y. D., Kim, K. Y., Park, D. H., Jang, Y., and Chung, K. H., Purification and molecular cloning of a novel serine protease from the centipede, *Scolopendra subspinipes* mutilans. *Insect. Biochem. Mol. Biol.,* 34, 239-250 (2004).

Vergote, D., Sautiere, P. E., Vandenbulcke, F., Vieau, D., Mitta, G., Macagno, E. R., and Salzet, M., Up-regulation of neurohemerythrin expression in the central nervous system of the medicinal leech, *Hirudo medicinalis*, following septic injury. *J. Biol. Chem.*, 279, 43828-43837 (2004).

Xu, C. Q., Brone, B., Wicher, D., Bozkurt, O., Lu, W. Y., Huys, I., Han, Y. H., Tytgat, J., Van Kerkhove, E., and Chi, C. W., BmBKTx1, a novel Ca^{2+}-activated K^+ channel blocker purified from the Asian scorpion *Buthus martensi Karsch*. *J. Biol. Chem.,* 279, 34562-34569 (2004).