Article

# Purification and Molecular Identification of an Antifungal Peptide from the Hemolymph of *Musca domestica* (housefly)

**Ping Fu[1], Jianwei Wu[1, 2] and Guo Guo[1]**

**Antibacterial and antifungal peptides found in houseflies (*Musca domestica*) in large number are indispensable components of its immune defense mechanism. In this study the anterior tip of the larvae of housefly was cut off with a pair of fine scissors and hemolymph was collected and exuded in an ice-cold test tube. From the hemolymph an antifungal substance was isolated by solid-phase extraction combined with reverse phase-high performance liquid chromotography (RP-HPLC) and named as *Musca domestica* antifungal peptide-1 (MAF-1). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) showed its molecular weight was 17 kDa. UV absorption spectra revealed that this antifungal substance possessed the characteristics of protein peptides. Analysis by fingerprint-identification and tandem mass spectrometry suggested MAF-1 was an unknown protein. Edman degradation identified the sequence of 30 amino acids of its N-terminal which matched no peptide in the MASCOT search database, indicating MAF-1 was a novel insect antifungal peptide. Mass spectrometry showed the precise molecular weight of MAF-1 was 17203.384 Da. Its isoelectric point was acidic. *Cellular & Molecular Immunology*. 2009;6(4):245-251.**

**Key Words:** *Musca domestica*, antifungal peptide, purification, molecular identification

## Introduction

A great abundance of biologically active proteins and peptides with antibacterial, antifungal and antiviral properties are found in insects. Identification and isolation of these active substances and determination of their primary structures or DNA sequences are of vital importance both to the study of non-specific immune response mechanism of insect against pathogen invasion and to the application of these substances into bio-pharmaceutical industry that will ultimately benefit mankind (1-4).

Housefly is an important medical insect which has a highly effective immune defense mechanism and is rarely infected even reared in large-scale, high-density conditions (5-11). Scholars studied the hemolymph of housefly larvae and isolated a variety of antibacterial and antifungal peptides (12-17). Research of antifungal peptides (proteins) mainly focued on induction, separation and purification, and detection of biological activity (18-23). No report has been made on the isolation, purification and molecular identification of antifungal peptide elements from the hemolymph of housefly larvae. In this study, solid-phase extraction combined with reverse phase-high performance liquid chromotography (RP-HPLC) was applied in isolation and purification of the antifungal peptide from the hemolymph of house fly larvae. Mass spectrometry was used to determine its precise molecular weight, and Edman degradation was conducted to determine its N-terminal amino acid sequence. This study is expected to lay a foundation for further study of the housefly immune defense mechanism and the discovery of a new generation of antifungal biological agents.

## Materials and Methods

### *Materials*

*Candida albicans* ATCC 76615 (*Candida albicans* (Robin) Berkhout 1923) strain 09 was obtained from Shanghai Second Medical University (Shanghai, China). Acetonitrile (ACN) was purchased from American Tedia Corp. Trifluoroacetic acid (TFA) was purchased from Sigma Corp. The BCA protein analysis Kit was purchased from Pierce Corp.

### *Animals and collection of hemolymph*

The houseflies (*Musca domestica*) were bred by the

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Department of Parasitology, Guiyang Medical College (Guiyang, China). The third instar larvae of housefly was kept under humid condition at room temperature for up to 4 days, so it could be used for the isolation of hemolymph. The anterior tip of the larvae was cut off with a pair of fine scissors and hemolymph was collected and exuded in an ice-cold test tube. Typically close to 100 µL of hemolymph was collected from about 30 larvase.

***Assay of antifungal activity***

*C. albicans* ATCC 76615 was grown in Sabouraud medium (4% glucose, 1% peptone in distilled water, pH 5.8) at 37°C. Cells in the exponential phase of growth were suspended in distilled water at a density of $10^6$ cells/ml. Fifty µl of sample solution was added to 50 µl of this cell suspension, and the mixture was incubated at 37°C for 24 h. Then the mixture was spread on Sabouraud agar medium and after incubation for 24-36 h at 37°C, the numbers of colonies were counted (24).

***Purification of antifungal peptide of musca domestica***

A peptide with antifungal activity was purified from the hemolymph of *Musca domestica* larvae as follows. About 1 ml of hemolymph was diluted 10-fold with ultrapure water (Millipore), heated for 5 min at 100°C, and centrifuged at 12,000 ×g and 4°C for 30 min to remove unresolved substances. The resulting supernatant containing the antifungal activity was ready for further purification. Sep-Pak cartridge (Waters, USA) was conditioned with 0.05% trifluoroacetic acid. The resulting supernatant containing the antifungal activity was loaded onto a conditioned Sep-Pak cartridge. Elutions were performed with stepwise concentrations of 5 ml acetonitrile (ACN) (from 0 to 80%) in acidified water (0.05% TFA), and elution was controlled at the flow rate of 1 ml/min. Fractions eluted from every concentration were collected and dried under vacuum to evaporate the ACN totally. The dried samples were dissolved in distilled water for antifungal activity assay. The fractions obtained at different ACN concentrations containing antifungal activity were filtered through a nitrocellulose membrane filter of 0.22 µm pore size (Millipore), and the filtrate was analyzed using RP-HPLC on a Sephasyl $C_{18}$ column (Beckman, System Gold, USA, ODS 0.5 µm, 0.46 × 25 cm). Absorbed material was eluted with a linear gradient of 0-80% solution B (0.1% trifluoroacetic acid in acetonitrile) in solution A (0.1% trifluoroacetic acid in Ultrapure water) over 40 min at a flow rate of 0.8 ml/min. Each fraction corresponding to an absorbance peak was lyophilized, redissolved in distilled water, and tested for the antifungal activity. By this procedure, a peptide with antifungal activity was purified from the hemolymph of housefly larvae in protein concentration assay, BCA reagents were used (25).

***Determination of MAF-1 by ultraviolet spectrometry***

Purified MAF-1 was pooled and its absorption at wave-lengths between 190-600 nm was determined using an ultraviolet spectrophotometer.

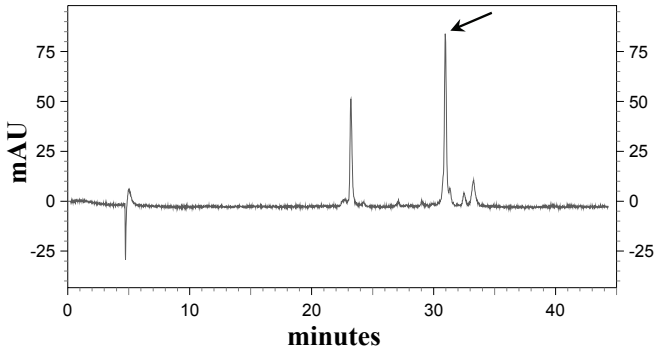


**Figure 1. RP-HPLC peaks of the fractions eluted with 50% and 60% Acetonitrile.** 50% and 60% ACN fraction from SPE was applied to RP-HPLC and eluted by a linear gradient of 0-80% ACN in 0.1% TFA over 40 min at flow rates of 0.8 ml/min. Arrow indicated the peak of MAF-1.

***Molecular identification of MAF-1***

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the purified MAF-1 with reference to the method of Hermann Schagger et al. (26). The concentrations of separation gel, stacking gel and sample gel were 16%, 4% and 10% respectively. After SDS-PAGE, the band of MAF-1 was identified by peptide mass fingerprinting (PMF) and tandem mass spectrometry. The mass spectrometry was performed on a Finnigan LTQ instrument (Finnigan, USA). Spray voltage was 4.5 kV and capillary temperature at 170°C. The sample was dissolved in 0.25% acetic acid/50% methanol at a concentration of approximately 10 pmol/µl. Scans were acquired in profile mode in the m/z range of 400 to 2,000. Peptide product ion spectra generated by the mass spectrometry were searched against the National Center for Biotechnology Information nonredundant protein database using the Mascot database search engine.

The MsBlast was carried out for searching consensus protein sequence composed from aligned sequences of

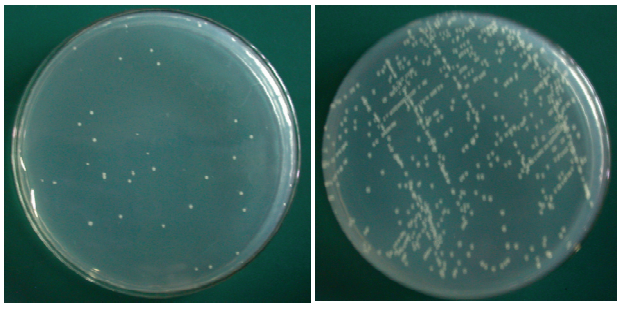


**Figure 2. Antifungal activity of MAF-1.** *C. albicans* was spread on Sabouraud agar medium with or without MAF-1. After incubation for 24-36 h at 37°C, the numbers of colonies were counted. The colony number of MAF-1 was obviously less than that of negative control group. Data were also presented in Table 1.

**Table 1.** Antifungal activity of MAF-1

| Sample | Concentration (μg/ml) | Colony number |
|---|---|---|
| MAF-1 | 72.92 ± 2.12 | 40 ± 9* |
| Control | 0 | 1258 ± 126 |

Data were shown as mean ± SD. *$p < 0.05$ compared with negative control group.

database using the peptide sequences deduced by mass spectrometry. The precise molecular weight of MAF-1 was determined by Matrix-assisted laser desorption/ionization time of flighty-mass spectrometry (MALDI-TOF MS) (Bruker, German). The purified MAF-1 from RP-HPLC was pooled and analyzed to determine the isoelectric point of the protein. Finally, the N-terminal sequence of MAF-1 was determined by the Edman degradation method. (PMF was accomplished by Beijing Genomics Research Centre. Protein characterization using tandem mass spectrometry, isoelectric point determination, precise molecular weight determination and N-terminal amino acid sequencing was accomplished by Research Centre for Proteome Analysis, Key Lab of Proteomics, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China.)

***Statistical analysis***

Statistical differences were analyzed using the SPSS 11.5 software. The $p$ value < 0.05 was statistically significant. All data were expressed as mean ± SD.

## Results

***Purification of an antifungal peptide of Musca domestica***

After heating and centrifugation of the collected hemolymph, the obtained supernatant was subjected to Solid Phase Extraction (SPE) followed by elution of ACN at different concentrations. The fractions obtained at the ACN concentration of 50% and 60% were found to have antifungal activity.

On RP-HPLC, two major absorbed peaks were observed about the fractions and one peak appeared at around a retention time (RT) of 31.0 min (Figure 1). Antifungal assay found that it had a strong antifungal activity against *C. albicans* (Figure 2, Table 1).

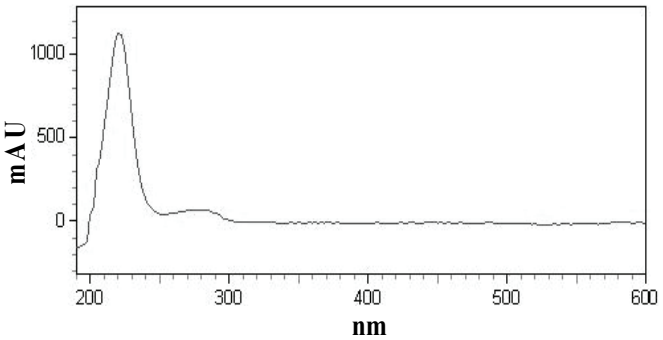


**Figure 3. The UV absorbance spectrum of MAF-1.** It showed a positive peak at 210-220 nm and a small negative peak at 250-285 nm and it had the characteristic absorption peak of protein.

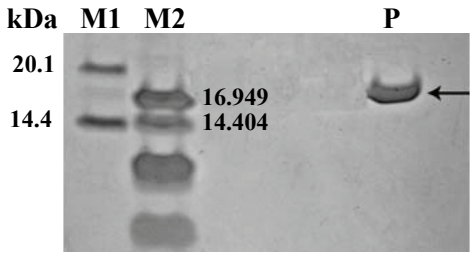


**Figure 4. SDS-PAGE electrophoresis of MAF-1.** Lanes M1 and M2, standard for molecular weight of protein; Lane P, MAF-1 (showed by arrow).

***Determination of MAF-1 by ultraviolet spectrometry***

The UV-absorption spectrum of MAF-1 showed a positive

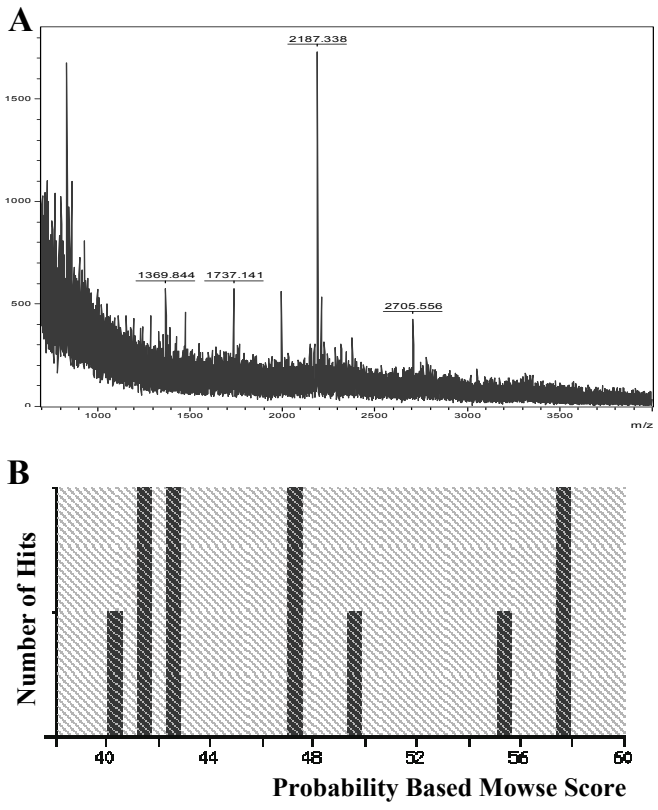


**Figure 5. The analysis of PMF in MAF-1.** (A) The mass chromatogram of PMF of MAF-1. (B) Protein score was -10*Log(P), where P has the probability that the observed match is a random event. Protein scores greater than 80 were significant ($p < 0.05$).

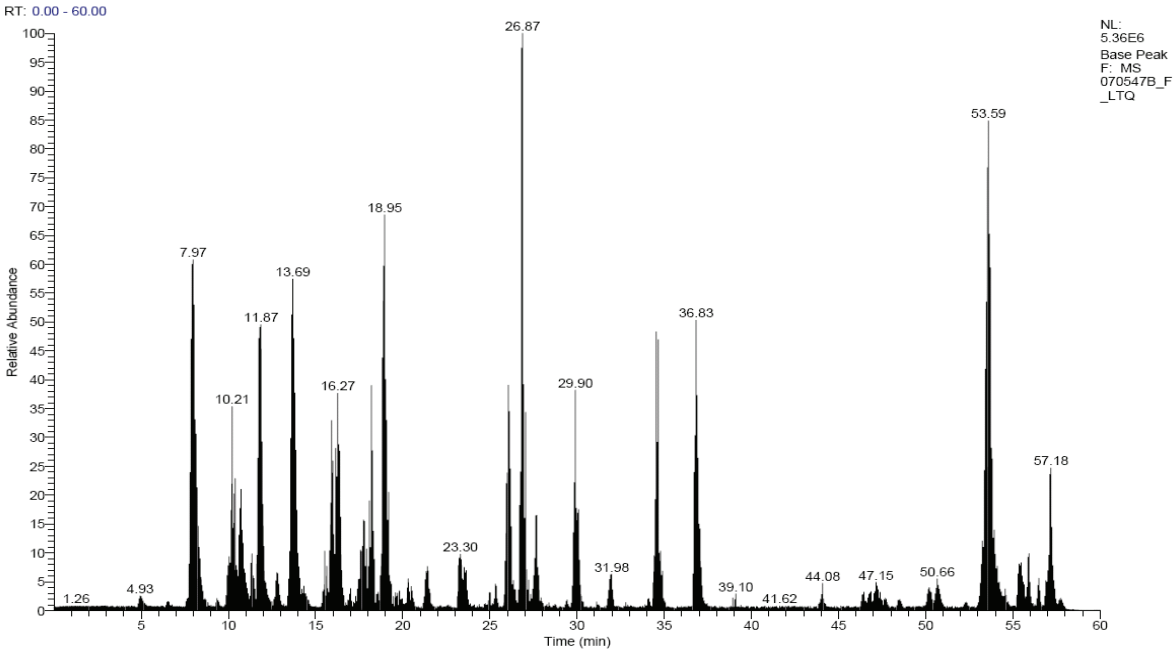


**Figure 6. Mass spectrometric analysis of MAF-1 on LTQ-FT mass spectrometer.**

peak at 210-220 nm and a small negative peak at 250-285 nm (Figure 3). The absorption at 210-220 nm resulted mainly from peptide bond while the absorption at 250-285 nm was attributed to the aromatic side chains of amino acids, including the phenolic group of tyrosine, the indole ring of tryptophan, the indole ring of histidine, phenylalanine and cystine, the absorption maxima of which are at 280.4 nm, 274.8 nm, 250-260 nm, 247-264 nm and 280 nm respectively. The UV-absorption spectrum of MAF-1 suggested that it had the characteristic absorption peak of protein.

***Molecular identification of MAF-1***

Analysis by SDS-PAGE showed that MAF-1 had only one clear band with molecular mass of about 17 kDa (Figure 4). MAF-1 band was obtained from SDS-PAGE gel and digested with trypsin. Then matrix-assisted laser desorption/ionization time of flight-mass spectrometry (MALDI-TOF MS) was applied to the analysis of peptide mass finger- printing (PMF) in MAF-1. The PMF data of MAF-1 was processed by Mascot search, but no matched data was found (Figures 5).

Then MAF-1 was identified by using tandem mass spectrometry and searching NCBI insect database (Figure 6, Table 2). The meta-analysis results showed that MAF-1 might be a novel protein.

MAF-1 was obtained from RP-HPLC and freeze-dried. Its molecule weight was determined accurately to be 17203.384 Da by MALDI-TOF-MS (Figure 7).

The purified MAF-1 from RP-HPLC was pooled and Model 111 Mini IEF cell was used to identify its isoelectric points. Two bands were observed in MAF-1, and the isoelectric points were 4.96 and 4.58 respectively (Figure 8). The result indicated that MAF-1 was an acidic peptide and the two bands were the result of space conformation alteration observed during the determination. But the specific reasons for the changes in conformational space still need

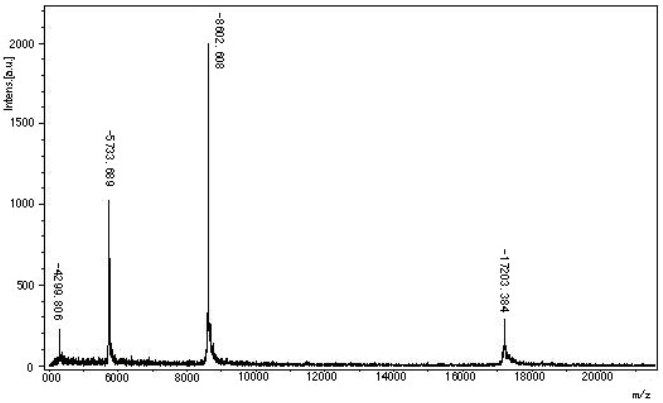


**Figure 7. The molecular weight spectrogram of MAF-1.**

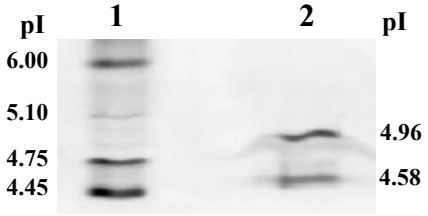


**Figure 8. The pI scanning image of MAF-1.** Lane 1, Marker; Lane 2, MAF-1.

**Table 2.** Result of Mass spectrometric analysis

| Sequence | MH+ | Cover Percent | Ions |
|---|---|---|---|
| K.ARLEHKLYLAR.E | 1370.6281 | 1.65% | 11/22 |
| K.EELVRLLR.C | 1028.2308 | 0.82% | 13/14 |
| K.EKEEEAEKK.K | 1120.1924 | 3.61% | 13/16 |
| K.FGANAILGVSLAVCK.A | 1520.792 | 6.55% | 19/28 |
| K.KEEKIEESTEEK.K | 1479.5689 | 4.12% | 12/22 |
| K.QPKEESKVELIK.C | 1428.6563 | 2.21% | 16/22 |
| R.KVGGEGGSLEVLPK.P | 1370.5768 | 0.63% | 13/26 |
| R.SSSAVEKK.A | 835.9259 | 0.58% | 9/14 |
| -.M*ADEELPAGWEK.R | 1392.5164 | 7.41% | 17/22 |
| -.MENDPSKQEK.A | 1206.3088 | 1.54% | 13/18 |
| K.AANSGVEPPM*PLSQMR.F | 1701.9502 | 2.95% | 11/30 |
| K.AVFLKIDVDK.C | 1148.3768 | 3.51% | 12/18 |
| K.EQPAADTATKALPEQK.K | 1698.8562 | 5.73% | 12/30 |
| K.ITKGGETINGK.A | 1118.266 | 2.56% | 13/20 |
| K.KLQSTPTPDRISSVPLTPEK.S | 2195.5021 | 1.68% | 13/38 |
| K.NELEVIELDTTPIK.K | 1614.8191 | 1.46% | 16/26 |
| K.PIVVGPKTEGEKPSGDK.K | 1738.9635 | 5.52% | 17/32 |
| K.QFLDRVMGQVESAK.E | 1608.8445 | 9.66% | 11/26 |
| K.QLAELNTYVPTK.A | 1377.5678 | 1.15% | 14/22 |
| K.QVGDALLLEGQQR.T | 1427.5884 | 1.21% | 14/24 |
| K.RCQDLASFM*ESLM*EETPK.I | 2205.4461 | 1.7% | 12/34 |
| K.RCQDLASFMESLM*EEVPK.T | 2187.474 | 18.18% | 16/34 |
| K.RLLKNDILM*FR.T | 1435.7635 | 1.48% | 12/20 |
| K.SIIDNQGEEAVK.L | 1303.401 | 3.3% | 16/22 |
| K.SPSEGRGGKNQK.S | 1245.328 | 2.23% | 15/22 |
| K.SSTTTSREQQK.T | 1253.302 | 2.7% | 11/20 |
| K.VWFQNRRMK.D | 1265.5155 | 2.95% | 11/16 |
| K.YEEELQTM*KEKQK.K | 1700.8923 | 2.65% | 12/24 |
| R.EALVKHK.K | 824.991 | 1.03% | 8/12 |
| R.EAYPGDVFYLHSR.L | 1554.6872 | 2.36% | 15/24 |
| R.EKESMPYLEAIIK.E | 1551.8297 | 2.64% | 13/24 |
| R.EMNFSPEKEK.K | 1239.3804 | 5.52% | 12/18 |
| R.GNPGPNPGEKEAAEKR.K | 1651.7629 | 0.28% | 13/30 |
| R.IIDDGMKKAAGVLK.H | 1459.7801 | 7.87% | 12/26 |
| R.INGLAKHEFIARK.D | 1497.7699 | 2.24% | 14/24 |
| R.KLTLTKGGPLAEGAELK.M | 1727.039 | 3.2% | 17/32 |
| R.KVRSFFISEEK.S | 1370.5785 | 3.45% | 12/20 |
| R.LHCTRLSLMIKNWDEFK.A | 2192.5632 | 6.23% | 12/32 |
| R.M*DNQASR.K | 837.8822 | 1.4% | 11/12 |
| R.NNFKLDLLK.L | 1105.3121 | 0.97% | 12/16 |
| R.PDSAGFALMVEGGR.I | 1407.5775 | 2.83% | 12/26 |
| R.QFACANK.K | 838.9249 | 0.78% | 12/12 |
| R.QFIDDMITNLNNK.E | 1566.7613 | 2.3% | 16/24 |
| R.SNFLNYCQPIISK.E | 1584.7914 | 3.04% | 13/24 |
| R.SNSAGQGSGSRSTGVIIGGSTSSR.P | 2211.2938 | 0.83% | 14/46 |
| R.STSVTSDKGIM*NILDKIPLLSK.T | 2377.7843 | 1.07% | 25/84 |
| R.TQGAIGEKEGKM*DHK.T | 1645.82 | 1.96% | 14/28 |
| R.VLERNMQQLIER.E | 1529.7905 | 2.82% | 14/22 |
| R.WVGGPEIELIAIATGGR.I | 1739.9963 | 3.15% | 17/32 |
| R.YLKGVIGEKQSGEK.L | 1536.7548 | 1.57% | 14/26 |

in-depth exploration and research.

Finally, the N-terminal residue of MAF-1 was sequenced with Edman method. Its N-terminal sequence was ESAPAPE VSGDAVFSAIQNG-LKNLGNAFFW. BLAST results further confirmed that MAF-1 was a novel antifungal peptide from insects (Table 3).

**Table 3.** Blast result

| Sequences producing significant alignments | | Score | E |
|---|---|---|---|
| ref\|YP_860418.1\| | hypothetical protein GFO_0364 [Gramella forsetii KT0803] | 32.5 | 3.5 |
| ref\|XP_803928.1\| | dispersed gene family protein 1 (DGF-1), putative [Trypanosoma cruzi strain CL Brener] | 32.5 | 3.5 |
| ref\|XP_803493.1\| | dispersed gene family protein 1 (DGF-1), putative [Trypanosoma cruzi strain CL Brener] | 32.5 | 3.5 |
| ref\|XP_806743.1\| | dispersed gene family protein 1 (DGF-1), putative [Trypanosoma cruzi strain CL Brener] | 32.5 | 3.5 |
| ref\|YP_823169.1\| | Erythromycin esterase [Solibacter usitatus Ellin6076] | 32.0 | 4.7 |
| ref\|YP_960137.1\| | hypothetical protein Maqu_2875 [Marinobacter aquaeolei VT8] | 31.6 | 6.4 |

The score of BLAST searching was lower than 40.

## Discussion

*Musca domestica* is widely distributed and found in all corners of the Earth. Its larvae and adults usually live on garbage, livestock manure and other areas that serve as breeding grounds where a variety of pathogens can be found. Housefly carries more than 60 kinds of harmful pathogenic micro-organisms which are contagious among human and animals, but not among houseflies. Even in large-scale, high-density artificial laboratory rearing conditions they have not been observed to contract infectious diseases in large number. This is mainly due to the housefly-specific immune defense mechanism formed by immunological agents innate to the housefly which include antimicrobial peptides, anti-fungal peptides, lectin, chitin of which the exoskeleton is composed and which are also found in other tissues and organs, *Alcaligenes faecalis* present in the larvae where it has functions related to metabolism, allantoin and other organic compounds. Among them quite a number of potent anti-microbial peptides play an important role in resisting invasions from various pathogenic microorganisms. These anti-bacterial and anti-fungal peptides include composition peptides found in houseflies without induction and inductive peptides whose production and increased expression requires induction. This study also proved that an antifungal peptide named MAF-1 residing in the hemolymph of housefly without induction

Wu JW, Gu LJ, et al. detected that the antifungal peptide isolated from the hemolymph of the housefly larva was thermally stable (18, 21). During our experiments, the hemolymph of the house fly larvae was boiled in water to remove the miscellaneous protein molecules. Solid-phase extraction combined with the separation technique of reverse phase-high performance liquid chromotography (RP-HPLC) proved to be a fast and easy method for isolating the peptide. Lijima et al. also used this method to isolate an antifungal peptide(AFP) from the hemolymph of a *Sarcophaga peregrine* (flesh fly) larva (24). It should be noted that, in general, separation of proteins by RP-HPLC would normally denature proteins, but small proteins (< 20,000 Da) can often be separated without significant degradation (27). In this study, the fractions obtained with SPE containing the antifungal activity was further purified by RP-HPLC. A peptide with a molecular mass of 17203.384 Da was competely purified and named as MAF-1 by us. The above described process of separating and purifying antifungal peptide from the hemolymph of the housefly larva have been proved to be efficient and effective.

Both peptide fingerprinting and Tandem Mass Spectrometry are capable of characterizing peptides and the results can be subjected to the current database for identification. Edman degradation can be used to determine the N-terminal amino acid sequence of undocumented peptides. The comprehensive analysis of MAF-1 in this study showed it was a novel antifungal peptide from *Musca domestica*. It is worth noting that two bands were observed in the determination of the isoelectric point for MAF-1. When a protein or peptide is confirmed as a pure substance by two or more tests, an isoelectric point of more than two bands can only be explained as the protein or peptide was going through conformational space alteration during the process of determination. The specific reasons for the changes in conformational space still await in-depth exploration and research. Research discoveries show antimicrobial peptides in insects are mostly strong alkaline and studies on houseflies in recent years also found weak acidic antibacterial peptide in houseflies (28).

The isoelectric points of the two bands of MAF-1 appear acidic in this study, suggesting this antifungal peptide is a kind of acidic bio-active peptide. This discovery has also further proved the complicate nature of the housefly defense system, in which a great varieties of antimicrobial active proteins are playing important roles.

## Acknowledgements

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