Synthesis and Self-Assembly of His-tag Hybrid of Substrate-Binding Short Domain in Transient Receptor Potential Vanilloid Type 1 for Vanillin Sensing Application

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The amino acid sequence of vanillin-binding site of transient receptor potential vanilloid type 1 from rat, Leu544-Tyr553, was extracted and hybridized with His-tag. The hexadecamer invariant chain peptide, Leu-Ala-Met-Gly-Trp-Thr-Asn-Met-Leu-Tyr-His-His-His-His-His-His (VBH), was prepared by solid-phase peptide synthesis. Circular dichroic spectral measurements determined the α-helix content to be 17%, which was consistent to that of short peptides. In a combined use of thiol-derivatized nitrilotriacetic acid (s-NTA) monolayers, the His-tag successfully attached the whole peptide on gold substrate surfaces through Ni²⁺-chelation (Γ_{VBH} = 224 ± 120 pmol cm⁻², n = 8). Moreover, various surface analyses including atomic force microscopy imaging, FT-IR spectroscopy, and quartz-crystal microgravimetry (QCM) revealed self-assembly (SA) of VBH at the S-NTA monolayer surfaces. QCM measurements also showed that vanillin, the major component of natural vanilla flavoring, binds to VBH SAs (K_{app} = 2.7 × 10³ M⁻¹). The affinity of host–guest binding remains limited but possesses a certain degree of selectivity; for cases of structural analogs that give a pleasant flavor, acetophenone showed rather weak affinity (K_{app} = 2.8 × 10² M⁻¹) whereas 4-heptanone did not bind at all. With these results VBH was concluded to be useful in vanillin sensing as a supramolecular affinity host.

Key words: Transient receptor potential channel, synthetic peptide, self-assembly, biosensor, vanillin.

1. INTRODUCTION

The transient receptor potential (TRP) superfamily consists of various types of cation-selective ion channels with six predicted transmembrane segments. Among the TRP family, vanilloid receptor 1 (TRPV1) is the best-characterized member[1], which can recognize pungent vanilloid molecules, noxious heat, and pH; upon the chemical/physical stimuli reception, the ion channel opens to trigger a biochemical chain event inside the cell. With the interesting sensing behavior, TRPV1 has been promoting some biosensor applications. For example Moon et al. reported capsaicin- and resiniferatoxin-sensing using TRPV1-confined vesicles[2]. Regarding the host-guest interaction, Gavva et al have clarified that Met547 and Thr550 are essential for vanilloid reception by single point mutations and have proposed a docking structure through the non-covalent interactions with the amino-acid residues involving Tyr511 and Trp549[3]. Additionally, Liao et al have determined the 3D-structure of TRPV1 at 3.4 Å resolution using electron cryomicroscopy[4]. In the present paper, we extracted the vanilloid-binding domain of rat TRPV1, Leu544-Tyr553, and hybridized with His-tag for self-assembly (SA) at the transducer electrode surfaces; a hexadecamer invariant chain peptide, Leu-Ala-Met-Gly-Trp-Thr-Asn-Met-Leu-Tyr-His-His-His-His-His-His (VBH), was prepared by solid-phase peptide synthesis (SPPS). We report

![Fig. 1 The primary structure of VBH (A) and its an optimized 3D-structure (B). In the bottom panel, the immobilization method based on chelation of Ni²⁺ between chemisorbed S-NTA and His-tag hybridized VB is schematically shown.](image-url)
characterization about some fundamental properties of the peptide SAs and demonstrate a biosensing application to vanillin by taking advantage of quartz-crystal microbalance measurements.

2. EXPERIMENTAL

2.1 Chemicals

For fmoc SPPS, Rink Amide AM resins (200–400 mesh) were purchased from Merck Millipore (Darmstadt, Germany). All fmoc protected amino acids (Fmoc-AA-OH) and the SPPS-related reagents including piperdine, O-[1H-benztetrazol-1-yl]-N,N,N',N'- tetramethyluronium hexafluorophosphate (HBTU), N-hydroxybenzotriazole (HOBr), disopropylethylamine (DIEA), and N-methylpyrrolidone (NMP), were obtained from Watanabe Chemical Industries, Ltd (Hiroshima, Japan). Similarly trifluoroacetic acid (TFA), triisopropylsilane (TIS), and 1,2-ethylenedithiol (EDT) were supplied from Wako Pure Chemical Industries Ltd (Osaka, Japan). For the Ni²⁺-chelator at gold-substrate binding at the gold-film surfaces deposited on the quartz-tips.

2.2 SPPS with Microwave Synthesis Protocol

The peptide compounds were prepared using a Discover® SP microwave synthesis system (CEM Co, Matthews, NC, USA)[6]. In the SPPS synthesis cycle, deprotection was performed using 20% piperidine/DMF in two stages with an initial deprotection of 60 s followed by 3 min at 75 °C. For coupling reaction, 3-fold excess of Fmoc-AA-OH was reacted with the activation method using HBTU/HOBt for 5 min at either 50 °C (Fmoc-Cyc-OH and Fmoc-His-OH) or 75 °C (the rest of the fmoc AAs). After the peptide sequence preparation, acetylation blocked the N-terminal amine (5 min at RT) and TFA/TIS/EDT cleavage solution gave the final product (30 min at RT). The microwave system was operated at 20 W all through the experiments. The obtained crude peptide was chromatographically purified on a C18-column using AcCN/0.05%–TFA as eluent, and the peptide fractions were collected for lyophilization. MALDI TOFF-MS using u-cyano-4-hydroxycinnamic acid as matrix analyzed the obtained products; m/z 2063.996 (M⁺. C₃₀H₁₂₁N₃₀O₂₁S₂ requires 2063.92); 2086.026 (M⁺. C₃₁H₁₂₅N₃₀NaO₂₁S₂ requires 2085.90).

2.3 Instrumentation

Peptide geometry was assessed by a semi-empirical method (PM3) using HyperChem® professional software package ver. 8. Fluorometric studies of host-guest interaction between the peptide hosts and vanillin in homogeneous solution were made using a RF-5300 PC spectrophotometer (Shimadzu Co, Kyoto, Japan). Circular dichroic spectra were acquired on a J-1500 spectropolarimeter (JASCO Co, Tokyo, Japan). For characterization of the peptide SAs, a multimode atomic force microscope, Nanoscope IV (Digital Instruments, Santa Barbara, CA, USA) observed the surface topographic images whereas an FT-IR 620/N spectrometer (JASCO Co, Tokyo, Japan) recorded the infrared reflection-absorption spectra. A QCA 934 Quartz Crystal Resonator Biosensing System (SEIKO EG&G Co, Ltd, Tokyo, Japan) determined the amounts of VBH adsorption as well as the guest-molecule binding at the gold-film surfaces deposited on the quartz-tips.

2.4 Preparation of VBH SAs and Procedure of Measurement[7]

For AFM, evaporated gold films having (111) texture (Agilent Technologies Inc., Santa Clara, CA, USA) were used. The substrate was immersed into S-NTA solution (10 mM in 50% EtOH) for 24 h at room temperature (RT) and then, by casting a 100-μL portion of 5 mM NiCl₂ solution onto the surface, was subjected for Ni²⁺ chelation for 1h at RT. Similarly VBH immobilization was made by exposing the substrate to a 100-μL portion of the solution (5 mM in 50%-DMSO/TE buffer) for 3 h at RT. The substrate was rinsed thoroughly with deionized water and dried under vacuum for 1 h. Tapping mode AFM using NCHR-10 cantilevers (Nanoworld AG, Neuchâtel, CH) allowed high-resolution topographic imaging of the sample surfaces. For IR, polycrystalline Au films resistively deposited onto TEMPAK Float® glass-plates were used (Kinoene Kogaku, Tokyo, Japan). The substrate was first treated by chemical etch using piranha solution (caution! Piranha solution is a strong oxidizing agent and extreme care is necessary), and VBH immobilization was done using the same procedure described above. For microgravimetric analysis, commercially available Au quartz crystals (AT-cut, 9 MHz) were used. The tips were first subjected for the piranha etches and then, mounted in a well-shaped PTFE quartz-tips.

3. RESULTS AND DISCUSSION

3.1 Characterization of VBH in Homogeneous System

As shown by MAIDI TOFF-MS, the microwave-assisted SPPS reported satisfactory outcomes. We found that, in light of our past experiences, the method generally gave the particular product in good yield and importantly within a short period of time compared to the conventional method. We routinely used the microwave-assisted SPPS for the preparation of the peptide host molecules.

With the constituent amino acids, i.e., Trp and Tyr, VBH gave strong fluorescence emission at 362 nm (λex = 280 nm). Since Trp’s primary sources for vanillin reception of TRPV1, the spectral feature of VBH will be
a new basis of fluorescent method of vanilloids by taking advantages of fluorescence resonance energy transfer. Regarding the steric aspects of guest binding, \textit{VBH} necessary takes helical structure and maintains it for specified time. CD spectral measurements gave data that ensures the requirement (Fig. 2). One can find two negative shoulders at 222 and 208 nm and additionally, an intense positive band at 192 nm, all of which are typical to \(\alpha\)-helical structure of the peptide bonds. The specific intensity ratio, \(\theta[220] / \theta[208]\) was found to be 0.85; the somewhat smaller data in comparison to that for pure \(\alpha\)-helix, \(\theta[220] / \theta[208] = 1\), implies the presence of another conformers. The His-tag sequence that possibly takes random structure was though to be the cause of weaker helicity. By using an empirical formula[8],

\[
\max[\theta]_{222} = -40,000 \times [1 - (2.5 / n)], \quad \text{where} \quad n = 16
\]

the mean residual ellipticity at 222 nm, 5428 deg cm\(^2\) dmol\(^{-1}\), gives the \(\alpha\)-helical content to be 17\%. The result was almost consistent to those for short peptides, typically ranging from 2.4\% to 25\%[9]. As seen in Fig. 1, a theoretical 3D structure of \textit{VBH} well depicts the experimentally observed results.

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Fig. 2. A representative CD spectrum observed for \textit{VBH} in homogeneous solution. The sample solution was 0.1 mg/mL \textit{VBH} in 50\%-TFE/TE (pH 8).

3.2 Molecular Aggregation of \textit{VBH} and Characterization

Fig. 3 shows typical surface topographic images obtained by the tapping-mode AFM measurement. Neither of the samples having the S-NTA/Au interfacial structure nor the \textit{VBH}/S-NTA/Au gave any local order in their AFM images; the results were consistent with a well-packed monolayer formation by the adsorbate. The displays of atomically flat Au(111) grains, on the other hand, proved differences. Namely well-defined step edges with the terrace structure blurred to a considerable extent after \textit{VBH} immobilization. The surface-attached \textit{VBH} molecules, which are mechanically flexible, might deform upon contacts with the AFM-tip leading the indistinct outlines for themselves. AFM imaging in a liquid environment may prevent the effect and therefore we will examine sometimes in the future.

IR-RAS measurements also characterized the \textit{VBH} SA by showing intense an IR band at around 1700 cm\(^{-1}\) – 1640 cm\(^{-1}\), which consisted of a couple of peaks (data are not shown). They were definitely attributed to the amide bonds containing in the polypeptide chain. We further characterized the absorption by quantitative curve fitting analysis; the particular absorption gives roughly six-component peaks including 1698 cm\(^{-1}\), 1673 cm\(^{-1}\), and 1652 cm\(^{-1}\) that will be ascribed to sheet, turn, and helix structures, respectively. Preliminary analysis of the specific peak area revealed that the helix content reached up to 40\% for \textit{VBH} in the SA-state.
one-half of the experimental ones. Stable Ni\(^{2+}\)-chelation between the NTA-moiety and the His-tag might ease the steric restrictions and let VBH molecules to bind to NTA-SA stoichiometrically. At last, Ni\(^{2+}\)/S-NTA complexation resulted in considerably large excess-adsorption, typically eightfold or more, with reference to that of the surface-attached S-NTA. Yet it has not been known exactly, but any chemical reaction might deposit Ni in some-sort of multilayer structure at the Au surfaces.

![Graph](image_url)

**Fig. 4** A representative time-course QCM analysis for a set of treatments and the vanillin-response obtained for the VBH/S-NTA/Au-tip. The encircled area is enlarged in the bottom panel. The tip was placed in 200-µL of deionized water and then, a 2-µL portion was replaced by (1) S-NTA solution and subsequently with (2) Ni\(^{2+}\) solution. After the solution was renewed to 5%-DMSO/TE (pH 8), a 10-µL portion was replaced with either of (3) VBH solution or (4) vanillin solution.

3.4 Host-Guest Binding of Vanillin and Comparison with Different Ketones

In **Fig. 4** a typical QCM response to vanillin is also shown. Due to its rather small molecular weight, the frequency responses remain limited but are fairly evidently. The amount of binding of vanillin was determined to be 57 pmol cm\(^{-2}\), which is equivalent to 66% of surface-attached VBH. Simply assuming a one-to-one binding, the specific vanillin concentration in the measurement solution (\(C_{\text{vanillin}} = 0.725 \text{ mM}\)) leads an apparent binding constant of \(2.7 \times 10^7 \text{ M}^{-1}\).

\[
K_{\text{app}} = \frac{[\text{VBH-vanillin}]_s}{([\text{VBH}]_s \times [\text{vanillin}]_s)}
\]

where subscript s denotes the immobilized state. For control, a Au-tip without VBH treatment did not give vanillin response at all. For comparison, two types of ketones including acetophenone and 4-heptanone were also examined; the former provides floral fragrance whereas pleasant scent later case is. Although they display similar odor characteristics, it was within 17\% \((K_{\text{app}} = 2.8 \times 10^5 \text{ M}^{-1})\) for acetophenone. Moreover, 4-heptanone did not bind at all. Unfortunately, our achievement described here remains exploratory study since various chemicals other than vanilloids also bind with TRPV1, e.g., anandamide, 2E-(2,3-dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-propenamide, and 2-aminoethoxydiphenylborane; further experiments will be necessary.

Currently vanillin has been used for the major component of natural vanilla flavoring and therefore, its analysis method has become considerably important. A variety of instrumental analysis [10–13] including electrochemical methods has been developed ever. We hope that our new affinity peptide host will find various applications in chemical sensing of vanillin.

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4. REFERENCES


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