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Short communication

Synthesis of an immobilized *Bombyx mori* pheromone-binding protein liquid chromatography stationary phase☆

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Abstract

The pheromone-binding protein from the silkworm moth, *Bombyx mori* (BmorPBP) has been covalently bonded to a liquid chromatographic stationary phase. The resulting column was evaluated using radiolabeled bombykol and the immobilized protein retained its ability to bind this ligand. The data also demonstrate that the BmorPBP column was able to distinguish between four compounds, and rank them in their relative order of affinity for the protein from highest to lowest: bombykol > bombykal > 1-hexadecanol > (*Z,E*)-5,7-dodecadien-1-ol, and that the immobilized BmorPBP retained its pH-dependent conformational mobility.

The results of this study demonstrate that pheromone-binding protein from the silkworm moth, *Bombyx mori* and an odorant binding protein (OBP) obtained from the female mosquito *Culex quinquefasciatoes* have been immobilized on a silica support with retention of ligand-binding activity. The data indicate that proteins from non-mammalian organisms can be used to create liquid chromatography affinity columns.
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1. Introduction

Life on the insect scale is a chemical dominion; therefore, insects rely on pheromones to communicate, and their senses of smell and taste dominate the search for both plant and animal hosts. Pheromone and odorant binding proteins (PBPs and OBPs) are a family of small soluble proteins (15 kDa) containing six α-helices enclosing a hydrophobic ligand-binding pocket [1]. These proteins appear to be the major link between the external universe of chemical signals to which insects are sensitive, and the odorant receptors (ORs) embedded in antennal neurons [2,3].

The identification and characterization of OBPs has led to speculation that these proteins may be fruitful research targets in the design of novel insect control tactics, particularly against disease-transmitting mosquitoes [1]. One approach to the development of a screen for potential attractants, pheromones, and repellents is the development of liquid chromatographic stationary phases containing the immobilized OBP or PBP target. The resulting chromatography columns can then be used in online screens. This approach has been suggested by the use of immobilized protein-based liquid chromatography columns in protein-binding studies, which have been prepared from mammalian serum albumins and α_1-acid glycoprotein (for a recent review see Ref. [4]). Drug receptors and transporters have also been immobilized and used in affinity screens [5]. However, to our knowledge, insect-derived binding proteins have not been immobilized and used in affinity chromatography studies.

This manuscript reports the initial synthesis and characterization of a liquid chromatography stationary phase containing an immobilized PBP obtained from the silkworm moth, *Bombyx mori*, the BmorPBP. A second insect-derived prortein, an odorant binding protein (OBP) obtained from the female mosquito *Culex quinquefasciatoes*, has also been immobilized with retention of binding activity.

☆ Mention of commercial products does not constitute an endorsement by USDA.

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2. Experimental

2.1. Reagents

2,4,6-Trichloro-1,3,5-triazine (cyanuric chloride), 3-aminopropyltriethoxysilane (APTS), sodium phosphate monohydrate, potassium chloride and glycine ethyl ester were obtained from Aldrich Chemical Company (Milwaukee, WI). Dibasic sodium phosphate (Anhydrous Sigma Ultra) was purchased from Sigma Chemical Company (St. Louis, MO).

2.2. Expression of pheromone and odorant binding proteins

The BmorPBP was produced by recombinant techniques following a previously described method [6] and the odorant binding protein (OBP) obtained from the female mosquito *Culex quinquefasciatoes* was also obtained following previously described procedures [7].

2.3. Immobilization of BmPBP

The immobilization was accomplished following a previously described procedure used with bovine serum albumin [8] in which 50 mg of silica (5 μm, 300 Å) from Chrom Expert (Sacramento, CA, USA) was activated with cyanuric chloride, packed in a HR-5/2 glass column to yield a 3 mm × 5 mm (i.d.) chromatographic bed and the resulting column was linked to a peristaltic pump (Minipulse 2, Rainin, Woburn, MA, USA). A solution of phosphate buffer (50 mM, pH 7.0) was delivered through the column at 50 μl/min for 1 h, followed by 1.6 mg of BmorPBP in 1 ml phosphate buffer (50 mM, pH 7.0), which was recycled through the column at 25 °C for 8 h. The column was then washed with an aqueous glycine ethyl ester solution (1%, w/w, pH 6.7) at 25 °C for 2 h, in order to cap any unreacted groups. A second batch of activated silica was only capped with glycine ethyl ester and used to create a control column.

2.3.1. Immobilization of OBP

The immobilization was carried out following the protocol for the BmPBP. The cyanuric acid activated silica was packed in a HR-5/2 glass column to yield a 3 mm × 5 mm (i.d.) chromatographic bed and the resulting column was linked to a peristaltic pump (Minipulse 2, Rainin, Woburn, MA). A solution of phosphate buffer (50 mM, pH 7.0) was delivered through the column at 50 μl/min for 1 h, followed by 1.5 mg of OBP in 1 ml phosphate buffer (50 mM, pH 7.0), which was recycled through the column at 25 °C for 4 h. The column was then washed with an aqueous glycine ethyl ester solution (1%, w/w, pH 6.7) at 25 °C for 2 h, to cap any unreacted groups and sequentially washed with 5 ml of each of the following solutions: phosphate buffer (50 mM, pH 7.0); phosphate buffer (25 mM, pH 7.0) containing 25 mM NaCl; deionized water; phosphate buffer (50 mM, pH 7.0). The column was then stored at 4 °C until further use.

2.4. Protein assay

A Micro BCA Protein Assay Kit® from Pierce (Rockford, IL, USA) was used to determine that 1.63 nmol of BmPBP protein had been immobilized per mg of silica and 1.21 nmol of OBP protein had been immobilized per mg of silica.

2.5. Frontal chromatographic studies

The BmorPBP column (or the control column) was placed in a chromatographic system consisting of a LC-10AD isocratic HPLC pump purchased from Shimadzu (Columbia, MD, USA) and an IN/US system β-ram Model 3 on-line scintillation detector from IN/US (Tampa, FL, USA) with a dwell time of 2 s and running Laura Lite 3 software. The mobile phase consisted of phosphate buffer (50 mM, pH 7.0) delivered at 0.2 ml/min at room temperature. Between every injection the column was washed for 18 h with phosphate buffer (50 mM, pH 4.5) containing 50 mM potassium chloride and 7% methanol, followed by an 18 h wash with phosphate buffer (50 mM, pH 7.0).

The compounds used in this study were (*Z,E*)-10,12-hexadecadien-1-ol (bombykol), 1-hexadecanol and (*Z,E*)-5,7-dodecadien-1-ol which were provided by Dr. Ashot Khrimian (USDA-ARS CAIBL, Beltsville, MD, USA), (*Z,E*)-10,12-hexadecadien-1-al (bombykal) which was synthesized from bombykol as previously described [9] and [^{3}H]-bombykol which was obtained from the reduction of bombykal by tritium containing reagents at Amersham Life Sciences (Piscataway, NJ, USA). The binding affinity of [^{3}H]-bombykol was determined with a series of concentrations: 80, 100, 120, 150, 200, 250, and 300 pM.

2.6. Zonal chromatographic studies

The studies were carried out with CquiOBP column using the chromatographic system Series 1100 liquid chromatography/mass selective detector, LC/MSD (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum de-gasser (G 1322 A), a binary pump (1312 A), an autosampler (G1313 A) with a 20 μl injection loop, a mass selective detector, MSD (G1946 B) supplied with atmospheric pressure ionization electrospray (API-ES) and an on-line nitrogen generation system (Whatman, Haverhill, MA, USA). The chromatographic system was interfaced to a 250 Mhz Kayak XA computer (Hewlett–Packard, Palo Alto, CA, USA) running ChemStation software (Rev B.10.00, Hewlett–Packard). The mobile phase was deionized water for chiral separation of D,L-lactic acid and ammonium acetate buffer (50 mM, pH 5.0–8.0) for pH dependence and ammonium bicarbonate 10 mM for displacement studies, respectively. The flow rate was 0.2 ml/min, and the experiments were carried out at 37 °C.

3. Results and discussion

In the current study, BmorPBP was initially recycled through the activated column for 4 h and 1.07 nmol of BmorPBP was immobilized per mg of silica support (53% of starting material) which was similar to what was obtained with BSA in which 1.4 nmol of BSA were immobilized in 2 h [8]. In this study, when the recycling time was increased to 8 h the amount of protein immobilized increased to 1.63 nmol per mg of silica

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