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**RESEARCH PAPER**

# Purification and Functional Reconstitution of Human Olfactory Receptor Expressed in *Escherichia coli*

Heehong Yang, Hyun Seok Song, Sae Ryun Ahn, and Tai Hyun Park

**Abstract**  Olfactory receptors (ORs), belonging to the G-protein coupled receptor (GPCR) family, are very difficult to be overexpressed, purified and reconstituted because of their hydrophobicity and complicated structure. These receptors bind to their specific ligands, thus their specificity is very useful for application as a bioelectronic nose. Furthermore, highly purified and well-reconstituted human olfactory receptor (hOR) can be used in various fields, such as in protein-interaction research, drug screening, and analysis of the hOR structure. In this study, human olfactory receptor, hOR2AG1, was produced with high purity and functionally reconstituted in detergent micelles. The hOR2AG1 was overexpressed in *Escherichia coli* (*E. coli*) with glutathione S-transferase (GST) and 6xHis-tag as an inclusion body. The hOR2AG1 fusion protein was solubilized in buffer containing sodium dodecyl sulfate (SDS) and purified using Ni-NTA chromatography. The GST domain was removed using proteolytic cleavage before elution from the column. After purification, the hOR2AG1 was successfully reconstituted using nonionic detergents and methyl-β-cyclodextrin. Finally highly purified and well-reconstituted hOR was obtained, and its biological characteristics were confirmed by using circular dichroism (CD) spectrum and tryptophan fluorescence assay. These results can be applied to develop protein-based sensing systems including a bioelectronic nose and to analyze the native hOR structure using solid-state NMR, X-ray crystallography, or neutron scattering.

**Keywords:** human olfactory receptor, purification, reconstitution, *Escherichia coli,* ligand binding

## 1. Introduction

Olfactory receptors (ORs), members of the G protein-coupled receptors (GPCRs) family, are integral membrane proteins comprised of seven transmembrane domains, an extracellular N terminus, and a cytoplasmic C terminus [1]. GPCRs are involved in diverse physiological processes, including sensory signaling, cell signal transduction, hormonal signaling and neuronal transmission [2]. They account for approximately 30% of all human drug targets for various diseases, containing targets for cancer, metabolic, inflammatory and central nervous system diseases [3]. Although they are considered to be highly important, the production of GPCRs has been the critical bottleneck in their study, as they are known to be very difficult to be overexpressed, solubilized, and purified because of their strong hydrophobicity and complicated structures [4]. Moreover, expression of eukaryotic membrane proteins in bacterial cell membrane is considered to be difficult because the membrane insertion mechanism is different, and there are inappropriate membrane interactions due to difference in charge distribution between eukaryotic and prokaryotic cells [5,6]. Therefore, when these membrane proteins have been expressed in bacterial cells, only milligram quantities of proteins could be produced as inclusion bodies [7,8]. In addition, reconstitution of the GPCRs in a native-like environment is necessary for analysis of the native GPCR structure using X-ray crystallography, solid-state NMR, or neutron scattering and to understand

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the kinetics and dynamics of the GPCRs [9]. Despite this difficulty, many groups have attempted to overexpress GPCRs in bacterial expression systems because of the many advantages it would confer, such as simplicity, convenience, low cost and high productivity [10,11].

ORs play an important role in recognizing thousands of odorant molecules and their specificity of binding particular ligands facilitates the discrimination of lots of odorant molecules [12,13]. These properties provide a powerful advantage for the application of olfactory biosensors in various fields, such as in quality assessment of food, clinical diagnosis, health control, and industrial environmental monitoring [14,15]. Recently, many studies have suggested that hORs can allow the development of a highly selective and sensitive bioelectronic nose, with human nose-like performance [16-19]. Development of a bioelectronic device using mouse OR produced from *Xenopus* oocytes was reported [20]; however, well-reconstituted hOR produced from *Escherichia coli (E. coli)* has not yet been applied to bioelectronic nose production. Above all, a purified and well-reconstituted protein-based biosensor would provide a great advantage in terms of being able to mimic the human olfactory system [21,22]. In addition, it is expected to have outstanding sensitivity, selectivity, stability, reusability, and monitoring of receptor/ligand binding in real time [23-25]. In this study, we demonstrate the successful overexpression, purification and reconstitution of hOR2AG1 from *E. coli*. This work can be applied to hOR and GPCR production, protein-based biosensors, and GPCR research of biophysical and structural analysis.

## 2. Materials and Methods

### 2.1. Overexpression of hOR2AG1 fusion protein in *E. coli*
The 6xHis gene was inserted at the C-terminus of the hOR gene, hOR2AG1 (GeneBank accession no. NC000011), in the pDEST15 vector (Invitrogen), which was used in our previous study [26]. The insertion was performed with an EZchange site-directed mutagenesis kit (Enzynomics, Daejeon, Korea). The BL21 (DE3) *E. coli* strain was transformed with the obtained pDEST15/GST-hOR2AG1-6xHis vector, and incubated in Luria-Bertani (LB) medium supplemented with 50 μg/mL ampicillin at 37°C overnight. Precultures were transferred to 1 L of LB medium in shaking flasks and grown until the OD$_{600}$ value reached 0.5. The expression of the hOR fusion protein was induced by the addition of 0.5 mM isopropyl β-D-thiogalactoside (IPTG), after which cells were incubated for 4 h. The cells were then harvested at 4°C by centrifugation (7,000 g, 30 min), and pellets were resuspended in PBS (phosphate-buffered saline, pH 7.4). The cells were lysed by sonication

with 5 s on/off for 10 min (Sonics Vibracell, CT, USA), and the sample was centrifuged (15,000 g, 4°C, 20 min). The supernatant was removed, the experiment was repeated and then the sample was resuspended in PBS. The expression of hOR2AG1 was confirmed by SDS-PAGE and western blot analysis. Western blots analysis was performed using anti-GST mouse antibody (Santa Cruz Biotechnology, CA, USA) or anti-His-probe mouse antibody (Santa Cruz Biotechnology, CA, USA) as a primary antibody, HRP-conjugated anti-mouse antibody (Millipore, MA, USA) as a secondary antibody and Luminata Forte western HRP substrate (Millipore, MA, USA).

### 2.2. Solubilization and column purification of hOR2AG1 fusion protein
The pellet obtained from the 6 L of bacterial culture in the shaking flasks was resuspended in 60 mL of PBS. The cells were lysed as described above, and the final cell lysate was solubilized in 60 mL of 0.1 M Tris-HCl (pH 8.0), 100 mM dithiothreitol (DTT), 20 mM sodium dodecyl sulfate (SDS), and 1 mM EDTA at room temperature. The solubilized sample was centrifuged, and the supernatant of the sample, containing fusion protein, was obtained. Next, the solubilized proteins were dialyzed in 0.1 M sodium phosphate (pH 8.0) and 10 mM SDS with a 10K MWCO dialysis cassette (Thermo Scientific, CA, USA). The solubilized proteins were filtered with a 0.2 μm bottle top filter (Thermo Scientific, CA, USA), then were applied to a 5 mL Ni$^{2+}$ column (GE Healthcare, NJ, USA) equilibrated in 0.1 M sodium phosphate (pH 8.0) and 10 mM SDS. The column was washed successively four times with buffer containing 0.1 M sodium phosphate and 10 mM SDS at pH 8.0, then changing to pH 7.0, and the fusion protein was eluted from the column using the same buffer at pH 6.0.

### 2.3. Removing of GST domain from hOR2AG1 fusion protein
Thrombin protease (GE Healthcare, NJ, USA) was used to cleave the GST domain. The fusion protein was bound to the 1 mL Ni$^{2+}$ column based on its C-terminal 6xHis-tag, and the column was washed four times with the column's volume of wash buffer (PBS, 10 mM SDS, pH 7.4). Fifty units of thrombin were added to the Ni$^{2+}$ column and incubated for 16 h at 20°C in a shaking incubator. After incubation, an additional washing condition was optimized using various wash buffers, and the washing condition (PBS, 1% SDS, 2% N-lauroylsarcosine (sarcosyl), pH 7.4) was chosen to remove the GST domain from the hOR. The hOR was then eluted from the column with the buffer (0.1 M sodium phosphate and 10 mM SDS, pH 6.0). Finally purified hOR was obtained and analyzed by SDS-PAGE,

and protein concentration was determined using BCA assay kit (Pierce, IL, USA) with bovine serum albumin as a standard.

### 2.4. Reconstitution of the purified hOR2AG1

To reconstitute the hOR, the eluted proteins were dialyzed against 0.1 M Tris-HCl (pH 8.0), 10 mM SDS, and 0.5 mM EDTA. The sample was diluted to 0.25 mg/mL in the final buffer (0.1 M Tris-HCl (pH 8.0), 3 mM SDS, 0.5 mM EDTA). After that, 1 mM glutathione disulfide (GSSG), 5 mM glutathione (GSH), 6 mM n-dodecyl-β-D-maltopyranoside (DDM), 6 mM 6-cyclohexylhexyl-β-D-maltoside (Cymal 6), and 6 mM methyl-β-cyclodextrin were added with stirring. After 48 to 60 h at -20°C, the sample was defrosted at 4°C, and 25 mM $CaCl_2$ was added before overnight incubation at 4°C. Finally, the sample was dialyzed against 100 mM Tris-HCl (pH 7.4), 300 mM NaCl, 10% glycerol, 1 mM DDM, 1 mM Cymal 6, and 1 mM EDTA. Reconstituted hOR2AG1 was maintained at 4°C before characterization steps.

### 2.5. CD spectrum and tryptophan fluorescence measurements

CD spectra were recorded on a chirascan-plus cd spectrometer (Applied Photophysics, Leatherhead, UK). Purified and reconstituted hOR2AG1 (250 μg/mL) was diluted with 100 mM Tris-HCl (pH 7.4), 300 mM NaCl, 10% glycerol, 1 mM DDM, 1 mM Cymal 6, and 1 mM EDTA. CD spectra between 190 and 260 nm of the hOR2AG1 sample and the blank buffer were recorded respectively, and background signal was subtracted. Tryptophan fluorescence quenching experiments were conducted using a LS 55 luminescence spectrometer (Perkin Elmer, CT, USA) (excitation 290 nm; emission 300 ~ 420 nm). Titration was carried out at room temperature using 500 nM reconstituted hOR2AG1 in the presence of 50 ~ 1,000 iM of the ligand. Fluorescence intensity of hOR2AG1 was measured in the presence of 1 mM of various odorant molecules.

## 3. Results and Discussion

### 3.1. Designing expression vector and overexpression of hOR2AG1 fusion protein

Fig. 1A shows the 6xHis gene insertion site and schematic diagram of the expression vector. In our previous study, the hOR2AG1 with GST was successfully overexpressed as a fusion protein at high-levels [26]. However, column purification and reconstitution of the hOR expression were unsuccessful because the GST-hOR2AG1 was expressed as an inclusion body. In that case, the GST did not have a functional affinity to the GST column. Therefore, the bacterial expression vector was redesigned as pDEST15/GST-
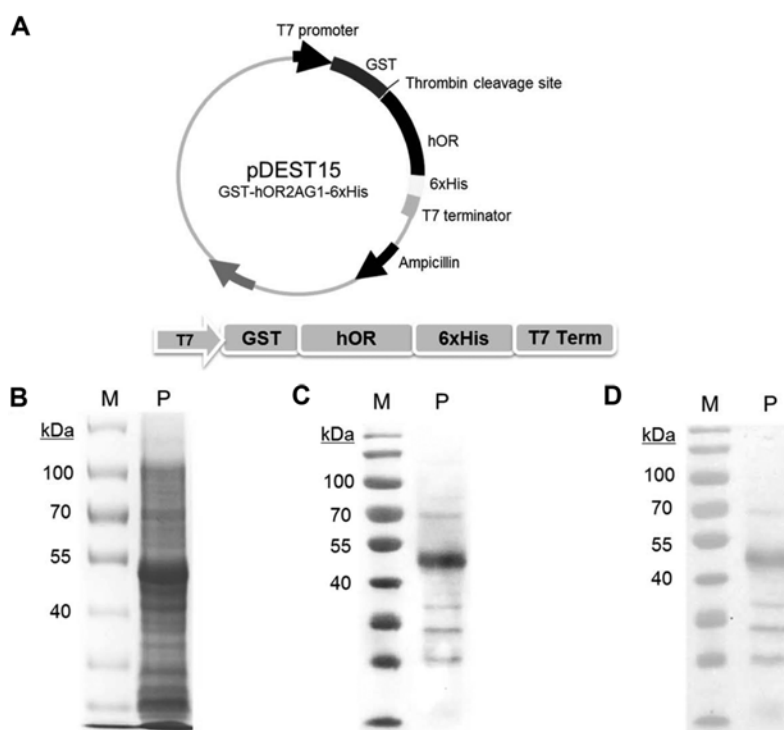


**Fig. 1.** Overexpression of human olfactory receptor, hOR2AG1 in *E. coli*. (A) Schematic representation of the pDEST15 vector containing GST, hOR2AG1, and 6xHis gene. 6xHis gene was inserted into the hOR2AG1 cloned vector by mutagenesis. (B) SDS-PAGE of the pellet of cell lysate after centrifugation. Western blot of the insoluble fraction of cell lysate using (C) anti-GST antibody and (D) anti-His-probe antibody after centrifugation. M, molecular weight marker; P, pellet of cell lysate after centrifugation.

hOR2AG1-6xHis by inserting the 6xHis gene into the cloning vector. Mutagenesis of the expression vector was successfully conducted, and the overexpression of hOR2AG1 with GST at the N-terminus and 6xHis-tag at the C-terminus as a fusion protein was confirmed by SDS-PAGE and western blotting.

Fig. 1B shows the results of the SDS-PAGE analysis for the hOR2AG1 fusion protein overexpressed from *E. coli*. The hOR was overexpressed at high levels and the band revealed at 53 kDa was mainly observed from the insoluble fraction of cell lysate (Fig. 1B). These results indicate that the hORs were overexpressed as an inclusion body. Figs. 1C and 1D show the western blot of the hOR2AG1 fusion protein using GST and His-probe antibodies, respectively. The GST-fusion protein was tagged at the N-terminus, and the 6xHis-tag was fused at the C-terminus of hOR2AG1. In both experiments, the bands were observed at the expected sizes in western blot analysis. These results demonstrate that full-length hOR2AG1 was successfully overexpressed.

Many reports have suggested that it is difficult for eukaryotic membrane proteins to be expressed in bacterial cell membranes due to the short loops containing positive charges in the eukaryotic membrane proteins [27]. In addition, when eukaryotic membrane proteins were expressed in *E. coli*, it was observed that the bacterial cells were easily lysed, due to difference in insertion mechanisms of the membrane proteins between prokaryotic and eukaryotic cells [6]. Despite these difficulties, some previous reports suggested that use of the pDEST15 vector containing GST gene allows high-level expression of GPCR in *E. coli* [28-30]. We tried to overexpress the hOR in *E. coli* using various expression vectors including pTrcHisC, pGEX4-T1, pET23a, pET21b(+), and pDEST15; however, only pDEST15 was successful in the overexpression of hOR [26].

## 3.2. Solubilization, purification and functional reconstitution of hOR2AG1

Fig. 2A shows the solubilization of the fusion protein expressed in *E. coli* as an inclusion body. The hOR2AG1 band at 53 kDa in the insoluble fraction indicated that it was completely dissolved in the solubilization buffer containing SDS. This indicates that hOR2AG1 fusion protein was successfully solubilized before the purification step. In bacterial expression systems, hORs are generally expressed as an insoluble form, thus, they need to be solubilized for purification and reconstitution. To solubilize the hOR effectively, appropriate detergents or chaotropic agents are required. In our previous study [26], various detergents and chaotropic agents were used in an attempt to solubilize the hOR. Sarcosyl, a strong ionic detergent, was found to allow efficient solubilization; however, it was difficult to remove the detergent for the subsequent reconstitution of hOR. Thus, in this research, the anionic detergent SDS was used to denature the hOR2AG1.

After solubilization, the fusion protein was dialyzed for successful affinity column purification, and the purification step was carried out using a $Ni^{2+}$ affinity column. Figs. 2B and 2C show the purified fusion protein by SDS-PAGE and western blotting, respectively. With $Ni^{2+}$ column purification, high-purity hOR2AG1 fusion protein was obtained (Fig. 2B). The purified fusion protein was observed in monomeric and dimeric forms, by both SDS-PAGE and western blotting. The amount of purified protein was estimated to be 1.16 mg from 1 L culture, calculated using the BCA total protein assay. It has been reported that GPCRs and other integral membrane proteins tend to form dimers or oligomers under the denaturing conditions for SDS-PAGE [31]. The productivity of the purified protein obtained was higher than that of the other previous reports [10,32,33], and the purity (> 95%) was much higher than that of our previous
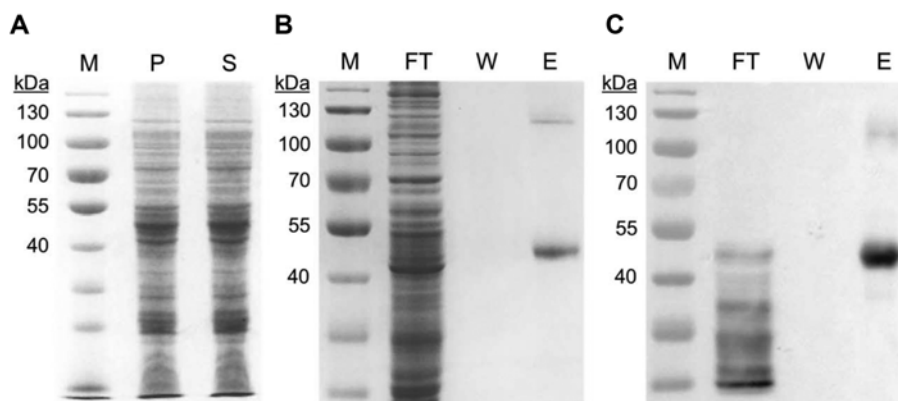


**Fig. 2.** Solubilization and column purification of GST-hOR2AG1-6xHis fusion protein. (A) SDS-PAGE of solubilization of hOR2AG1 fusion protein. (B) SDS-PAGE of the purified hOR2AG1 fusion protein. (C) Western blot of the purified hOR2AG1 fusion protein using anti-GST antibody. M, molecular weight marker; P, pellet of cell lysate after centrifugation; S, solubilized P after solubilization; FT, flow-through after $Ni^{2+}$ column; W, washed fraction; E, eluted fraction after $Ni^{2+}$ column purification.

report (~ 60%) [26].

The eluted hOR2AG1 fusion protein was in the denatured form, and was reconstituted after purification. The GST domain of the fusion protein was cleaved for the correct reconstitution of hOR2AG1 using thrombin protease. The thrombin cleavage was performed in various washing conditions in order to efficiently cleave the GST domain out of the fusion protein bound to the column matrix. Figs. 3A, 3B, and 3C show SDS-PAGE analysis for the thrombin-cleaved hOR2AG1, under different washing conditions (A: wash buffer; B: wash buffer + 1% SDS; C: wash buffer + 1% SDS + 2% sarcosyl). In Fig. 3A, the major band from the eluted fraction was observed around 27 kDa, indicating that removal of the GST domain was not performed well. Fig. 3B shows that the amount of the GST was reduced; however, it was not completely removed. While conditions (A) and (B) were not very effective for the removal of the GST domain from the eluted fraction, it can be observed in Fig. 3C that (C) allowed for almost complete removal, and the dimeric- and oligomeric- sized bands of hOR2AG1 were clearly observed. This indicates that condition (C), containing strong ionic detergent at a high concentration, is an appropriate condition for washing off the GST domain. It also shows that the hOR2AG1 was appropriately cleaved by thrombin protease, to obtain the hOR2AG1 at a high-purity (> 95%). When the GST was removed from the hOR2AG1, the dimeric form of hOR was predominantly observed in SDS-PAGE. It has been reported that some proteins could form dimers or oligomers after proteolytic cleavage [34]. Many GPCRs exist, and may function as monomeric, dimeric and oligomeric complexes [35]. In many cases, strong ionic detergents such as SDS and sarcosyl have been added to wash buffer to effectively wash off the GST domain, since it is usually strongly

associated with the receptor and very difficult to be washed off the column [36].

After purification, the receptors were reconstituted by refolding in detergent micelles. Purified hOR2AG1 has a linear form, therefore needs to be refolded to its original form. Detergent micelles have been previously used for efficient folding of GPCR [37], and ORs have also been successfully reconstituted by the refolding strategy using detergent micelles, which create artificial membrane-like environments [9]. In this study, DDM and Cymal 6, typical nonionic detergents, were used for forming the detergent micelles. Reduced and oxidized glutathione (GSH/GSSG) were used for oxido-shuffling reagents [38] and methyl-β-cyclodextrin, a cyclic oligosaccharide, was used as an artificial chaperone and to strip off the SDS [39,40].

### 3.3. Characterization and functional study of hOR2AG1

The function of hOR2AG1 was confirmed using CD spectrum analysis and the tryptophan fluorescence quenching method, after the purification and reconstitution process. In order to evaluate the quality of the hOR2AG1 reconstituted in detergent micelles, the CD spectrum of the hOR was measured, as shown in Fig. 4A. Table 1 shows the secondary structure of hOR2AG1, predicted using program CDNN secondary structure analysis. The α-helical content was about 64.4%. This result agrees with the α-helical feature demonstrated in other functional ORs [36,41-43]. It has also been predicted that the seven transmembrane domains of ORs are mainly comprised of α-helical structures [44-46].

As an approach to analyze the interaction of OR with the odorant molecule known as its ligand, the tryptophan fluorescence quenching method was used. The interaction of hOR2AG1 with the ligand amyl butyrate (AB) was observed by recording the intrinsic fluorescence of the
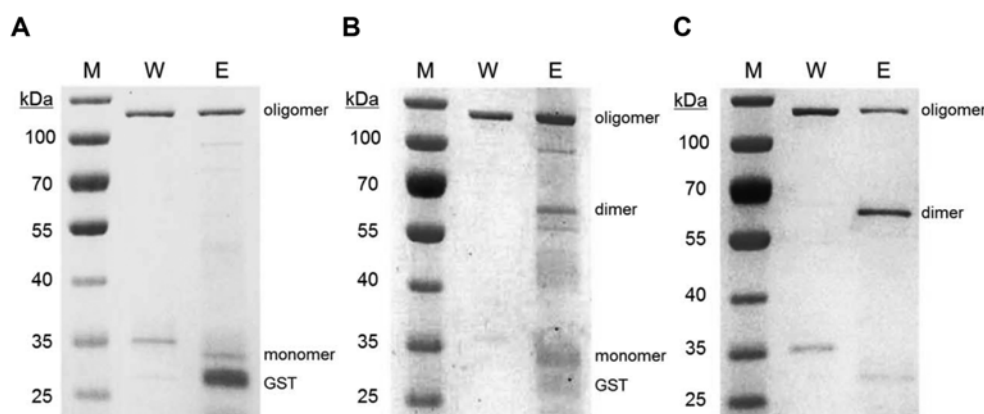


**Fig. 3.** Thrombin cleavage of GST-hOR2AG1 bound to the column. SDS-PAGE of thrombin-cleaved fusion protein with different washing conditions of (A) wash buffer, (B) wash buffer + 1% SDS, and (C) wash buffer + 1% SDS + 2% sarcosyl, which was applied to the bound receptor. The GST domain of the fusion protein strongly associated with the receptor was difficult to be washed off the column completely. Thus, SDS and sarcosyl were added to the wash buffer to ensure complete wash off of the GST domain. M, molecular weight marker; W, washed fraction; E, eluted fraction after thrombin cleavage.
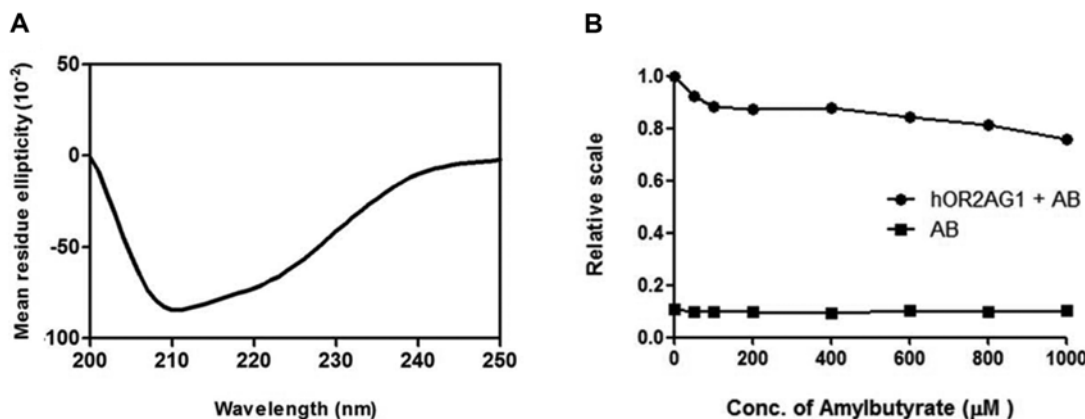
**A**



**B**



**Fig. 4.** Characterization and functional assay of hOR2AG1. (A) CD spectrum of hOR2AG1. (B) Tryptophan fluorescence quenching of hOR2AG1 by the binding of AB.

**Table 1.** Secondary structure analysis of the reconstituted hOR2AG1

|             | 200 ~ 260 nm | 205 ~ 260 nm | 210 ~ 260 nm |
|-------------|--------------|--------------|--------------|
| Helix       | 57.8         | 64.4         | 63.4         |
| Antiparallel| 4.0          | 3.5          | 3.3          |
| Parallel    | 4.1          | 3.5          | 4.1          |
| Beta-Turn   | 13.4         | 12.6         | 12.4         |
| Rndm. Coil  | 17.3         | 16.4         | 17.6         |
| Total Sum (%)| 96.7        | 100.3        | 100.7        |

receptor at increasing AB concentrations. Fig. 4B shows that the tryptophan fluorescence intensity decreased with increasing concentrations of AB. With an increase in the AB concentration up to 1,000 μM, the tryptophan fluorescence intensity of hOR2AG1 decreased by about 25%. On the other hand, the intensity of the buffer solution without hOR did not change, even with the addition of AB. Two tryptophan sites of the hOR2AG1 were putatively located in the center of transmembrane 4 region [43]. This position is considered to be closely related to the ligand binding sites of hORs [44]. Thus, we assumed that if the ligand bound to the hOR, the tryptophan fluorescence could be changed. Due to the strong hydrophobicity of hOR, tryptophan prefers to be buried in the protein hydrophobic cores when the ligand binds to the receptor. Hence, fluorescence intensity from the hydrophobic residue on the receptor was quenched in the case of ligand binding in solution. Therefore, these results suggest that the tryptophan fluorescence from hOR was quenched by the specific interaction between the receptor and odorant. In this tryptophan fluorescence measurement, the equilibrium dissociation constant ($K_d$) was estimated to be 54.6 μM. This value agrees with other odorant-hOR dissociation constants obtained in previous experiments using detergent micelles [42].

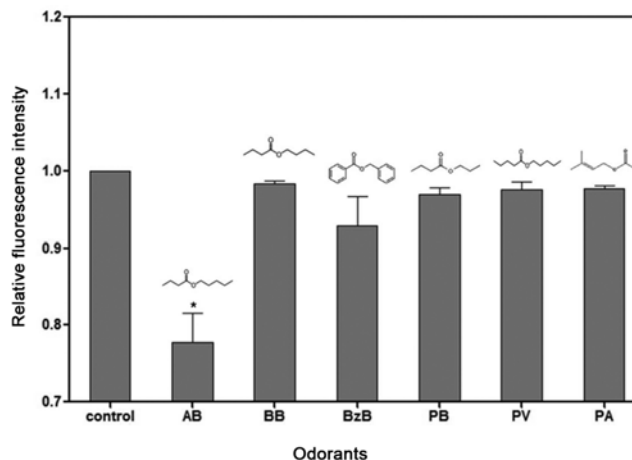The binding properties of hOR2AG1 were investigated



**Fig. 5.** Effect of various odorant molecules on the tryptophan fluorescence quenching of hOR2AG1 ($^*p < 0.05$). AB, amyl butyrate; BB, butyl butyrate; BzB, benzyl benzoate; PB, propyl butyrate; PV, pentyl valerate; PA, prenyl acetate.

using six different odorant molecules with ester functional groups, which have slightly different structures. Fig. 5 demonstrates that the fluorescence was quenched significantly only by the addition of AB. These results show that the hOR2AG1 selectively binds with its specific ligand. Therefore, we can conclude that the receptor was highly purified and effectively reconstituted using the detergent micelles. Thus, the purified and reconstituted hOR can be used in various fields, like for analyzing the receptor structure, monitoring the ligand binding in real time, and bio-sensing systems conjugated with nano-scale devices.

## 4. Conclusion

We successfully overexpressed the GST-hOR2AG1-6xHis at high-levels in *E. coli*. The GST domain of the fusion

protein was removed after proteolytic cleavage, and high-purity hOR2AG1 was obtained. Purified hOR was correctly reconstituted in detergent micelles and the biological characteristics were confirmed by CD spectrum analysis and the tryptophan fluorescence quenching method. As a result, hOR was shown to regain its native structure with high selectivity. This work can be broadly applied to the production of hORs in *E. coli* for use in hOR-based biochemical, biophysical and structural analysis. In addition, GPCRs including the ORs produced by this method can be utilized as recognition elements for protein-based biosensors by integration with sensor platforms.

## Acknowledgements

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