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## Putative Roles of Bovine Colostral Odorant-binding Protein (bcOBP) for Pheromone Transport and Sexual Behavior

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### ABSTRACT

A novel protein, bovine colostral Odorant-Binding Protein (bcOBP) was found in bovine colostrums. This protein belongs to the lipocalin superfamily, most of them are secretory proteins. Amino acid sequences of bcOBP have CxxxC motif, which is well conserved among mammalian pheromone binding proteins. Its biological function is unclear, but pheromone transport is considered. Vomeronasal organ, whose important role is supposed to be in pheromone detection was suspected to express bcOBP mRNAs. The expression was found in vomeronasal organ, nasal mucosa and olfactory epithelium. Surprisingly, the expression was also found in lacrimal gland. The presence of bcOBP mRNAs in the area around the olfactory sense and in lacrimal gland might imply a functional role on pheromone transport and on sexual behavior between male and female like Exocrine gland-secreting peptide 1 (ESP1), respectively.

**Key words:** Animal behavior, CxxxC motif, milk science, biochemistry, cow

### INTRODUCTION

Olfaction involves the binding of small, hydrophobic, volatile molecules to receptors of the nasal neuroepithelia (Buck and Axel, 1991). It generates a cascade of neurological events that transmit the information to the olfactory bulbs projecting into the brain. The first step in this process is the solubilization of these hydrophobic molecules in the hydrophilic nasal mucus. Odorant-Binding Proteins (OBPs) are thought to transport these molecules within the mucus (Pelosi, 1996). The OBPs were first identified in the nasal mucosa (Pelosi *et al.*, 1982; Bignetti *et al.*, 1985; Pevsner *et al.*, 1985) in which human was able to find OBPs bind volatile compounds and expressed.

These proteins belong to the lipocalin superfamily of which biochemical structures are well suited for this function. This superfamily comprises >100 small proteins secreted in various biological fluids (Pervaiz and Brew, 1987). They contain eight consecutive  $\beta$ -sheets forming a barrel-shaped fold in which hydrophobic pocket is present (Flower, 1996).

Lipocalins in the mucus of the oral and nasal sphere epithelia act as biosensor proteins for the detection of environmental signals, especially pheromones. Odorants, which are chemically diverse, are distinguished at the neuroepithelium level using combinations of hundreds of receptors (Malnic *et al.*, 1999). Some lipocalins specifically bind volatile pheromones, which can be regarded as a sub-class of odorants. These proteins are the mouse and rat urinary proteins, the hamster aphrodisin and salivary lipocalins in mouse and pig (Bocskei *et al.*, 1992; Singer and Macrides, 1993; Marchese *et al.*, 1998).

In 2009, a novel protein in bovine colostrum was found by Japanese research groups, which named bovine colostrum Odorant-Binding Protein (bcOBP) (Fukuda *et al.*, 2009). This protein, which consists of 172 amino acid residues including a putative 16 AA signal peptide, belongs to lipocalin superfamily considered as secretory proteins (Fukuda *et al.*, 2009). The OBP derived from nasal mucosa showed low sequences similarity (52%) to bcOBP (Fukuda *et al.*, 2009). Amino acid sequences of bcOBP have CxxxC motif, which is well conserved among mammalian pheromone binding proteins (Vincent *et al.*, 2001; Srikantan *et al.*, 2005; Stopkova *et al.*, 2009). They proposed hypothesis that the function of bcOBP might be pheromone transport (Fukuda *et al.*, 2009) and hence expected that vomeronasal organ (VNO) express bcOBP mRNAs.

## MATERIALS AND METHODS

**RNA isolation and reverse transcription:** Total RNA was purified with Trizol reagent (Invitrogen) and treated with RQ1 RNase free DNase (Promega) to remove genomic DNA contamination. One microgram of total RNA was used for reverse transcription reaction with ReverTraAce (Toyobo) and random primer (Invitrogen), according to the manufacturer's instructions. The PCR was performed with *Ex Taq* (Takara). Quantitative Polymerase Chain Reaction (PCR) was performed with Taqman RT-PCR using *Premix Ex Taq* (Takara) and analyzed with the 7300 real-time PCR system (Applied Biosystems).

**Probe sequences used in quantitative PCR:** 6-carboxyfluorescein (FAM), 6-carboxytetramethylrhodamine  $\beta$ -actin (TAMRA), Forward, 5'-TGCCATGTATGTGGCCATCC-3'; reverse, 5'-CGCTCGGCTGTGGTGGTAA-3' probe, 5'-FAM-ACTCTGGGGACGGGGTCA-TAMRA-3' bcOBP, Forward, 5'-GCACATGCCAGTTTTTCTCA-3'; reverse, 5'-GGAATCCCCCTCTCATTGTT-3'; probe, 5'-FAM-AAGGTTCCGCCAAAGGAACCA-TAMRA-3'.

**Primers for RT-PCR:** Glycerol-3-Phosphate Dehydrogenase (G3PDH) Forward, 5'-CGACCACTTTGTCAAGCTCA-3'; reverse, 5'-TCAGGGCCTTAGAGATGGAA-3' bcOBP Forward, 5'-GCACATGCCAGTTTTTCTCA-3'; reverse, 5'-TTGGCGGAACCTTCAGTTAC-3'.

**Tissue collection:** We used female cows. All experiments were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals of the Obihiro University of Agriculture and Veterinary Medicine, Japan.

## RESULTS

**bcOBP mRNAs are expressed in the area around the olfactory sense:** There was a hypothesis that a functional role of bcOBP was considered to pheromone transport (Fukuda *et al.*, 2009). Vomeronasal organ, whose biological function is supposed to be in pheromone detection was suspected to express bcOBP mRNAs. An mRNA of bcOBP was known to be expressed in mammary glands (UniGene: Bt.90525). To investigate bcOBP mRNA expression patterns around the olfactory sense, we isolated mRNAs from bovine tissue samples. RT-PCR analysis revealed that the expression was found in vomeronasal organ (VNO), nasal mucosa and olfactory epithelium (Fig. 1). Our results supported the hypothesis that bcOBP might be involved in pheromone transport. Surprisingly, the expression was also found in lacrimal gland (Fig. 1). This result proposes the hypothesis that a functional role on sexual behavior between male and female like ESP 1.

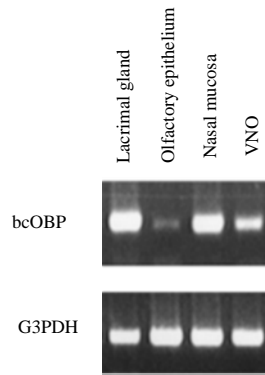


Fig. 1: RT-PCR analysis of bcOBP gene expression in several tissues. Primers for bcOBP and G3PDH are specific for transcripts from the respective endogenous locus. G3PDH was used as a positive control

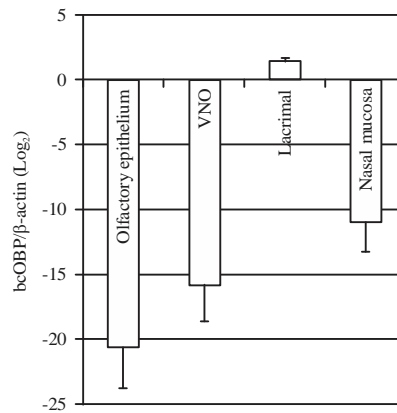


Fig. 2: Quantitative PCR analysis of bcOBP transcription in several tissues. Transcript levels were normalized to β-actin. Data is shown in the averages and the standard deviations (n = 2)

**Quantification of bcOBP mRNA expression levels:** To identify the amount of mRNA expression of bcOBP, TaqMan quantitative PCR (qPCR) was performed for the tissues in the area around the olfactory sense and for lacrimal gland. The qPCR analysis showed that the highest expression level in lacrimal gland has been observed (Fig. 2). Among olfactory sense tissues, the expressions are altered about  $\pm 5$  or  $\pm 10$  (Log<sub>2</sub> conversion) (Fig. 2). In EST profile, the expression level of bcOBP mRNAs between olfactory sense tissues and lacrimal gland showed no expression at all (UniGene: Bt.90525). Our results demonstrated that bcOBP mRNA expression levels between olfactory sense tissues and lacrimal gland were not zero, but detectable. These results support the two hypothesis, (1) bcOBP might be related to sexual behavior and (2) bcOBP might be related to pheromone transport.

## DISCUSSION

Prior to this study, a novel protein ESP1 was found in male mouse tear fluids and its biological function is to enhance female sexual receptive behavior through a specific vomeronasal receptor

(Haga *et al.*, 2010). This study provided us a new hypothesis that bcOBP might be related to sexual behavior between male and female. This study synthesized a biological model, which is consist of three steps, (1) bcOBP is expressed in female lacrimal gland, (2) Male cows recognize it in female through vomeronasal receptors and (3) Male sexual behavior will be enhanced. Our results demonstrated that bcOBP mRNA is abundantly expressed in lacrimal gland (Fig. 1 and 2), indicating that step (1) is correct. However, it was impossible to confirm step (2) and (3), because we could not obtain male cows and samples and not conduct experiments using them. Our model is still inconclusive and hence further study is required for proof of step (2) and (3).

In mammalian pheromone binding proteins (Vincent *et al.*, 2001; Srikantan *et al.*, 2005; Stopkova *et al.*, 2009), Cxxx motif, which was first discovered in insect pheromone binding proteins (Du and Prestwich, 1995), are well conserved as well as bcOBP. Those mammalian pheromone binding proteins are intended to be distributed in olfactory sense (Guiraudie-Capraz *et al.*, 2005). The distribution pattern is corresponded between bcOBP (Fig. 1 and 2) and other pheromone binding proteins, thus, bcOBP might be involved in pheromone transport.

## CONCLUSION

This study provides a basis for further research into the ligand specificities and other crucial knowledge.

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