An Odorant-binding Protein in Bovine Colostrum

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1. General features of OBP

OBP was first found in 1985 in bovine nasal mucosa as an abundant protein, which is capable of binding bell pepper odor, 2-isobutyl-3-methoxy pyridine. This protein has a hydrophobic internal cavity in the β-barrel domain, and it can bind low molecular weight (usually less than 300 Da) hydrophobic molecules in the cavity with broad specificities. Generally its affinities to the ligands are in the micromolar range. OBP has been found in the nasal mucosa of several vertebrates to date, including human beings.

OPB is a relatively small globular protein, of which molecular mass is around 19 kDa. This protein has a β-barrel core domain, which consists of 8 anti-parallel β-sheets, and has a loop domain, which consists of one alpha-helix and one beta-sheet. Generally, there are two disulphide bonds observed like this, and one of them forms a bridge between the barrel core and the loop domain. I did not mentioned in this picture, but usually there is a 3_{10}-helix exists at the N-terminal region. So far, only the bovine nasal OBP is known to lack the conserved disulphide bond, which enables the protein to form dimeric structure by so-called domain swapping. All other OBPs are supposed to be monomers.

OBP is also known to belong to the lipocalin superfamily, of which member proteins share a well-conserved 3D structure but widely distributed on their amino acid sequences and biological functions such as odorant carrier, odorant scavenger, regulator of the olfactory receptor, transducer of the olfactory receptor, and pheromone transporter. There are three conserved motifs of the lipocalins. Among the so-called kernel lipocalins, all these three motifs are conserved. On the other hand, among the outlier lipocalins, including OBPs, just one or two out of these three motifs are conserved.

2. Sensory organs in nasal cavity

There are two sensory organs in the nasal cavity, one is olfactory epithelia, OE, which is

exposed to the nasal cavity directly, and the other is vomeronasal organ, VNO that is a blind tube which is connected to the nasal cavity through a narrow canal. The odor or pheromone molecules floating in the air are aspirated into the nasal cavity, and then pass through the mucosal layer, of which depth is tens of micrometers, to reach to the olfactory receptor, which is expressed on the surface of olfactory cilia protruding from the olfactory sensory neuron. The neuron axons in OE are projected to the main olfactory bulb and those in VNO are projected to the accessory olfactory bulb. Initially, it was supposed that OE functions as odor perception, on the other hand, VNO functions as pheromone perception. However, this is still controversial. In this context, OBP is believed to promote the solubilization of hydrophobic molecules like as odor and pheromone to penetrate the mucosal layer.

3. Partial purification and characterization of OBP in bovine colostrum

Bovine colostral OBP (bcOBP) was partially purified by chloroform/methanol extraction, gel-filtration on Bio-gel P2 column, and anion-exchange chromatography on Q-sepharose. The partially purified was subjected to SDS-PAGE and peptide mass fingerprinting. The protein in the gel was assigned to be a hypothetical protein LOC517854. According to the description of this protein in the database and its sequence similarity, it turned out that this is a similar protein to bovine nasal odorant-binding protein. It is predicted that bcOBP has a putative signal sequence, which consists of sixteen N-terminal amino acid residues. The molecular weight and pI value of putative mature protein were calculated to be 17888.9 and 4.51, respectively. By BLAST homology search, two other bovine proteins which are similar to bcOBP have been found. The highest similarity, 84%, was observed on allergen bos d 2, which is known to be secreted in sweat. bcOBP shows 52% of the sequence similarity against nasal OBP. Furthermore, bcOBP possesses the conserved four cysteine residues which are related to the formation of the disulfide bonds.

4. Gene cloning and expression of bcOBP

We performed gene cloning of bcOBP by an overlap extension PCR technique. The putative signal peptides were eliminated. The recombinant bcOBP contains N- or C-terminal His_6-tag and thrombin cleavage site. Two-step purification by affinity chromatography and gel filtration gave a pure recombinant bcOBP, giving ca. 5 mg/L of the yield. The molecular weight of the recombinant bcOBP was estimated by gel-filtration on Toyopearl HW-55F column using standard proteins as described in here. As the result, the molecular weight of the recombinant bcOBP was estimated to be 35 kDa. From this result, it was suggested that the recombinant bcOBP might forms a dimmer. However, bcOBP does not lack the conserved cysteine residues, which connect the barrel core and the loop domain, indicating that bcOBP might be a monomer.

Using 1-aminoanthracene as ligand, the binding ability of the recombinant bcOBP was evaluated. The recombinant bcOBP showed around 0.3 micromole of the dissociation constant. This value is quite comparable to those of both the wild type and the recombinant bovine nasal OBP, ranging from 0.6 to 0.7 micromoles. Judging from these results, we concluded that the recombinant bcOBP was correctly folded and functionally active.

5. Establishment of hybridoma cell line producing monoclonal antibody against bcOBP

Using the recombinant bcOBP, of which His-tag was removed via thrombin cleavage site, as antigen, three 4-weeks-old balb/c female mice were immunized intraperitoneally. The tighter of the three individuals' serum increased gradually, and before the 3rd immunization, the tighter reached to the plateau, giving the maximum OD of 2.15 at the wavelength of 492 nm. Splenocytes were collected from the immunized mice and fused with myeloma cells. Single hybridoma cells which can produce good amount of antibodies were selected by limiting dilution. As the result, we successfully obtained three cell lines producing sufficient amount of the monoclonal antibody against the recombinant bcOBP. The antibody isotype was determined using this commercial kit. As indicated here, the monoclonal antibody produced was apparently IgM kappa. For further use, the monoclonal antibodies were purified from the cell culture supernatant by 80%-saturated ammonium sulfate precipitation and affinity chromatography.

6. Analysis of expression profile of bcOBP in the early lactation period

First, to confirm the binding ability of the monoclonal antibody to both the recombinant and the native bcOBP, Western blotting was performed. Furthermore, the cross-reactivity of the antibody against β-lactoglobulin isoforms, which are most abundant in whey at around the similar molecular weight of bcOBP, was elucidated at the same time. As the results, the binding of the monoclonal antibody against the recombinant and the native bcOBP in whey was obvious. Furthermore, there was no cross-reactivity against β-lactoglobulin isoforms. Unexpectedly, the positive band in whey shows higher molecular weight than that of the recombinant bcOBP.

To figure out the expression profiles of bcOBP in milk during the early lactation period, we collected milk samples over time from 5 individual cows. From the milk samples, fat and cells were removed by centrifuge, and caseins were removed by acid precipitation. The whey fractions were subjected to Western blot analysis. The concentration of bcOBP maintained until 14 hours after the first milking, and then drastically decreased. After 48 hours, the concentration became about 10-fold less than that of the maximum. Afterwards, no significant change was observed up to 240 hours. From this observation, it is suggesteed that bcOBP presumably play biologically significant roles in a quite early lactation period. According to the standard curve which was obtained using the recombinant bcOBP, the concentration of the native bcOBP in the

colostrum of the first milking, here, was estimated to be about 7.5 mg/L.

7. Possible biological roles of bcOBP

Assuming from the previous studies, OBP most presumably acts as a pheromone carrier. In this context, the expression profile of bcOBP may support the hypotheses that it may functions as a mediator of the establishment of appropriate relationship between mother and her neonate through the individual recognition immediately after birth, or that it may evoke suckling behavior of the neonate.

8. Summary

We confirmed the presence of OBP in milk by Western blot analysis. The concentration of bcOBP in the first milking was estimated to be 7.5 mg/L. bcOBP concentration drastically decreased about 10 times less in 48 hours of the first milking. The biological roles of bcOBP still remain to be elucidated.