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Olfactory biosensor for insect semiochemicals analysis by impedance sensing of odorant-binding proteins on interdigitated electrodes

Yanli Lu [a], Yao Yao [a], Qian Zhang [a], Diming Zhang [a], Shulin Zhuang [b], Hongliang Li [c], Qingjun Liu [a,*]

[a] Biosensor National Special Laboratory, Key Laboratory for Biomedical Engineering of Education Ministry, Department of Biomedical Engineering, Zhejiang University, Hangzhou 310027, P.R. China
[b] College of Environmental and Resource Sciences, Zhejiang University, Hangzhou 310058, P.R. China
[c] College of Life Sciences, China Jiliang University, Hangzhou 310018, P.R. China

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ABSTRACT

Insects can sensitively and selectively detect thousands of semiochemicals at very low concentrations by their remarkable olfactory systems. As one of the most important olfactory proteins, odorant-binding proteins (OBPs) from insects are the most promising candidates for fabricating biosensors to detect biochemical molecules in the chemical ecology as well as for other biotechnological applications. In this study, we designed an olfactory biosensor by immobilizing OBPs from oriental fruit fly on interdigitated electrodes to detect semiochemicals. After successfully separated and purified, OBPs were immobilized by the special designed polyethylene glycol (PEG), SH–PEG–COOH, to produce a robust sensing membrane. Based on electrochemical sensing, interactions between OBPs and different semiochemicals emitted from host plants of the insect, such as the isoamyl acetate, β-ionone, and benzaldehyde, could be sensitively detected. With related amino acid residues in the hydrophobic cavities distinguished, the interaction forces between semiochemicals and OBPs were analyzed by molecular docking. Integrated biological olfaction proteins of insects, OBPs based biosensors could not only advance the progress in the understanding of chemical communication systems of insects, but also show promising potentials for biosensing applications in many fields.

1. Introduction

Semiochemicals are chemical substances that are emitted by plants, insects and mammals, such as fruity scents, floral odors, and sex or alarm pheromones. They carry messages that are used to communicate between individuals, including pheromone for intraspecific communication and allelochemical for interspecific communication of plans and animals (Sbarbati and Osculati, 2006; Schulz, 2010; Mensah and Moore, 2011; Apps, 2013). Been sensitively and selectively detected by the highly-adapted olfactory systems of animals, semiochemicals play central roles in chemical communications in the natural world by influencing the behaviors of animals, especially insects, which embraced a new research area known as chemical ecology (Pickett et al., 2013; Wehrenfennig et al., 2013). Detecting and monitoring the chemicals that are involved in interactions between living organisms are very important for the understanding of how they can maintain an excellent coordination in complex ecosystems. At the same time, investigating of the molecular mechanisms of olfaction for semiochemicals maybe will offer great benefits for bio-inspired methodologies of artificial olfaction.

Up until now, approximately 3500 animal semiochemicals have been identified, more than 3000 of which are insect semiochemicals, while only a few are mammals semiochemicals (Apps, 2013). Insects are known for their remarkable olfaction ability, which is crucial for them to distinguish, comprehend and estimate the overall situation and even for their survival, reproduction, and social communication (Fan et al., 2011; Forêt and Maleszka, 2006; Lu et al., 2014). Therefore, semiochemicals of plants and animals can be detected by insects at low concentrations over a long distance, even dozens of meters away (Wehrenfennig et al., 2013).

Generally, high-resolution gas chromatography (HRGC), fluorescence probes, electrophysiology and electroantennography (EAG) could be used to sensitively detect semiochemicals. However, there were some drawbacks for practical applications, such as either the advanced instruments used in the experiments were expensive, or the procedures were time consuming and cumbersome due to the labeling of fluorescence probes (Czerny et al.,

* Corresponding author. Fax: +86 571 87951676.
E-mail address: qjliu@zju.edu.cn (Q. Liu).

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2011; Li et al., 2013; Pickett et al., 2013; Wehrenfennig et al., 2013). Therefore, low-cost and ease of operating approaches with fast response should be developed. Some recent researches indicated that the insect olfaction with broad receptive ranges for chemosensing could provide distinguished responses to a variety of chemicals even if these chemicals do not occur in the animal's natural environment, such as volatile compounds emitted from cancer cells (Strauch et al., 2014). The exceptional olfactory ability had inspired researchers to develop biosensors, which were mainly based on insect behaviors or isolated organs such as antenna (Schroth et al., 1999; Schöning and Poghossian, 2002; Fernández-Grandon et al., 2011), for different analytes detection, like 2,46-trinitrotoluene (TNT) and other explosives (Repasky et al., 2006; Rains et al., 2008), and even for diseases control and diagnosis (Carey and Carlson, 2011; Strauch et al., 2014). Because of the difficulties in interpreting quantifiable behavior responses and the short life time of insect organs (Fernández-Grandon et al., 2011; Wehrenfennig et al., 2013), odorant molecules of natural olfactory systems should be integrated into artificial systems to build specific sensors for semiochemicals detection.

For insects, the prominent selectivity and sensitivity of the olfactory systems are achieved by discriminating filters of the small soluble binding proteins and the odorant receptors (Leal, 2005; Fan et al., 2011; Hansson and Stensmyr, 2011). These soluble binding proteins, which are mainly odorant-binding proteins (OBPs), not only can be expressed and purified easily, but also are highly stable to temperature, pH, solvents and proteases (Wei et al., 2008; Pelosi et al., 2014; Lu et al., 2014). With these excellent properties, biosensors based on mammal OBPs, such as bovine OBP and porcine OBP, had already been studied for detection of ethanol, octenol, and carvone, and obtained high specificity and sensitivity (Hou et al., 2005; Ramoni et al., 2007; Capone et al., 2009; Pietrantonio et al., 2013). For insects, however, only few applications of insect OBPs based biosensors were reported for semiochemical detection. Studies showed that OBPs of honeybee and peptide sequence derived from OBPs of insects (*Drosophila* and *Apis mellifera*) have been used to detect floral odors, alcohols and explosives (Lu et al., 2014, Sankaran et al., 2011; Kuang et al., 2010).

In this paper, an OBP of oriental fruit fly was expressed and purified. Though a self-assembled monolayer, the polyethylene glycol (PEG), the insect OBP was immobilized on the interdigitated electrodes to establish an olfactory biosensor. It could sensitively detect semiochemicals such as asisoamyl acetate, β-ionone, and benzaldehyde that emitted from the fly's host plants by electrochemical impedance. Combined with analysis of molecular docking, the sensors could be used to reflect affinities between insect OBPs and their semiochemicals.

2. Experiment

2.1. Expression and purification of the protein

The expression and purification of the active recombinant BdorOBP2 were cloned from the full-length cDNA of oriental fruit fly, *Bactrocera dorsalis*. Briefly, using reverse transcription-polymerase chain reaction (RT-PCR) and pET-30a (+)/BL21 (DE3) prokaryotic expression system, BdorOBP2 from antenna of oriental fruit flies was cloned and expressed by being transformed into *Escherichia coli* BL21 competent cells. The bacteria were induced by isopropyl β-D-1-thiogalactopyranoside (Merck, Darmstadt, Germany) to initiate the expression of recombinant proteins. After harvesting the bacterial cells, the crude cell extracts, pellet and supernatant were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to check if the recombinant proteins were expressed. Afterwards, the inclusion body of BdorOBP2 was severely precipitated in 1.5 M urea in ddH_2O and finally freeze-dried. The protein was resuspended (67 μg/ml) in phosphate buffered saline (PBS; pH=7.4) and saved under 4 °C for the following biosensor experiments.

2.2. Semiochemicals and reagents

The fruity scents, such as isoamyl acetate, β-ionone, and benzaldehyde, which could specifically bind to BdorOBP2, were used as semiochemicals for biosensing. 4-allylveratrole and butanedione were also tested as the negative control, which were two kinds of widely used odorants in biosensors. Taking 1% methanol as co-solvent, these semiochemicals were prepared with PBS solution for different concentrations: 10^{-7} M, 10^{-6} M, 10^{-5} M, 10^{-4} M, and 10^{-3} M.

The solvents and reagents, potassium ferricyanide/ferrocyanide ($K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$), PBS, 2-(4-Morpholino) ethanesulfonic acid (MES), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), and α-thio-ω-carboxy poly (ethylene glycol) (MW 2100 Da, COOH–PEG–SH in short) for protein immobilization and impedance detecting were all purchased from Sigma-Aldrich, USA.

2.3. Fabrication of interdigitated electrodes

Interdigitated electrodes, which have uniform electric fields and high electrode coverage, were used to measure impedance changes when semiochemicals binding to BdorOBP2. The fabrication process of the electrode arrays using semiconductor technology had been described in the previous paper (Zhou et al., 2013; Lu et al., 2014). Briefly, A four-inch sterilized Pyrex glass 7740 was chosen as the insulating substrate. After sputtering a layer of Cr

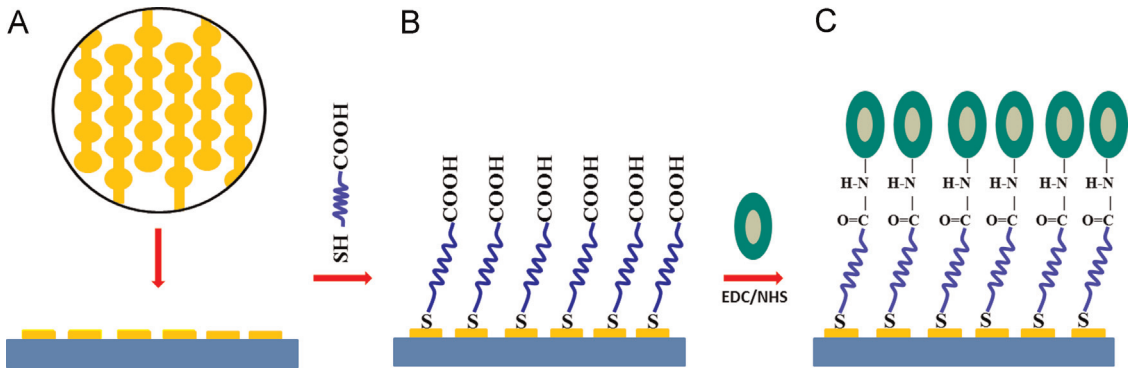

Fig. 1. OBPs immobilized on PEG modified interdigitated electrodes. (A) Sketch map of the interdigitated electrodes, (B) COOH–PEG–SH forms Au–S bonds with gold electrodes, and (C) BdorOBP2 forms covalent amino bonds with PEG-electrodes by EDC/NHS coupling.

(20 nm thick) on the glass, a layer of Au (200 nm thick) was sputtered with the same method. Subsequently, through conventional lithography and etching techniques, the interdigitated electrodes, interconnects and pads were patterned from this composited metallic layer. The shape of the electrodes is "circle-on-line" (Fig. 1A). The shape of the detecting channels was circle, diameter of which was 8 mm. There were 36 pairs of electrodes in each channel. The longest pair of electrodes was 7.8 mm, which was in the middle of the channel. The diameter of each circle on the electrodes was 90 μm, while the distance between two adjacent electrodes was 20 μm. The line part of the electrodes was 30 μm. Afterwards, the sensing chip was packaged with PCB board. A perspex with eight pairs round holes was used as the impedance detecting well in the experiments. Finally, the perspex was adhered on the board with liquid adhesive, epoxy resin, when the round holes were aligned with the electrodes.

2.4. Protein immobilization by self-assembled PEG

For surface modification, the electrodes were thoroughly rinsed with ethanol and ultrapure water respectively to remove the organic residues off the substrate and then dried in nitrogen. Subsequently, 200 μL of COOH–PEG–SH solution (1.5 mg/ml) was added to the electrodes for approximately 24 h to form stable Au–S covalent bonds, which could offer a robust and sensitive membrane. After the unbound COOH–PEG–SH was washed away with ultrapure water, a self-assembled monolayer (SAM) of PEG was established on the electrodes (Fig. 1B).

BdorOBP2 was immobilized to PEG-electrode via EDC/NHS coupling. EDC and NHS solutions were prepared in 0.1 M MES buffer (pH=5, contained 0.5 M NaCl) and 0.1 M PBS buffer (pH=7.4), respectively. Equal volume of EDC (8 mg/mL) and NHS (16 mg/mL) solutions, 60 μL, were successively added onto the electrodes to activate the carboxyl group of COOH–PEG–SH for 15 min. After pH was increased to 7.2–7.5 with sodium bicarbonate, 50 μL of BdorOBP2 solution was added into the plate wells containing COOH–PEG–SH on the electrodes. Finally, the solution in the plate well was mixed by an oscillator for about 30 s and incubated them for 2 h to allow forming amide linkages between BdorOBP2 and COOH–PEG–SH (Fig. 1C). Then after rinsing with PBS buffer to remove the unreacted EDC/NHS solutions and unbound proteins, the device with immobilized BdorOBP2 was saved under 4 °C for the following biosensor experiments. All of the above immobilizing processes were performed at room temperature (22 °C).

2.5. Impedance detecting of semiochemicals

Zahner ZENNIUM electrochemical workstation (Zahner Elektrik, Germany) was used for impedance measurement. The tested frequency was set from 0.1 Hz to 100 kHz with 5 mV alternating voltage. Semiochemicals at different concentrations were added to the plate wells containing 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1:1) for recording the electrochemical impedance spectroscopy. At each concentration, electrochemical impedance spectroscopy was recorded for about 40 min to insure steady binding (Liu et al., 2013). The charge transfer resistance (R_{ct}) of Randles model could be extracted by the software of Zview (Scribner Associates Inc., USA), which was easy for processing large number of tested data. After each measurement, the tested solutions were removed out of the plate wells, and 400 μl PBS was added to soak the electrodes for 15 min to eliminate the residual semiochemicals for the recovery of the proteins. Parallel experiments for each semiochmiecals were tested at least three times in different channels. All of the impedance detections were also performed at room temperature (22 °C).

2.6. Molecular docking of the semiochemicals binding

Based on the amino acid sequence of BdorOBP2, the three-dimensional model of BdorOBP2 was modeled by I-TASSER server (Zhang, 2008). To predict the binding modes of different semiochemicals to BdorOBP2, the semiochemicals were docked into the binding cavity of BdorOBP2 using the Molegro Virtual Docker (MVD) Version 4.2. (Molegro, CLC bio, Denmark) The binding pocket covered a site with a user-defined origin and a radius of 15 Å. MolDock SE was used as the searching algorithm, and MolDock Score was used for the energetic evaluations between the protein and the semiochemicals.

3. Results

3.1. OBP expression, purification

Due to the unavailability of commercial proteins of OBPs, the recombinant plasmids of pET32a-BdorOBP2 were transferred into competent *E. coli* BL21 (DE3) for the expression of recombinant proteins. After harvesting the bacterial cells, they were crushed, and the lysates were analyzed by electrophoresis to check if the recombinant proteins were successfully expressed. The target proteins with a molecular weight of about 37 kDa were acquired (Fig. 2). To obtain the recombinant proteins that had engaged in binding activities with semiochemicals, the protein were purified through Ni^{2+}-NTA affinity chromatography column from the above protein lysate. Finally, when the purity of the protein was above 90% based on the gray level analysis software (Glyco Band-Scan, Prozyme, USA), they were then ready to be used in the biosensor assay.

3.2. The immobilization of proteins

As one of the most powerful and sensitive techniques, electrochemical impedance spectroscopy was chosen for characterizing properties of the biosensor during the electrode fabrication and the protein assembly (Fig. 3A). The most important parameter of impedance spectroscopy is a semicircle portion at high frequencies corresponding to the electron transfer limiting process, the diameter of which is equal to the charge transfer resistance (R_{ct}) of Randles model (Lisdat and Schäfer, 2008; Alfinito et al., 2010). For the situation of electrodes in contact with electrolyte, the Randles

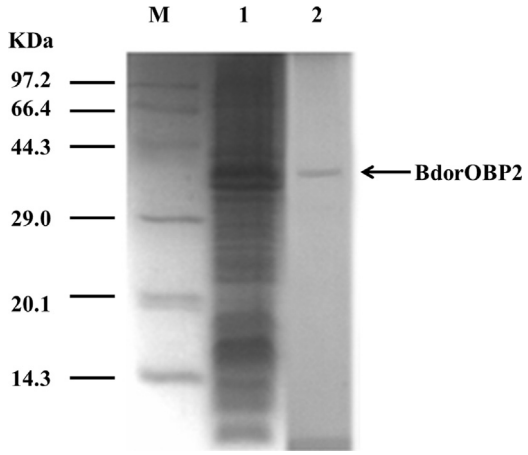

Fig. 2. Expression and purification of recombinant BdorOBP2 by the analysis of SDS-PAGE. M represents protein molecular weight marker. Lanes 1 and 2 show the expression and purification of the recombinant protein, respectively. The protein was shown as a black arrow.

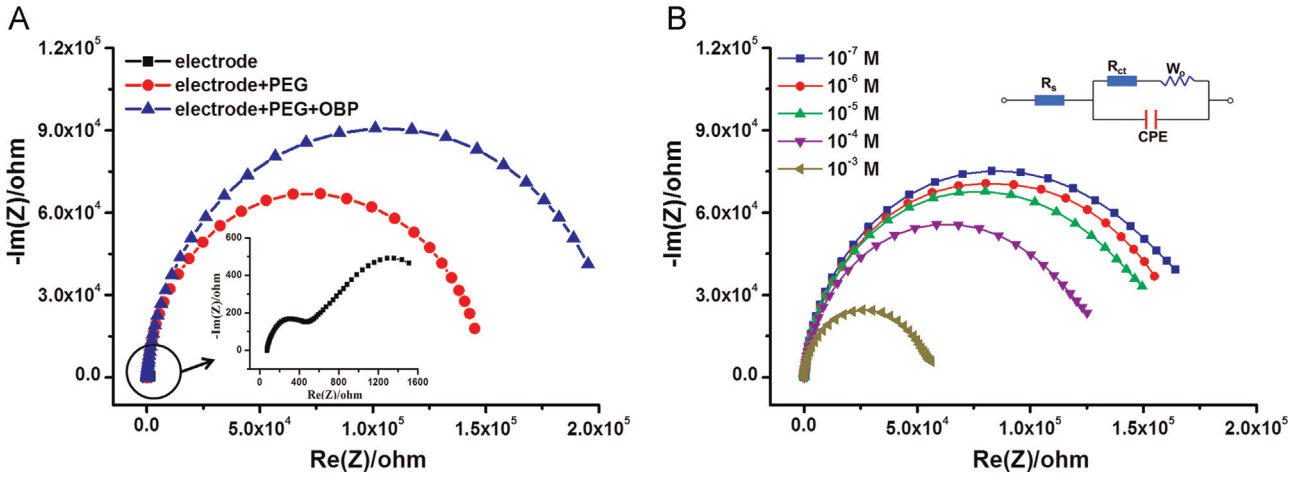

Fig. 3. Impedance spectra of BdorOBP2 for semiochemical detection. (A) Impedance spectra of BdorOBP2 immobilized on PEG modified electrodes. The blank line with squares represent the impedance spectrum of the bare golden electrode, the red line with circles is the impedance spectrum of PEG modified on electrodes, and the blue line with triangles is the impedance spectrum of BdorOBP2 immobilized on the PEG-gold electrodes. (B) Impedance spectra of semiochemical (isoamyl acetate) at different concentrations. Errors bars: ± standard deviation (SD) calculated from 3 measurements carried out on 3 impedance channels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

circuit could be used to simplify this complex system, which was shown in Fig. 3B. R_s and Z_w represent bulk properties of the electrolyte solution. CPE and R_{ct} both depend on the dielectric and insulting features at the electrode/electrolyte interface. They were affected by the property changes occurring at the interface.

Compared with bare gold electrodes, the immobilization of PEG changed the dielectric features at the electrode/electrolyte interface, which resulted in an increase of R_{ct}. Moreover, BdorOBP2 attachment on PEG modified electrodes led to a more difficult electron transfer process, which made a larger increase of R_{ct}. The

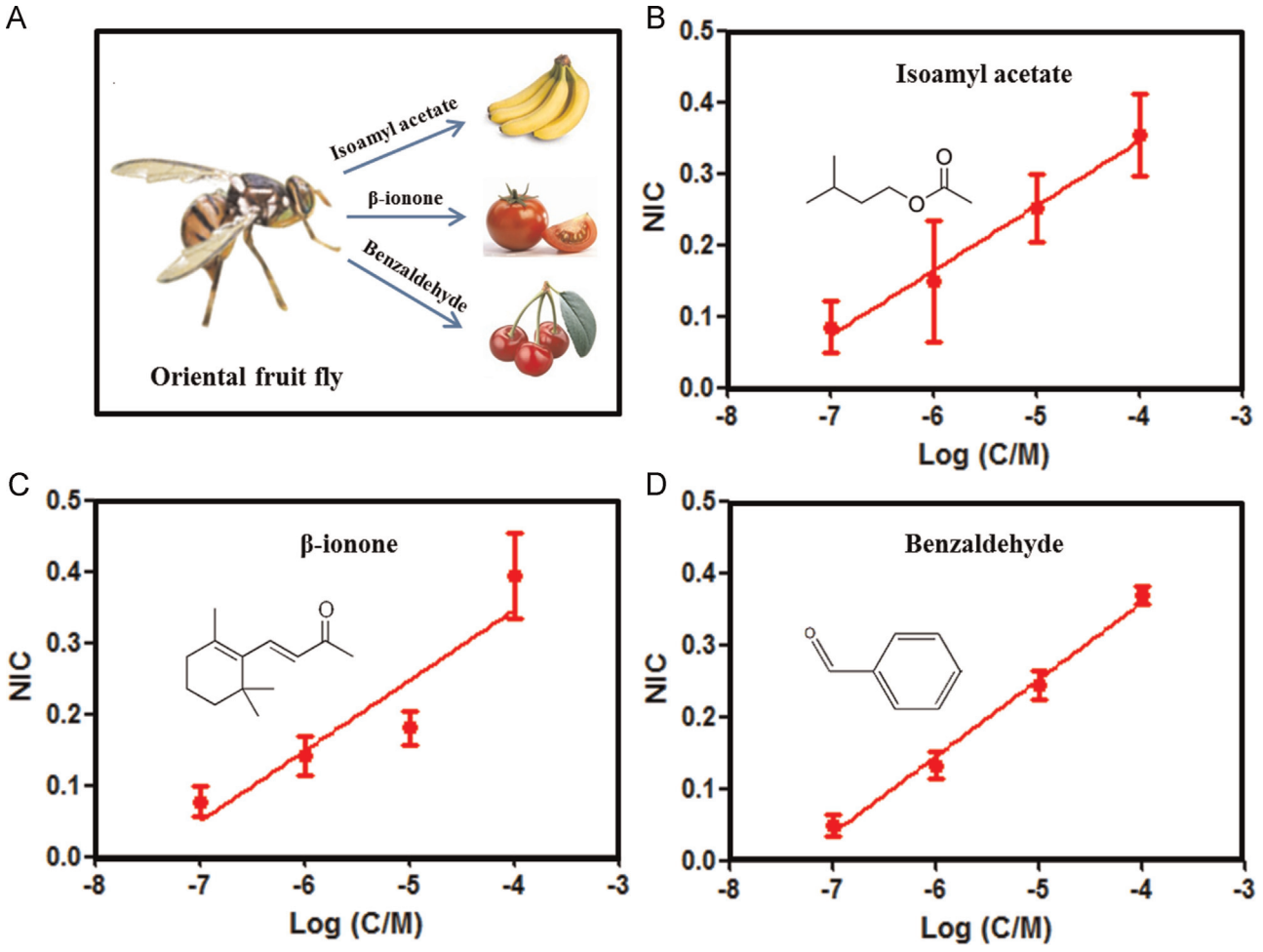

Fig. 4. Normalized impedance changes (NICs) of BdorOBP2 from oriental fruit fly interacting with different semiochemicals (A): isoamyl acetate (B); β-ionone (C); and benzaldehyde (D).

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impedance changes suggested that BdorOBP2 have been successfully immobilized on PEG modified electrodes for the semiochemicals detection.

3.3. Impedance sensing of semiochemicals

Despite that the electrode devices could be reused and retain their good stability even for more than 6 months, the stability of this OBPs based sensor was greatly affected by the immobilization and activity of the protein. Generally, the protein based sensor could retain good stability after 2 weeks storage under 4 °C. In the detection, there was no significant impedance change, usually less than 5%, when using the same sensor over 2 days. With BdorOBP2 immobilized on the electrodes, R_{ct} decreased apparently when the system was treated with different kinds of semiochemicals. Changes of R_{ct} had a close relationship with the concentrations of fruity scents. In order to visualize and determine R_{ct}, we simulated impedance spectra of the electrochemical experiment by Zview. As a typical R_{ct} recording example, impedance spectra of isoamyl acetate was displayed in Fig. 3B.

The results showed that the interactions between OBPs and semiochemicals enhanced the charge transfer process. This decrease of R_{ct} indicated a recovery in the efficiency of the mass transfer phenomenon and changes in dielectric and conductive properties of PEG and proteins on the electrodes. With the concentrations of semiochemicals increasing, conformation changes of OBPs led to a larger decrease of R_{ct}. Through simulating every impedance spectrum of the fruity scents, we could find that the normalized impedance change (NIC) of R_{ct} had a relationship with the logarithm of the concentrations. NIC was described as $(Z_b\text{-}Z_a)/Z_b$, where Z_b and Z_a represent the R_{ct} before and after the stimulation of the fruity scents for quantitative detection, which also could be used to offset relative errors in each test. The statistical NICs of BdorOBP2 to different fruity scents were shown in Fig. 4, each data point of which was recorded from a set of three different channels of electrodes. From the concentration-dependent curves of different fruity scents, the impedance biosensor showed high sensitivities to those special semiochemicals, isoamyl acetate, β-ionone, and benzaldehyde, with the proteins successfully immobilized by PEG. In order to assess the reproducibility of the impedance biosensor, the relative standard deviations (RSDs) for different semiochemicals were also calculated in detail (Table A1 in the Appendix). The results in Table A1 and in Fig. 4 indicated that the RSDs of isoamy acetate were higher than that of β-ionone and benzaldehyde. Moreover, under the treatment of higher concentrations (10^{-5} M and 10^{-4} M), the variability of the biosensor was lower than that of the concentrations of 10^{-7} M and 10^{-6} M.

The tested semiochemicals, isoamyl acetate, β-ionone, and benzaldehyde, are mainly the scents of banana, tomato and cherry that are attractive to the oriental fruit fly. As one of the most widely distributed fruity scents in nature, isoamyl acetate showed an excellent affinity to BdorOBP2. The linear equation was $NIC = 0.0908 \times \log C + 0.7093$, $R^2 = 0.9895$, within the sensitive test

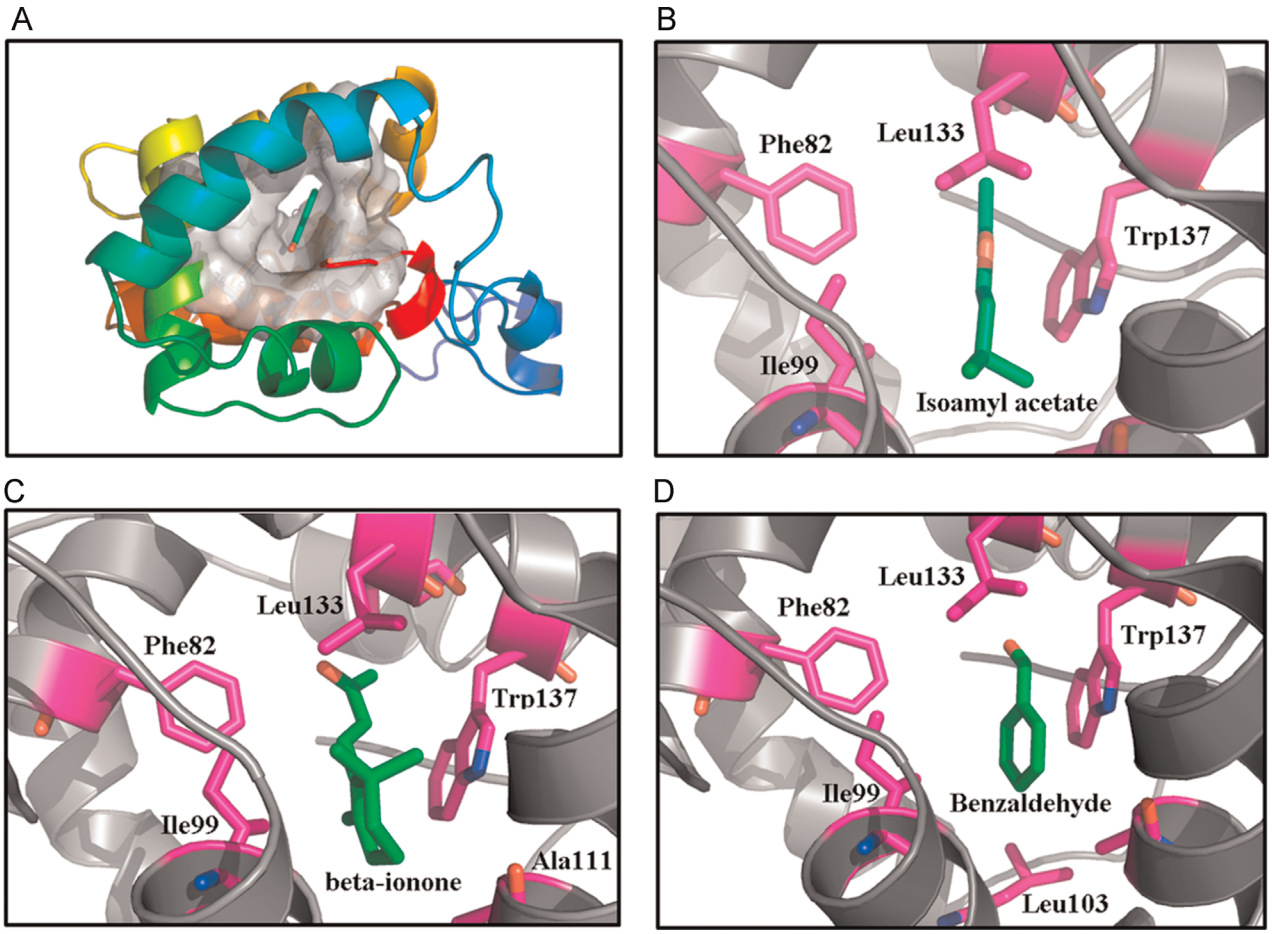

Fig. 5. Molecular docking of the semiochemicals binding to BdorOBP2. The structure of BdorOBP2- semiochemical (isoamyl acetate) complex (A); semiochemicals of isoamyl acetate (B); β-ionone (C); and benzaldehyde (D) in the binding pocket of the protein. Semiochemicals colored by atoms were represented as green sticks. The BdorOBP2 were represented as gray ribbons. Related amino acids in the inner cavity delineated by purple sticks. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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range from 10^{-7} M to 10^{-4} M. For β-ionone, the linear response was NIC=0.0988 × log C+0.7422, R^2=0.8659. Compared with isoamyl acetate and β-ionone, the slope of the linear response (NIC=0.1071 × log C+0.7879, R^2=0.9918) was higher than that of isoamyl acetate and β-ionone. It indicated that BdorOBP2 showed a relatively higher sensitivity and affinity towards benzaldehyde in impedance sensing. In the impedance measurement for the blank control, the obtained standard deviation was about 0.01. Based on the 3δ/slope method, the detection limits of these three semiochemicals, isoamyl acetate, β-ionone, benzaldehyde, could be calculated into 3.3×10^{-8} M, 6.2×10^{-8} M, and 8.4×10^{-8} M, respectively. The detection limits of all these three semiochemicals were between 10^{-8} and 10^{-7} M. In order to verify the specificity of the olfactory biosensors, another two negative semiochemicals, 4-allylveratrole and butanedione were also tested. The NICs of these two semiochemicas were less than 0.05, which indicated that they cannot interact with BdorOBP2 (Fig. A1 in the Appendix).

With these fruity scents successfully been detected by the impedance sensing, we inferred that the sensitivity and selectivity of this OBPs based biosensor to different semiochemicals were excellent, which could be used to reflect the affinities between OBPs and semiochemicals. Therefore, this biosensor could achieve the first step in semiochemical detection of insects, which was important for the researches about chemical communications in chemical ecology.

3.4. Molecular docking analysis of the semiochemicals

With a cavity prediction algorithm, the fast and accurate identification of potential binding modes and poses could be obtained by molecular docking (Thomsen and Christensen, 2006). The structure of BdorOBP2 and the interactions of different semiochemicals, isoamyl acetate, β-ionone, and benzaldehyde, in the binding cavity of BdorOBP2 were shown in Fig. 5.

Apparently, there was a central hydrophobic pocket in the three-dimensional structure of BdorOBP2 that can bind or transport hydrophobic molecules. There were a variety of chemical groups exposed in the cavity, which provided an optimal environment for the interactions between semiochemicals and the protein. When the semiochemicals bound in the cavity of BdorOBP2, they formed a specific conformation, which were stabilized by different hydrophobic, polar, electrostatic, and π-stacking interactions. All of these interactions could be represented by a theoretic binding energy (MolDock Score), a higher absolute value of which indicated a stronger binding strength of BdorOBPs and semiochemicals.

From the docking results, we could know that four amino acid residues in the cavity, Phe82, Trp137 Ile99 and Leu133, play important roles in forming the BdorOBP2-semiochemical complex. For the BdorOBP2-isoamyl acetate complex, isoamyl acetate lay exactly in the hydrophobic middle of several hydrophobic groups, such as benzene rings of Phe82 and Trp137. At the same time, electrostatic interactions between isomaly acetate and the negative charged side chain of Ile99 and Leu133 were also important in maintaining the stable structure. The MolDock Score of BdorOBP2 and isoamyl acetate was about −42.54 kcal/mol, which indicated a relatively strong interaction.

The structure of β-ionone could be divided in to two parts, a hexatomic ring and a short chain, function groups of which could supply more interactions with BdorOBPs, such as the hydrophobic interactions between the hexatomic ring of β-ionone and benzene rings of Phe82 and Trp137. Furthermore, because of the strong electron-withdrawing property of the oxygen atom, the carbonyl group of β-ionone can form a strong interaction with the negative charged side chain (Leu133). The MolDock Score of BdorOBP2 and β-ionone was about −64.18 kcal/mol, which was higher than that of isoamyl acetate. As for benzaldehyde, there is a benzene ring in the structure, which could form π-stacking interactions with Phe82 and Trp137. Because of the simple chemical structure and chemical group, other interactions (Van der Waals, hydrophobic and electrostatic forces) might make little contributions to the binding, so the MolDock Score of BdorOBP2 and benzaldehyde was only −37.70 kcal/mol, which could also reflect the lower affinity and sensitivity of BdorOBPs to benzaldehyde. Compared MolDock Scores with results of impedance sensing, it would be helpful for exploring the configurations and physiological functions of proteins to different semiochemicals.

4. Discussion

4.1. Semiochemicals detection by insects

Insects are the most diverse taxonomic class in the animal kingdom. Among the thousands insects species, most of them have an enormous impact on global agriculture as pests and on public health as disease vectors. Adapted to an ever changing chemosensory world for survival, except for optesthesia, their specialized olfaction was highly evolved to sensitively and specifically detect semiochemicals in the natural world (Carey and Carlson, 2011; Clifford and Riffell, 2013; Schott et al., 2013).

As one of the most economically damaging polyphagous pests of fruit crops, and a quarantine pest in the world, the oriental fruit fly, *Bactrocera dorsalis*, depend on chemical cues to regulate their host-finding behaviors (Shen et al., 2011; Jayanthi et al., 2012). Female fruit flies can travel long distances to find fruit crops to lay their eggs through detecting semiochemicals that emitted by host plants, just like isoamyl acetate, β-ionone and benzaldehyde emitted by bananas, tomatoes and cherries, which were studied in this research. Therefore, a clear understanding of the interactions between fruit flies and different semiochemicals can inspired us to investigate specific biosensors for semiochemical detections, which may offer an easy opportunity for insect pest control.

4.2. Insect OBPs based olfactory biosensor

After semiochemicals entering through pores on the sensilla of the antennae or maxillary palps of insects, they are transported across the sensillum lymph by OBPs to the olfactory receptors on the sensory neurons (Rützler and Zwiebel, 2005; Logan and Birkett, 2007; Ko et al., 2010). For analysis of semiochemicals binding with the olfactory molecules in vitro, the most important thing is to obtain the active recombinant proteins. As G protein-coupled receptors with seven transmembrane α helix, however, the membrane protein of olfactory receptors were very difficult to be isolated and purified (Liu et al., 2006; Du et al., 2013; Kaiser et al., 2008).

But to OBPs, which are secreted in large quantities surrounding the olfactory receptors, could be easy expressed and purified. In the oriental fruit fly, we cloned the OBP cDNA from *B. dorsalis*, which was named BdorOBP2, and then heterologously expressed its protein in prokaryotic cells. Finally, the recombinant protein BdorOBP2 was purified by affinity chromatography. The mature protein of BdorOBP2 includes six conserved cysteine residues, which are the hallmark of insect OBPs. After being successfully expressed and purified in large quantities, the insect OBPs that were stable to temperature, pH and solvents, show promising prospects in establishing specific biosensors for chemical molecules sensing.

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4.3. Semiochemicals impedance sensing and molecule docking

Compared with COOH–PEG–SH (MW=2100) and OBPs (MW > 10 KD), semiochemicals were small molecules, the molecular weights of which were just around hundreds (β-ionone, 192.3; isoamyl acetate, 130.2; benzaldehyde, 106.1). Therefore, although semiochemicals could bind to the proteins and block the charge transfer just like COOH–PEG–SH and OBPs, the effects were little. However, conformation of OBPs, especially the backbones and the cavities, could be changed when semiochemicals bonded into the cavities of OBPs. It could enhance the protein internal dynamics and might lead the local buffer ions pass through the pockets effortlessly (Lescop et al., 2009). Moreover, changes of protein conformation might increase the contact areas between mobile ions and electrodes, resulting in a much easier electron transfer process (Lu et al., 2014). Therefore, compared with the effects of semiochemicals, the conformation changes of OBPs had a great influence on the dielectric and conductive properties of the interdigitated electrodes.

With the help of self-assembly PEG, BdorOBP2 were immobilized on the gold electrodes for the semiochemicals detection in this study. As one of the most widely used inert and biocompatible linkers in surface engineering (Anne et al., 2002; Bhargav et al., 2012), PEG could form densely packed self-assembled monolayer on gold electrode, which comprised a uniform morphology with the ethylene glycol units in a helical conformation and oriented predominantly perpendicular to the surface (Herrwerth et al., 2003; Sun et al., 2006). When BdorOBP2 was immobilized by PEG, the impedance changes caused by conformationally altered proteins could be easily detected as they were all arranged orderliness through amido bonds, which provided an effective method for semiochemicals detection.

Compared with other biosensors that were based on OBPs to detect semiochemicals, such as surface acoustic wave (SAW), quartz crystal microbalance (QCM), surface plasmon resonance (SPR), and cantilever sensors (Pietrantonio et al., 2013, 2014; Sankaran et al., 2011; Vidic et al., 2008; Manai et al., 2014), the biosensor in our study had its own superiorities. The performances of different sensors were summarized in Table 1. Taking OBPs of mammals or insects as sensing elements, these biosensors could successfully detect different semiochemicals. Although the detection limits of SAW and SPR were lower than that of the impedance sensing, the impedance sensor showed a wider linear range, 10^{-7}–10^{-4} M, which was near 10–10,000 ppm. To the sensitivity, SAW and QCM were almost in the approximate ranges, dozens of Hz per ppm. The sensitivity of cantilever sensor and this impedance sensor could be represented by the relative variation of normalized changes, which were near 2.6–4.2 and 10^{-2} per molar concentration, respectively. Both of them also showed good sensitivities for the semiochemical detection.

Through calculation, the three-dimensional models and exact function groups of the protein to different semiochemicals can be modeled (Lescop et al., 2009; Liu et al., 2013). Based on molecular docking, fast and accurate identification of potential binding modes were obtained, which showed that amino acid residues of BdorOBP2, Phe82, Trp137, Ile99 and Leu133, played important roles in the binding process. Various chemical structures and functional groups of different semiochemicals could be analyzed at the same time. Under the stimulation of fruity scents of isoamyl acetate, β-ionone, and benzaldehyde, which respectively belong to the classes of esters, ketones and aldehydes, the different conformational changes of the protein could be sensitively detected by impedance sensing. However, 4-allylveratrole and butanedione, which respectively belong to ethers and small soluble ketones, could not lead obvious conformation changes of the proteins. The results were in accord with the behavior researches of the insects (Clifford and Riffell, 2013). Therefore, with the prominent chemosensory abilities of the proteins, this OBPs-based impedance biosensor that made full use of the conformational change properties could provide an easy, rapid and sensitive approach for chemicals detection and analysis.

4.4. Applications of the insect OBPs for biosensing

The practical goals of previous semiochemical researches focused on developing methods of exterminating and controlling insect pest to protect plants and forests by utilizing related semiochemicals, such as insect attractants, to disturb their olfaction judgments (Agelopoulos et al., 1999; Mensah and Moore, 2011). Excitingly, recent studies showed that insects could even detect chemicals that do not occur in the insects′ natural environment, such as TNT and volatile compounds emitted from cancer cells (Marshall, et al., 2010; Strauch et al., 2014), which inspired us to broaden the applications of insect semiochemicals researches.

Except for that the surrounding environment were very complex and uncontrollable, there were many other difficulties for the practical applications, such as pre-concentration and separation of different odors, control of temperature and humidity, and the miniaturization of apparatuses. In order to overcome these difficulties, researchers put their efforts in developing different sensors, such as QCM, SAW, FET and MEA (Pietrantonio et al., 2013; Sankaran et al., 2011; Schöning and Poghossian, 2002; Oh et al., 2011). Based on different sensing membranes, such as mammal OBPs, polypeptide, cells or olfactory organs, these biosensors could sensitively and specifically detect semiochemicals. However, the sensitivity or the life time of these biosensors was lower than protein based biosensors with impedance detecting. Moreover, the impedance detection system could be easily miniaturized to achieve portable detection systems (Jiang et al., 2014). It also made the impedance biosensor be a good candidate for practical applications in the near future.

Table 1
The properties of this biosensor compared with others reported.

Type of biosensor	Biosensing elements	Semiochemicals	Linear range	Detection limit	Sensitivity	References
Electrochemical Impedance	OBPs from oriental fruit fly (*Bactrocera dorsalis*)	Isoamy acetate, β-ionone, benzaldehyde	10^{-7}–10^{-4} M	10^{-8}–10^{-7} M	10^{-2}/M (normalized impedance change)	This study
Cantilever	Porcine OBPs	2-Isobutyl-3-methoxypyrazine	0–1.5 × 10^{-3} M	0.1 × 10^{-3} M	2.6–4.2/M (normalized frequency change)	Manai et al. (2014)
Surface Acoustic Wave (SAW)	Bovine OBPs	Octenol, carvone	5–80 ppm	0.1–0.2 ppm	10–50 Hz/ppm	Pietrantonio et al. (2013, 2014)
Quartz Crystal Microbalance (QCM)	OBPs from Drosophilidae (*Drosophila*, LUSH)	Alcohols	0–100 ppm	1–3 ppm	20–40 Hz/ppm	Sankaran et al. (2011)
Surface Plasmon Resonance (SPR)	OBP-1F and OR1740	Helional	–	10^{-9} M	–	Vidic et al. (2008)

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In our study, by directly targeting the insects′ sense of smell, interactions between OBPs and different semiochemicals could advance the progress in the understanding of chemical communication systems of insects, which showed strong potentials for applications in many fields, such as control of semiochemical pollution, insect vector-borne diseases, olfactory-based insect control technology, and even cancer diagnosis (Carey and Carlson, 2011; Wehrenfennig et al., 2013; Strauch et al., 2014). With more functional amino acid residues or peptides interpreted by molecular docking from the natural sequences of OBPs, we believe that along with insects OBPs, mutant proteins or peptide chains could be synthesized to be used as promising recognition elements in the future biosensors for practical applications.

5. Conclusions

In this study, OBPs from oriental fruit fly, *B. dorsalis*, have been successfully expressed and purified. With the help of a self-assembled monolayer of PEG on the interdigitated electrodes, OBPs were immobilized as the sensing membrane. Thus, a sensitive electrochemical impedance olfactory biosensor was established for the detection of specific semiochemicals, such as isoamyl acetate, β-ionone, and benzaldehyde from different host plants of the insect. Through molecular docking, the related amino acid residues and interaction forces between proteins and semiochmicals were discriminated. Based on OBPs of insect, the electrochemical impedance biosensor showed potential applications in lots of sensing fields, such as insect pest control, military and health care.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.09.098.

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