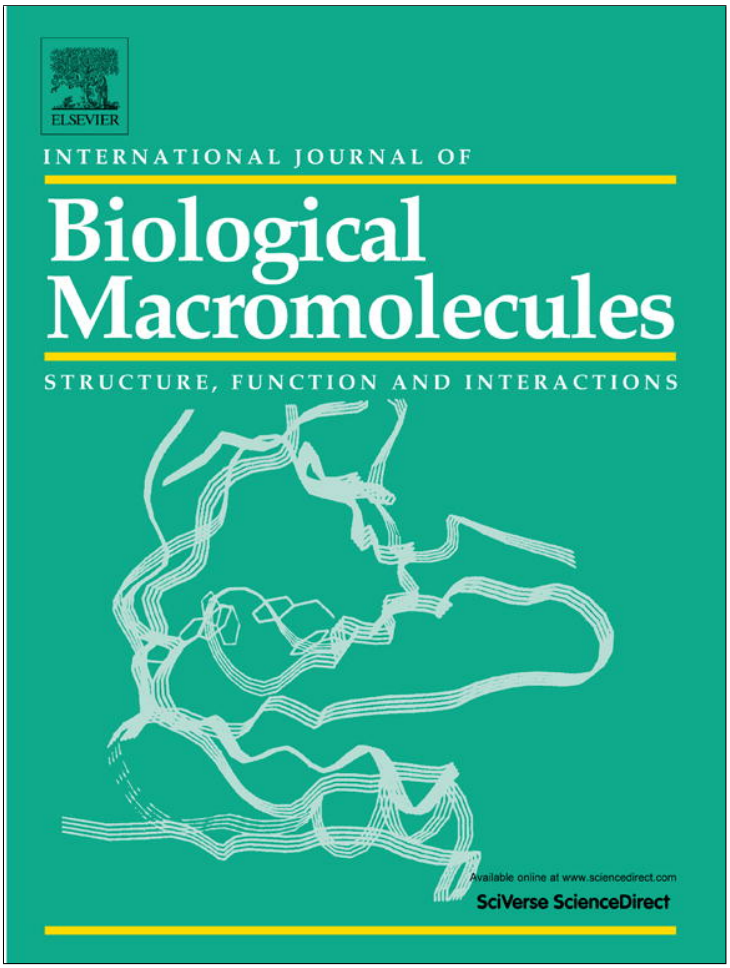

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Molecular recognition of floral volatile with two olfactory related proteins in the Eastern honeybee (*Apis cerana*)

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ABSTRACT

The honeybee relies on its sensitive olfaction to perform the foraging activities in the field. In the antennal chemoreception system of honeybee, odorant-binding proteins (OBPs) and chemosensory protein (CSPs) are major two protein families capable of binding with some plant volatiles and chemical ligands. However, the chemical binding interaction of plant odors with OBPs and CSPs in the honeybee olfactory system is still not clear yet. Hence, complex fluorescent spectra, ultraviolet absorption spectra, circular dichroism spectra and molecular docking were used to investigate the binding property of AcerASP2 (an OBP of *Apis cerana*) and AcerASP3 (a CSP of *Apis cerana*) with β-ionone, one of ordinary floral volatiles in botanical flower. As a result, β-ionone had a strong capability to quench the fluorescence that the two proteins produced, and their interaction was a dynamic process that was mainly driven by a hydrophobic force. AcerASP2 had a larger hydrophobic cavity than that of AcerASP3 and the conformation of AcerASP2 was changed less than AcerASP3 when binding with β-ionone. Our data suggests that OBPs like AcerASP2 might make a large contribution toward assisting the honeybee in sensing and foraging flowers, and *A. cerana* has evolved a good circadian rhythm to perceive a flower's odor following the fluctuation of temperature in the olfactory system. This significantly extends our knowledge on how to strengthen the honeybees' pollination service via manipulation of target proteins in the olfactory system.

1. Introduction

The honeybee exhibits complex social behaviors such as hive building, feeding of immature (larval) bees, and locating and foraging food resources, which is driven by its sensitive chemosensory functionality [1]. Of these, the gathering of pollen and nectar is an evolutionary trade between bees and flowers. During this process, honeybees get a source of food while accomplishing pollination for the plants [2]. The total economic value of crop pollination worldwide has been estimated at €153 billion, which is very important to feed the increasing population in the world [3]. As an eastern species of the Apis genus, *Apis cerana* possesses a sensitive olfactory system that is directly involved in the pollination process. So, understanding of how the olfactory system of *A. cerana* recognizes the flower volatiles is of vital importance for the improvement of pollination services.

In the olfactory chemosensillar lymph of insects, there exist mainly two families of soluble binding proteins, odorant-binding proteins (OBPs) and chemosensory proteins (CSPs). They are characterized as generally acidic and of low molecular weight (about 20 kDa), and have a binding affinity to some biochemical ligands, such as numerous plant volatiles and pheromones [4]. Although the physiological property and structural information of OBPs and CSPs have been well documented in other insects [5], their chemical interactions and binding models with plant volatiles are still scarcely known. Recently, though some characteristics of the two olfactory related proteins (one OBP and one CSP) have been reported in *A. cerana* [6,7], their chemical recognition mechanisms which stimulate binding with floral odors still remain an untouched research area.

As one of the floral scent components in a botanical flower [8], it has been well characterized that β-ionone (Chemical structure see Fig. 1A) could be combined with OBP4, OBP5 and CSP4 of *A. mellifera* [9], CSP1 [10] and OBP5 [11] in *Microplitis mediator*. Recently, with the availability of the full-length sequence of AcerASP2 and AcerASP3 [6,7] (the sequence alignment as seen in Fig. 1B), a strong binding affinity has been reported between β-ionone and AcerASP2

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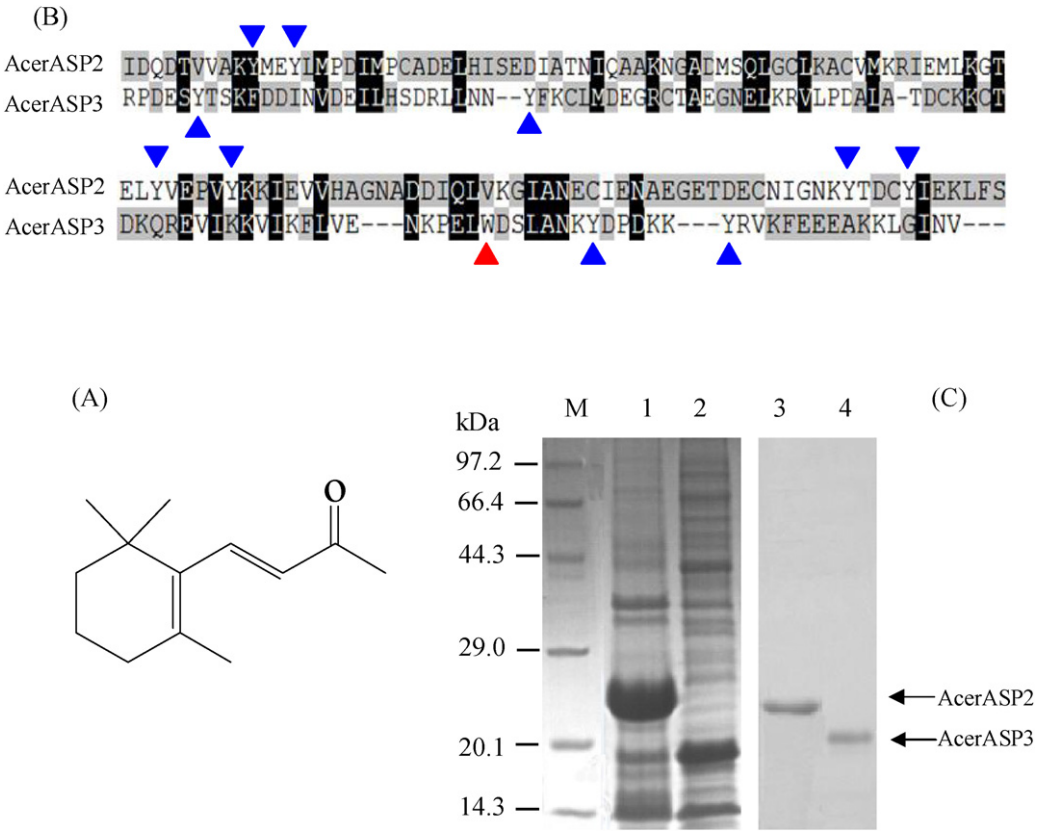

Fig. 1. Structure of β-ionone (A), the sequence alignment of mature amino acids sequences of AcerASP2 and AcerASP3 (B) and expression and purification of the two proteins by the analysis of SDS-PAGE (C). The mature protein of AcerASP3 only contains one tryptophan residue labeled by one red triangle near the C-terminal, and there exist 6 and 4 tyrosine residues labeled by blue triangles in AcerASP2 and AcerASP3, respectively. In (C), M represents protein molecular weight marker. Lanes 1 and 3 show that the expression and purification of recombinant AcerASP2 protein, respectively. Lanes 2 and 4 shows that the expression and purification of recombinant AcerASP3 protein. The both proteins are shown as black arrows, while the molecular weight of AcerASP2 is a little larger than AcerASP3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(OBPs families) and AcerASP3 (CSPs families) of *A. cerana* [12]. Since β-ionone is a widely used and representative chemical ligand for the analysis of the binding interaction with OBPs and CSPs [9–11], this provides us a platform to analyze the molecular recognition of the floral volatile with two olfactory related proteins in the eastern honeybee (*A. cerana*).

Therefore, to gain a deeper understanding of how the olfactory related proteins respond to the stimulation of floral odors, we analyzed the binding interaction of AcerASP2 and AcerASP3 with β-ionone using multiple fluorescent spectra, circular dichroism (CD) spectra and molecular docking. It will be beneficial to clarify the chemical mechanism through which floral volatiles are carried by the olfactory related proteins across the hydrophilic lymph of chemosensilla to the olfactory receptors of honeybees

2. Experiment

2.1. Preparation of recombinant proteins

For an analysis of protein binding with floral volatiles, it is important to obtain the active recombinant proteins. To this end, the recombinant proteins of AcerASP2 (*A. cerana*'s OBP) and AcerASP3 (*A. cerana*'s CSP) were prepared from *Escherichia coli* BL21 (DE3) competent cells, and the expression of two proteins were induced by Isopropyl-beta-D-thiogalactopyranoside (IPTG) and the recombinant proteins were purified as in our previously described method [6,7]. After the concentration measurement with bovine serum albumin (BSA), the purified recombinant proteins AcerASP2 and AcerASP3 were diluted into 1×10^{-6} mol L^{-1} with phosphate buffered saline (PBS; pH 74) as a stock solution. The testing floral volatile, β-ionone (purity >96%, ACROS, Belgium), was dissolved into 1×10^{-3} mol L^{-1} with methyl alcohol (TEDIA, USA) as the stock solution. The solutions were kept at 4 °C in the dark for further use. All of the chemicals used were of analytical reagent grade and Milli-Q water was used throughout.

2.2. Apparatus

All fluorescent measurements were conducted on an RF-5301PC Spectrofluorophotometer (Shimadzu, Japan) equipped with a xenon lamp source and quartz cells. UV spectra were obtained on a UV-2501 UV Spectrophotometers (Shimadzu, Japan) equipped with quartz cells. The CD spectra were recorded on a Jasco-815 CD spectrometer (Jasco, Japan) using a 10 mm path length quartz cuvette. A highly sensitive electronic thermostat water-bath (PolyScience, USA) was used to control the working temperature of samples. The pH was measured with a PB-10 digital pH-meter (Sartorius, Germany) with a combined glass-calomel electrode. The chemical material was weighed with BS224S electronic analytical balance (Sartorius, Germany).

2.3. Spectral experimental procedure

The fluorescent quenching spectra were used to study the binding interaction between chemical molecules (e.g. organic drugs) and the binding proteins in sera (e.g. BSA) [13,14]. To record the fluorescent quenching spectra of the two proteins binding with floral volatile (β-ionone), the stock solutions of AcerASP2 and AcerASP3

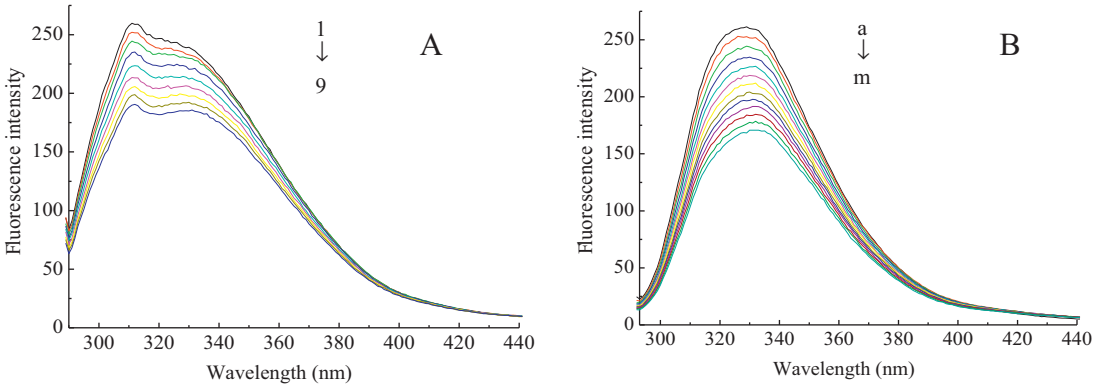

Fig. 2. Fluorescence spectra of AcerASP2 (A) and AcerASP3 (B) with β-ionone titrating c (AcerASP2) = 0.6×10^{-6} mol L^{-1}; c (AcerASP3) = 0.26×10^{-6} mol L^{-1}, pH = 74; λ_{ex} = 280×10^{-6} nm, c (β-ionone)/(mol L^{-1}): For AcerASP2, (1) 0; (2) 27; (3) 43; (4) 60; (5) 76; (6) 92; (7) 10.9; (8) 125; (9) 141; For AcerASP3, a, 0.0; b, 17; c, 31; d, 45; e, 58; f, 72; g, 86; h, 10.0; i, 114; j, 127; k, 141; l, 155; m, 168.

were diluted into working solutions with the respective concentrations of 0.6×10^{-6} and 0.26×10^{-6} mol L^{-1}, afterwards β-ionone was titrated into each solution in the quartz cell. When the mixture of AcerASP2 and AcerASP3 and β-ionone were excited at 280 nm, the fluorescent emission spectra were recorded in the wavelength of 290–440 nm with a slit width of 5 nm.

For the quenching mechanism and thermodynamical study, the emission spectra were recorded with sequential titrating at different temperatures, which was provided by placing the cell into the thermostatwater-bath for 3 min during each titrating interval. The UV absorption spectra of the two proteins binding with β-ionone were measured at the wavelength of 220–400 nm using a quartz cell filled with the PBS buffer (pH 74). The CD spectra of the two recombinant proteins that were binding with β-ionone were recorded to monitor the conformation change of proteins at the wavelength of 190–250 nm at room temperature under constant nitrogen flush. The molar ratios of β-ionone to the two proteins were varied as 0:1, 1:1, 16:1 and 32:1, respectively. All observed CD spectra had the baseline subtracted with PBS buffer (pH 74), and the results were taken as CD ellipticity in order to measure the conformation of the proteins.

2.4. Molecular docking analysis

The characteristic mode in the binding interactions of the organic compound and proteins could be visually analyzed by molecular docking analysis [15]. Since the tertiary structure of AcerASP2 and AcerASP3 are experimentally unavailable, they were predicted by SWISS-MODEL Workspace [16] using the crystal structure of ASP2 (PDB entry code, 1tuj) [17] and CSP*sg*4 (PDB entry code, 2gvs) as the templates. Based on the simulated tertiary structures of AcerASP2 and AcerASP3, β-ionone was docked into the predicted binding cavity of AcerASP2 and AcerASP3 through molecular docking method using a Molegro Virtual Docker (MVD) 42 program [18]. The grid-based cavity prediction algorithm was used to determine the potential binding cavities of the two proteins. MolDock Optimizer was chosen as the searching algorithm and MolDock Score was used as the scoring algorithm [18].

3. Results and discussion

3.1. Expression and purification of AcerASP2 and AcerASP3

Due to the unavailability of commercial proteins of AcerASP2 and AcerASP3, the recombinant plasmids of pET30/AcerASP2 and pET30/AcerASP3 were transferred into competent *E. coli* BL21(DE3), and the bacteria were induced by IPTG to initiate the expression of recombinant proteins. Afterwards the proteins were crushed, and the protein lysates were analyzed by 15% SDS-PAGE to check if the recombinant proteins were expressed. Both the target proteins with a molecular weight of about 20–25 kDa were acquired (Fig. 1C, lanes 1 and 2), with the molecular weight of AcerASP2 being

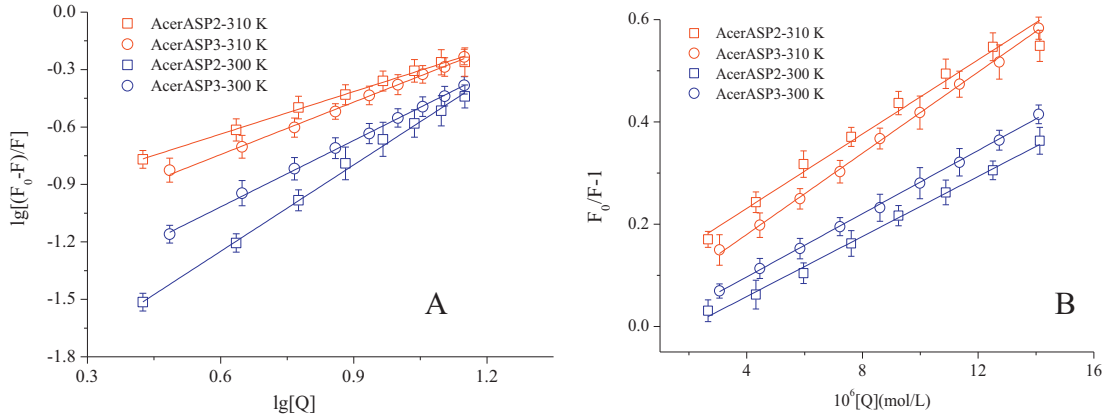

Fig. 3. Plots of log[$(F_0 - F)/F$] versus log[Q] of AcerASP2 and AcerASP3 in case of the static quenching (A) and plots of Stern–Volmer equation $F_0/(F-1)$ versus 10^6 [Q] of AcerASP2 and AcerASP3 in case of dynamic quenching (B). Higher temperature (310 K) and lower temperature (300 K) are shown by red and blue color, respectively. AcerASP2 and AcerASP3 are shown by square and circle, respectively. c (AcerASP2) = 0.6×10^{-6} mol L^{-1}; c (AcerASP3) = 0.26×10^{-6} mol L^{-1}, pH = 74; λ_{ex} = 280 nm. The increasing concentration of β-ionone are in accordance with to Fig. 2 when it is titrating. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a little larger than AcerASP3 due to AcerASP2 having more amino acid residues (Fig. 1B). To obtain the recombinant proteins that had engaged in binding activity with β-ionone, the two proteins were purified through Ni^{2+}-NTA affinity chromatography column from the above protein lysate. Finally, when the purity of both the proteins were above 90% based on the gray level analysis software (Glyco Band-Scan, Prozyme, USA, Fig. 1C, lanes 3 and 4), they were then ready to be used in the following binding assay. The final concentrations of the two proteins were quantified by the bicinchoninic acid (BCA) assay [19].

3.2. Binding property analysis

When β-ionone was titrated into the recombinant AcerASP2 and AcerASP3 solutions, their respective fluorescent spectra were obtained (Fig. 2). The fluorescent intensity of AcerASP2 and AcerASP3 regularly dropped at 312 and 330 nm in tandem with the increasing concentrations of β-ionone, and the emission peaks of each protein maintained a very similar pattern at the different concentrations of β-ionone, except for the slightly red shift of AcerASP3 (Fig. 2B). This indicates that non-fluorescent complexes were generated when β-ionone reacted to the two proteins, and thus it indicates an ability of β-ionone to bind with AcerASP2 and AcerASP3.

It is reported that the emission of fluorescence is mainly derived by the tryptophan and tyrosine residues that are contained in the proteins, and the fluorescent quantum yields of tryptophan are slightly higher than tyrosine [20]. Therefore, if the proteins have the same tryptophan and tyrosine residues but vary in number, their characteristics of fluorescent emissions spectra are distinct accordingly. Due to the fact that AcerASP2 has only six tyrosine, and AcerASP3 has one tryptophan and four tyrosine residues (Fig. 1B), the maximal fluorescent intensity of AcerASP2 and AcerASP3 were decreased at about 310 and 330 nm (Fig. 2), respectively. The longer maximal emission wavelength of AcerASP3 implies that its tryptophan contribute more fluorescent emissions than that of two tyrosine in AcerASP2. Furthermore, for the proteins without tryptophan like AcerASP2, tyrosine is thought to be an alternative amino acid to quantify the fluorescent quenching power of proteins.

3.3. Fluorescent quenching mechanism

There are two types of fluorescent quenching modes of proteins, i e dynamic and static quenching. Both of them have temperatures dependent on the property, such as in the dynamic and static quenching. The dynamic quenching means that the higher temperature results in faster diffusion and dissociation of weakly bound complexes, hence larger amounts of collision occur. In contrast, static quenching only produces stable complexes and results in the dissociation of complexes at higher temperatures [13]. Static quenching is usually analyzed by the double logarithm equation [21]:

$$\log\left[\frac{F_0 - F}{F}\right] = \log K_A + n\log[Q] \quad (1)$$

where F_0 is the fluorescent intensity in the absence of a quencher and F is the fluorescent intensity in the presence of a quencher at [Q] concentration. K_A is the apparent association constant, and n is the number of binding sites per protein. Both n and K_A at different temperatures were obtained once as a plot of $\log[(F_0 - F)/F]$ versus $\log[Q]$ (Fig. 3A, Table 1). The level of K_A in AcerASP2 and AcerASP3 was increased as the temperature rose, indicating that the binding interactions of β-ionone with the two proteins are likely dynamic quenching processes. In addition, when comparing n of β-ionone with the two proteins at different temperatures, n of AcerASP2 was always more than that of AcerASP3 regardless of the temperature

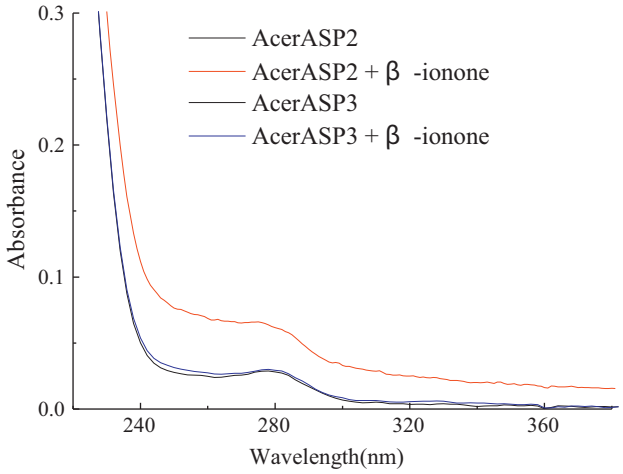

Fig. 4. UV spectra of AcerASP2 and AcerASP3 with β-ionone added red line and blue line are shown as AcerASP2 with β-ionone (the same $c = 0.6 \times 10^{-6}$ mol L^{-1}) and AcerASP3 with β-ionone (the same $c = 0.26 \times 10^{-6}$ mol L^{-1}), respectively. c (AcerASP2) = 0.6×10^{-6} mol L^{-1}; c (AcerASP3) = 0.26×10^{-6} mol L^{-1}, pH = 74; scanning range from 220 to 380 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

change. This suggests that OBPs may play a larger role than CSPs in entomic olfaction, despite their having a similar mode of involvement into the olfactory function of honeybee.

Since the two proteins were not observed as static quenching, the binding interactions of β-ionone with the two proteins were analyzed by dynamic quenching, which is commonly analyzed by the well-known Stern–Volmer equation [22]:

$$\frac{F_0}{F} = 1 + K_q\tau_0[Q] = 1 + K_{sv}[Q] \quad (2)$$

where F_0 and F are the fluorescent intensity of the absence or presence of a quencher at [Q] concentration, respectively. K_q is the quenching rate constant of the biomolecule, τ_0 is the average lifetime of the molecule without a quencher with a value of 10^{-8} s [23], and K_{sv} is the Stern–Volmer dynamic quenching constant. If the quenching process is dynamic, the dependence of F_0/F on the quencher concentration [Q] should be linear with a slope equal to the value of K_{sv} according to the Stern–Volmer equation. Based on this, the K_q (L mol^{-1} s^{-1}) of β-ionone binding with two proteins was obtained (Fig. 4B and Table 2). The K_{sv} at 310 K was higher than that at 300 K, suggesting that the interactions of β-ionone with the two proteins are still likely dynamic. This is because dynamic quenching usually results from the diffusion of molecules and the diffusion rate will increase as the temperature rises [24]. Specifically, K_{sv} increased proportionally with the temperature in dynamic quenching

To further confirm the quenching mechanism of proteins with plant odors, UV spectra were analyzed (Fig. 4). Once the same concentration of β-ionone was added to AcerASP2 ($c = 0.6 \times 10^{-6}$ mol L^{-1}) or AcerASP3 ($c = 0.26 \times 10^{-6}$ mol L^{-1}), their UV spectral patterns were quite similar. This suggests that the interactions of β-ionone with the two proteins are also dynamic processes. This is because this type of quenching usually influences the state of excitement of fluorescent molecules, but not the absorption spectra, while the shape of the spectra will change if static quenching occurs [25]. The K_q values were slightly greater than the maximal quenching rate constant of diffusion collision, which might be caused by the ionic salts in the solution. Taken together, all the fluorescent quenching analysis suggests a dynamic collision quenching occurring as the β-ionone bound to AcerASP2 and AcerASP3. This is particularly interesting because it reveals that the olfactory efficiency of *A. cerana* foraging behavior is

Table 1
Apparent association constant (K_A) and the number of binding sites and at different temperature.

Proteins	Temperaure (K)	Double logarithm equation		
		K_A (L mol^{-1})	n	R
AcerASP2	300	0.714×10^4	1504	0.9964
	310	8437×10^4	0.8891	0.9942
AcerASP3	300	1868×10^4	11768	0.9992
	310	5779×10^4	0.8629	0.9943

temperature dependent. That is, the higher the environmental temperature, the faster the volatilization and diffusion of floral volatiles, thus this is the driving force to stimulate the honeybee to fly out searching for a nectar source; since the binding interaction of olfactory related proteins increase with floral volatiles.

3.4. Acting force determination

The binding properties of β-ionone with AcerASP2 and AcerASP3 are influenced by several interactions between macromolecules and organic micromolecules such as hydrophobic interaction, electrostatic force, hydrogen bonds, and Van der Waals interactions etc., which can be deduced by the thermodynamic equations as follows [14]:

$$\Delta G = -RT \ln K = \Delta H - T\Delta S \quad (3)$$

$$\Delta H = \frac{RT_1T_2 \ln(K_{0,2}/K_{0,1})}{T_2 - T_1} \quad (4)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (5)$$

where ΔG, ΔH, and ΔS are the free energy change, enthalpy change, and entropy change. If the temperature changes slightly, the enthalpy change is always regarded as a constant. As a result, the values of ΔG, ΔH, and ΔS for β-ionone that had interacted with the two proteins were calculated (Table 2). Theoretically, when $\Delta H < 0$ or $\Delta H \approx 0$ and $\Delta S > 0$, the main acting force is an electrostatic force; when $\Delta H < 0$ and $\Delta S < 0$, the main acting force is van der Waals or a hydrogen bond and when $\Delta H > 0$ and $\Delta S > 0$, the main force is hydrophobic [26]. Here $\Delta H > 0$ and $\Delta S > 0$, which indicates that the acting forces between β-ionone with AcerASP2 and AcerASP3 are mainly driven by a hydrophobic interaction. Moreover, $\Delta G < 0$ in the two binding interactions, this suggests that spontaneous binding interactions of β-ionone and the two proteins occur. Collectively, our data suggests that floral volatiles could spontaneously bind to OBPs and CSPs to pass through the hydrophilic lymph in chemosensilla of the honeybee. This gives sound evidence that most olfactory related proteins can bind with hydrophilic chemical volatiles by hydrophilic force, thus the honeybee has an evolutionary strategy to do foraging activities timely and spontaneously at the blossom time of the plant.

3.5. Contribution of tyrosine and tryptophan by synchronous fluorescent spectra

To further analyze the fluorescent contribution to AcerASP2 and AcerASP3 from the two major fluorescent amino acids, tyrosine and tryptophan, and the polar change in the binding interactions, synchronous fluorescent spectra can be used and obtained by scanning excitation and recording emission spectra simultaneously [27]. It works under the condition of a fixed $\Delta\lambda$, the difference between the wavelength of monochromatic excitation and the emission light. With a value of $\Delta\lambda = 15$ and 60 nm, it will separately correspond to the fluorescent contribution of tyrosine and tryptophan of the proteins [28]. As Fig. 5 shows, the maximal fluorescent emission intensity of tyrosine and tryptophan in the two proteins was quenched regularly with no changing pattern of the emission spectra when β-ionone titrated. Therefore, it is clear that tryptophan contributes more fluorescent emissions than tyrosine in AcerASP3 Notably, Tyr26 (Tyr28 in AcerASP3) was found to form the bottom of one channel formed by Helix-A near nitrogen terminus, preventing its continuity with a nearby internal cavity of the CSPMbraA6 in *Mamestra brassicae* [29], tyrosine is supposed to be involved in the binding cavity of AcerASP3 in this study. On the other hand, tyrosine contributes to the total fluorescent emission in AcerASP2 due to the absence of tryptophan. Recently, Tyr 10, 113, and 117 residues are reported to be members of the side chains at the bottom of the roughly spherical internal cavity of the ASP2 of *A. mellifera* (radius of 7–8 Å) [17]. This is inline with our results and suggests that some tyrosines are close to the binding site in AcerASP2. In addition, the maximum emission wavelength of two amino acids did not exhibit an apparent blue or red shift (Fig. 5), suggesting no polar changing close to the binding sites when β-ionone interacted with the two proteins. This suggests a quite stable binding status when floral volatiles interacted with olfactory related proteins, which is beneficial for the quick recovery and carrying efficiency of the olfactory proteins after transportation of floral odors in the pollination process of the honeybee.

3.6. CD spectra analysis

To investigate whether the binding of β-ionone could induce the conformational changes of AcerASP2 and AcerASP3, the CD spectra were measured. Both of the proteins had one positive ellipticity at 192 nm and two negative ellipticities at 208 and 222 nm, respectively, which are the typical symbols of the presence of abundant α-helices [30]. As Fig. 6 shows, both of the proteins had evident α-helice structures, and AcerASP2 seemed to have more abundant α-helice structures than AcerCSP3, which is similar to the homology recombinants of ASP3c [31] and CSP*sg*4 [32]. When β-ionone was added to the solution of the two proteins with increasing concentrations, the negative ellipticity of the two proteins significantly decreased (shifting to zero levels) without any significant shift. Meanwhile the negative falling ellipticity of AcerASP3 seemed

Table 2
Fluorescence quenching constants (in equation of Stern–Volmer) and the thermodynamic parameters at different temperature.

Temperature (K)		K_{sv} (L mol^{-1})	K_q (L mol^{-1} s^{-1})	R	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	ΔG (kJ mol^{-1})
AcerASP2	300	1.15×10^4	1.15×10^{12}	0.9955	190.980	710.369	−22,131
	310	6.4×10^4	6.4×10^{12}	0.9738			−29,235
AcerASP3	300	1.984×10^4	1.984×10^{12}	0.9953	87,336	372,888	−24,531
	310	5.891×10^4	5.891×10^{12}	0.9991			−28,260

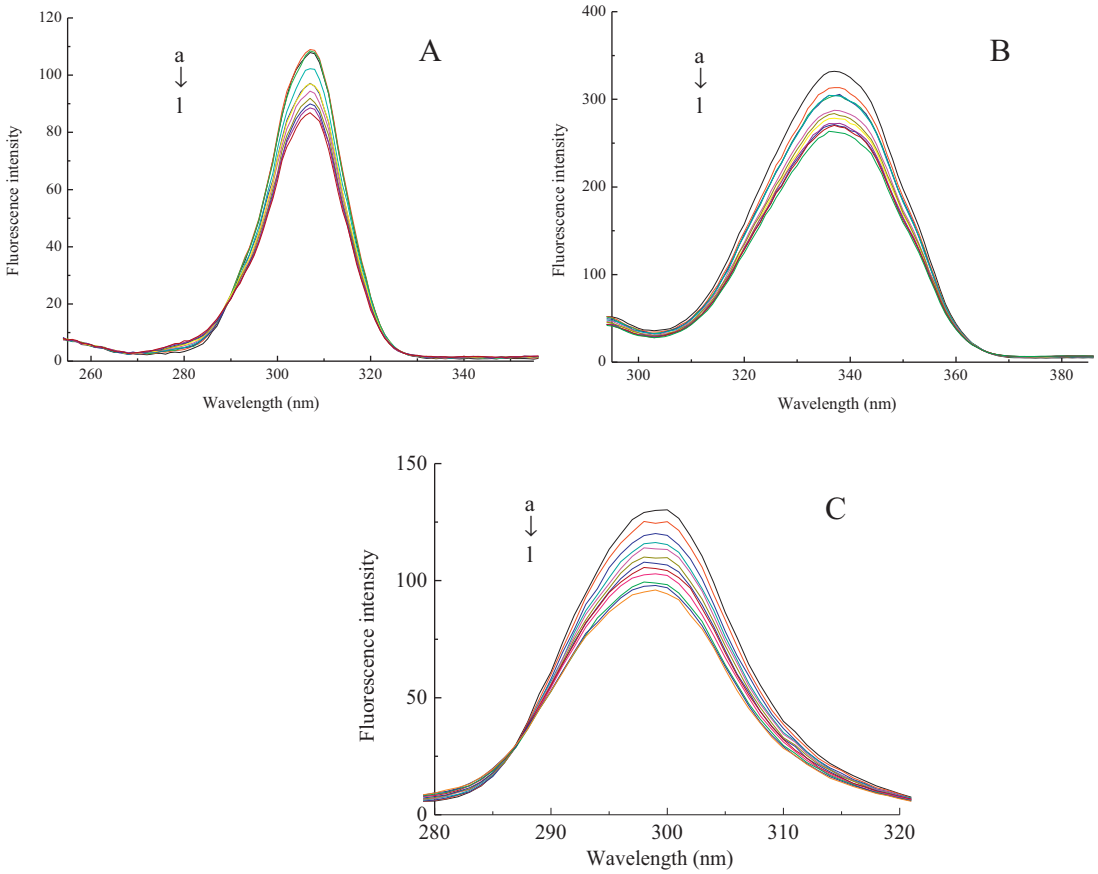

Fig. 5. Synchronous fluorescence spectra of AcerASP3 to tyrosine (A, $\Delta\lambda = 15$ nm) and tryptophan (B, $\Delta\lambda = 60$ nm), and AcerASP2 to tyrosine (C, $\Delta\lambda = 15$ nm). c (AcerASP2) = 0.6×10^{-6} mol L^{-1}; c (AcerASP3) = 0.26×10^{-6} mol L^{-1}, pH = 74; $\lambda_{ex} = 280$ nm. The increasing concentration of titer of β-ionone are a, 0.0; b, 0.6 (AcerASP2), 0.26 (AcerASP3); c, 10; d, 20; e, 30; f, 40; g, 50; h, 60; i, 70; j, 80; k, 90; l, 10.0.

to be larger than AcerASP2, which indicates that a percentage of α-helices decrease in the two proteins, thus the conformation of AcerASP2 and AcerASP3 is spread with increasing concentrations of β-ionone. Due to the potential larger binding cavity, AcerASP2 could keep a stable binding conformation with β-ionone, which thus suggests that OBPs are likely to have a stronger capability as odor recognizers than CSPs in the olfactory system of the honeybee.

3.7. Molecular docking analysis

To further investigate β-ionone interactions with AcerASP2 and AcerASP3 we viewed at the atomic level, the potential hydrogen bonds produced between β-ionone and the two proteins. Their binding modes were predicted by molecular docking analysis. Based on the available structure of ASP2 (PDB entry code, 1tuj) [17] and CSP*sg*4 [33], the tertiary structure of AcerASP2 and AcerASP3 was predicted by SWISS-MODEL Workspace. As Fig. 7 shows, a 3-D electrostatic solid model of AcerASP2 and AcerASP3 was constructed, both of the two proteins have one predicted cavity, while the cavity of AcerASP2 seemed to be slightly larger and deeper than that of AcerASP3 (Fig. 7A and C). A likely reason is that AcerASP2 has a larger molecular weight and potential binding cavity than AcerASP3 (Fig. 1B). The well embedded molecular pose of β-ionone in the predicted binding cavity of the two proteins through

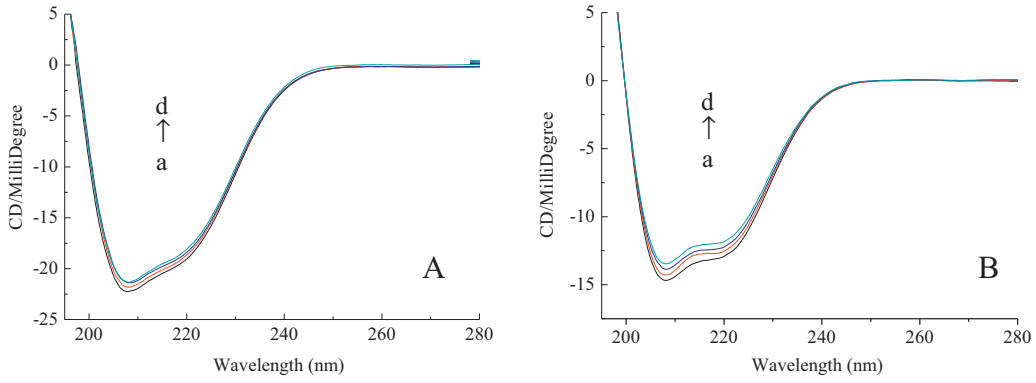

Fig. 6. Circular dichroism spectra of AcerASP2 (A) and AcerASP3 (B) with β-ionone titrating c (AcerASP2) = 0.6×10^{-6} mol L^{-1}; c (AcerASP3) = 0.26×10^{-6} mol L^{-1}, pH = 74; Scanning range from 195 to 280 nm. a, 0; b, 0.5; c, 80; d, 160.

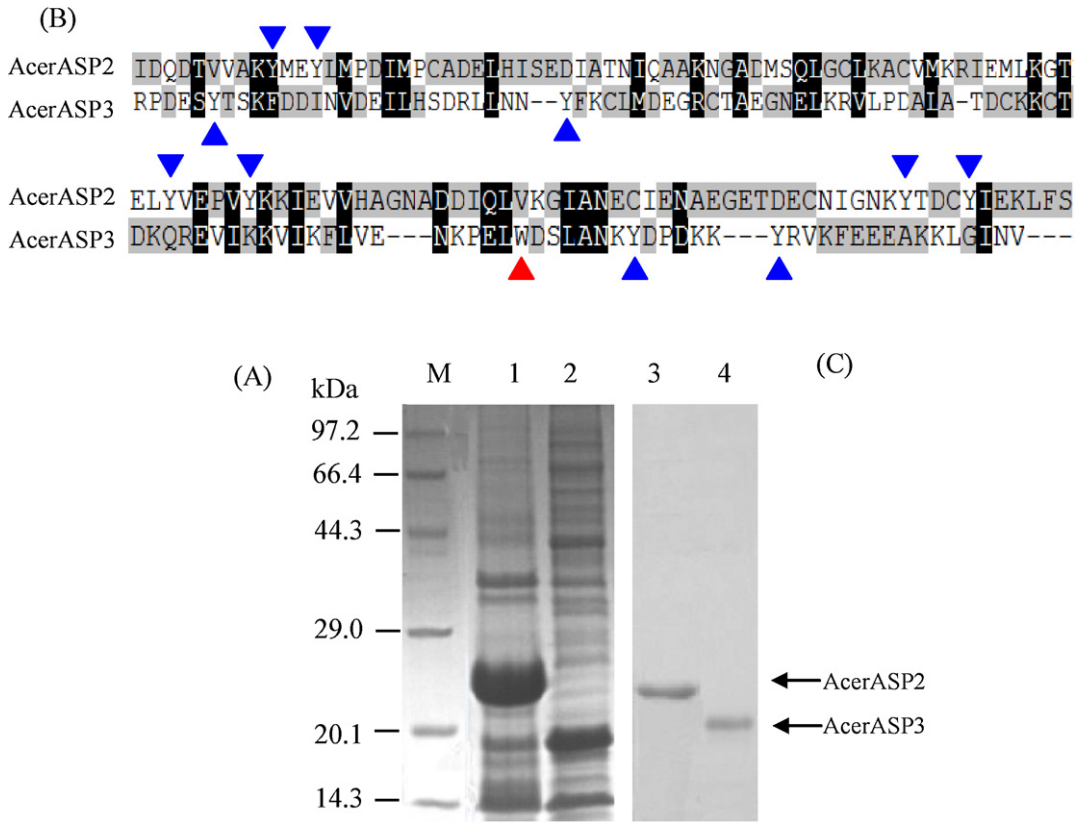

Fig. 7. Molecular docking analysis of the both proteins binding with β-ionone. β-ionone is predicted to embed in the hydrophobic binding cavity of 3-D electrostatic solid style of AcerASP2 (A) and AcerASP3 (C). Two hydrogen bonds occur between the carbonyl group of β-ionone with the amino group of AcerASP2 (C Lys 51) and AcerASP3 (D Tyr 6), respectively.

a strong hydrogen bond between the oxygen atom of β-ionone and the nitrogen atom of Lys 51 in AcerASP2 and Tyr 6 in AcerASP3 (Fig. 7B and D). Here Tyr 6 in AcerASP3 maybe also contribute the fluorescent quenching of AcerASP3, which was in accordance with the results of synchronous fluorescent spectra in AcerASP3. It suggests that the floral volatiles have evolved the most ideal and efficient way to attract the honeybee's visit, and vice versa.

4. Conclusions

In this study, we examined the detailed chemical mechanism of molecular binding recognition between a floral volatile (β-ionone) and two olfactory related proteins in *A. cerana*. The binding interactions of β-ionone with AcerASP2 and AcerASP3 are of a dynamic mode, i.e. the higher temperature is beneficial to the binding of olfactory proteins with floral odors, it thus promotes the diffusion of plant volatiles to strengthen the binding efficiency between odors and proteins. The binding of plant odors to olfactory proteins is spontaneous and hydrophobic effects are the major binding forces through some important hydrogen bonds in the binding cavity between the volatiles and the two proteins. AcerASP2 has a better binding capability with floral odors. Our study reveals that the honeybee has an evolutionary strategy to recognize the floral volatiles at blossom time of a plant through the functionality of specific olfactory proteins.

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