ZOOLOGICAL SCIENCE 10: 287–294 (1993) © 1993 Zoological Society of Japan

Identification and Immunological Properties of an Olfactory System-Specific Protein in Kokanee Salmon (*Oncorhynchus nerka*)

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ABSTRACT—An olfactory system-specific protein of 24 kDa (N24) was identified in kokanee salmon (*Oncorhynchus nerka*) by the comparison of proteins restricted to the olfactory system (olfactory epithelium, olfactory nerve and olfactory bulb) with those found in other parts of the brain (telencephalon, optic tectum, cerebellum, hypothalamus) and hypophysis. A polyclonal antibody to N24 was raised in a rabbit, and the specificity of the antiserum was examined by Western blotting analysis; the antiserum recognized only one 24 kDa band in the olfactory system but not in other parts of the brain in kokanee salmon, sockeye salmon (*O. nerka*), masu salmon (*O. masou*) and chum salmon (*O. keta*). Immunocytochemical analysis revealed that positive immunoreactivity occurred exclusively in the olfactory nerve and in some olfactory neuroepithelial cells. Both at the time of imprinting of maternal stream odorants and at the time of homing to the maternal stream, the immunoreactivity of N24 in fish in the maternal stream was stronger than that in seawater fish. The present study indicates that an olfactory system-specific protein may be importantly related to both imprinting and homing mechanisms in salmonids.

INTRODUCTION

The imprinting and homing behaviors of salmon in relation to their maternal stream constitute one of the most interesting phenomena in salmon biology. Since Hasler and Wisby [10] introduced the olfactory hypothesis for anadromous salmonids, olfactory imprinting and homing mechanisms have been studied in many behavioral experiments (e.g., [9, 24]). Several neurophysiological approaches using electrophysiological techniques have also pointed to the olfactory recognition of maternal stream water during spawning migration in several salmonids [8, 26]. It is now widely accepted that in juvenile salmon the olfactory system plays important roles in imprinting of the maternal stream odorants during downstream migration, and that in adult salmon the early olfactory exposure allows recognition of the maternal stream odorants during upstream spawning migration (homing). However, few attempts have been made to investigate biochemical aspects of the olfactory system in any salmonid species.

There are numerous literatures indicating a close relation between olfactory-specific proteins and recognition and discrimination of odorants in higher vertebrates[1, 13, 16]. Several olfactory-specific proteins have been identified and used as molecular markers to study olfactory function, such as the olfactory marker protein in the mammalian olfactory bulb [11, 14], carnosin in tetrapod olfactory systems [3, 15], and olfactomedin in frog olfactory neuroepithelium [23].

The present study was desinged to compare

Accepted February 2, 1993
Received December 14, 1992
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proteins restricted to the olfactory system (olfactory epithelium, olfactory nerve and olfactory bulb) with those found in other parts of the brain (telencephalon, optic tectum, cerebellum, hypothalamus) and hypophysis of several salmonid species. In addition, two immunological analyses (Western blotting and immunocytochemistry) were conducted to investigate the possible role of an olfactory system-specific protein in olfactory functions and to evaluate its possible usage as a molecular marker in the study of olfactory mechanisms.

MATERIALS AND METHODS

Fish

Male and female mature kokanee salmon (*Oncorhynchus nerka*; land-locked sockeye salmon), 3 to 5 years old, were caught in Lake Toya from October 22 to November 19, 1991. Mature sockeye salmon (*O. nerka*) of both sexes, 3 to 5 years old, were collected from the Chitose Branch of the Hokkaido Salmon Hatchery on October 29, 1991. Adult chum salmon (*O. keta*) of both sexes, 3 to 6 years old, were captured at sea and in fresh water at two different stages of sexual maturation (pre-spawning fish about 10–20 days before final maturation and spawning fish) on September 25, 1991. Wild one-year-old juvenile masu salmon (*O. masou*) were caught once a month in the Shakotan River from November 21, 1991 to May 25, 1992.

Fish were anesthetized with 10% ethyl p-aminobenzoate, and their heads were removed by decapitation and placed on ice. Under a dissecting microscope, olfactory organs and brains were dissected into olfactory epithelium, olfactory nerve, olfactory bulb, telencephalon, optic tectum, cerebellum, hypothalamus and hypophysis. Each tissue was washed quickly in ice-cold salmon Ringer (0.15 M NaCl, 0.31 mM KCl, 0.03 mM $MgSO_4 \cdot 7H_2O$, 0.34 mM $CaCl_2 \cdot 2H_2O$, 0.4 mM glucose, 0.4 mM Hepes buffer, pH 7.5) containing aprotinin (1 μg/ml), phenylmethylsulfonyl fluoride (PMSF; 1 mM) and ethylenediaminetetraacetic acid (EDTA; 1 mM). The tissue was homogenized in about 1.5 volumes of salmon Ringer and centrifuged at 10,000$\times g$ for 10 min. The supernatant fluid was stored at -30°C and used for gel electrophoresis.

Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [12] was performed using a Protean II slab gel system (Bio-Rad, South Richmond, CA, USA). Samples containing 50 μg protein, as determined by Lowry's method, were heated at 100°C for 2 min in double volumes of SDS sample buffer containing 2-mercaptoethanol, and applied to slab gels consisting of 7.5–17.5% logarithmic gradient of polyacrylamide and 5% stacking gel. Electrophoresis was carried out at 12.5 mA for stacking gel followed at 25 mA for gradient gel. Molecular weight markers (Pharmacia Fine Chemicals, Uppsala, Sweden) for determination of low molecular weight were run in a separate lane. Gels were fixed and stained for 2 hr in 0.1% Coomassie Brilliant Blue in 5% ethanol-5% acetic acid solution, destained overnight in 10% acetic acid, soaked in 20% ethanol-5% acetic acid-2.5% glycerin, and dried with gel dryer.

Two-dimmensional (isoelectric-SDS) PAGE (2D-PAGE) [19] was also carried out to obtain the evidence whether an olfactory system-specific protein appeared single spot or not.

Antiserum production

A protein band corresponding to an olfactory system-specific protein, designated as N24, was prepared from olfactory bulbs of mature kokanee salmon of both sexes. The band was excised carefully with scissors, and eluted from gel slices using an Electro-Eluter (Bio-Rad, South Richmond, CA, USA) following the manufacturer's instructions. An elution buffer was constituted with 25 mM Tris, 192 mM glycine and 0.1% SDS, and elution was done at 10 mA for 2 hr.

A polyclonal antiserum was raised in a rabbit using lymph node injections. Initial injection of 35 μg eluted N24 was made into inguinal lymph nodes in Freund's complete adjuvant. Subsequent booster injections of 90 μg N24 emulsified with Freund's incomplete adjuvant were made intradermally into the back of rabbit at four weekly intervals. One week after the final booster injection, a test bleeding was taken from the ear vein, and the serum was assayed by Western blotting analysis.

Western blotting

Protein samples were resolved by SDS-PAGE and transferred to Immobilon PVDF membranes (Millipore, Bedford, MA, USA) by the method of Towbin *et al.* [25]. The PVDF membranes were incubated for 1 hr with 1% skim milk in Tris-buffered saline (TBS, pH 7.4) to reduce non-specific protein absorption, and allowed to react for 2 hr with polyclonal antiserum against N24 (working dilution from 1:100 to 1:5000) in a solution of 3% bovine serum albumin and 1% normal goat serum in TBS. After rinsing with TBS, the membranes were incubated for 1 hr with horseradish-peroxidase-labeled goat anti-rabbit IgG (Cappel Laboratories, West Chester, PA, USA) at a dilution of 1:1000 in TBS, and developed in 4-chloro-1-naphthol solution (60 mg in 100 ml TBS containing 0.01% H_2O_2). The reaction was stopped after 3–5 min by washing in distilled water, and the membranes were air-dried.

Immunocytochemistry

The olfactory system and other parts of the brain of mature kokanee salmon were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) for 18 hr at 4°C. After rinsing with 0.1 M phosphate buffer containing 10% sucrose, tissues were embedded in Tissue-Tek OCT compound (Miles Laboratories, Elkhart, IN, USA) and stored at −30°C, to be sectioned later at 10-μm thickness in a cryostat. Tissue sections were immersed in 0.3% hydrogen peroxide in methanol for 30 min to eliminate endogenous peroxidase activity. After rinsing with phosphate-buffered saline (PBS), sections were incubated with 1% normal goat serum in PBS for 15 min, and immunoreacted with anti-N24 serum (working dilution 1:2500 or 1:5000) for 2 hr at room temperature. The strepto-avidin-biotin complex (sABC) method (Histofine, Nichirei, Tokyo, Japan) was used for the immunocytochemistry. In order to confirm specificity, normal rabbit serum or PBS was substituted for anti-N24 serum.

RESULTS

Soluble extracts of the olfactory system and other parts of the brain from mature kokanee salmon were resolved by SDS-PAGE (Fig. 1A). Several protein bands were observed in extracts of the olfactory system but not in other parts of the brain or the hypophysis. Among these proteins, the most clearly visible protein band (arrowhead in Figure 1A), which had a molecular weight of 24 kDa, was designated N24, and appeared single spot by the analysis of 2D-PAGE (manuscript in preparation). This protein was eluted from gel slices for antibody generation.

In Western blotting analysis, a polyclonal antiserum to N24 at dilutions of 1:500 and 1:1000 recognized only the band whose position was identical to N24 in extracts of kokanee salmon olfactory bulb. At a dilution of 1:100, presumably weak non-specific reactions were observed in bands of molecular weight around 40 kDa. At a dilution of 1:5000, a faint reaction only was observed in the 24 kDa band. At a dilution of 1:2500, the antiserum reacted with the 24 kDa protein in the olfactory system, which was absent from other parts of the brain and hypophysis in kokanee salmon (Fig. 1B).

Electrophoretic patterns of soluble extracts of the olfactory system and telencephalon of chum salmon, sockeye salmon and masu salmon were similar to those of kokanee salmon, and a similar olfactory system-specific protein of molecular weight 24 kDa was recognized in all these salmonid species (Fig. 2).

During parr-smolt transformation in masu salmon, although there were no distinct changes in electrophoretic patterns of the olfactory system and telencephalon between parr and smolt, the immunoreactivity of N24 to the antiserum was more intense in parr than in smolt (Fig. 3). Similarly, during spawning migration of chum salmon from coastal sea to maternal river, electrophoretic patterns of olfactory nerve, olfactory bulb and telencephalon showed no prominent changes. However, the cross-reactivity of anti-N24 serum was different in fish from the sea and the river; the antigenicity of N24 was more intense in the maternal river fish sample (Fig. 4).

The olfactory rosette of mature kokanee salmon stained with Nissl solution showed olfactory epithelium and several branches of the olfactory

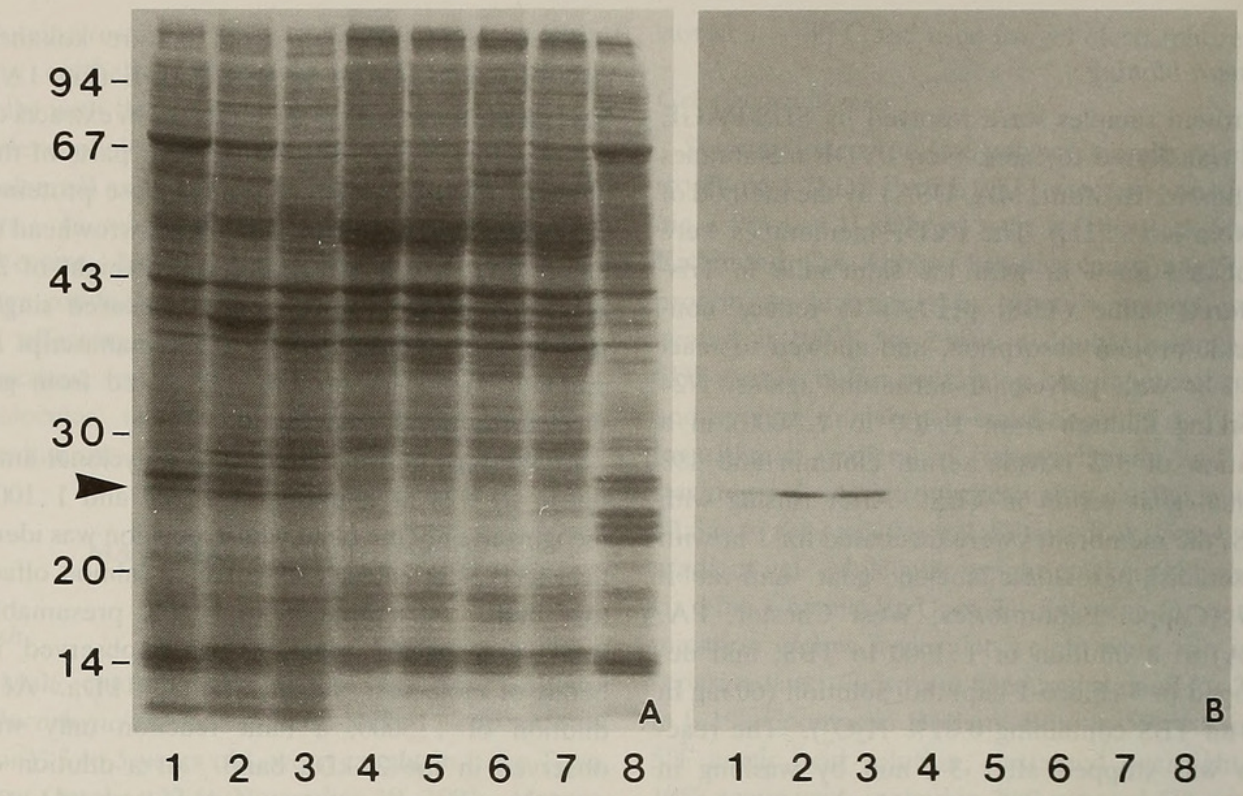

FIG. 1. SDS-PAGE (A) and Western blotting (B) of soluble extracts of the olfactory system (1, olfactory epithelium; 2, olfactory nerve; 3, olfactory bulb), other parts of the brain (4, telencephalon; 5, optic tectum; 6, cerebellum; 7, hypothalamus) and hypophysis (8) from kokanee salmon. Arrowhead in A identifies an olfactory system-specific protein of molecular weight 24 kDa (N24). Anti-N24 serum (1 : 2500) recognizes a band of molecular weight 24 kDa in the olfactory system but not in other parts of the brain. Numbers at left are Mr standards $\times 10^3$.

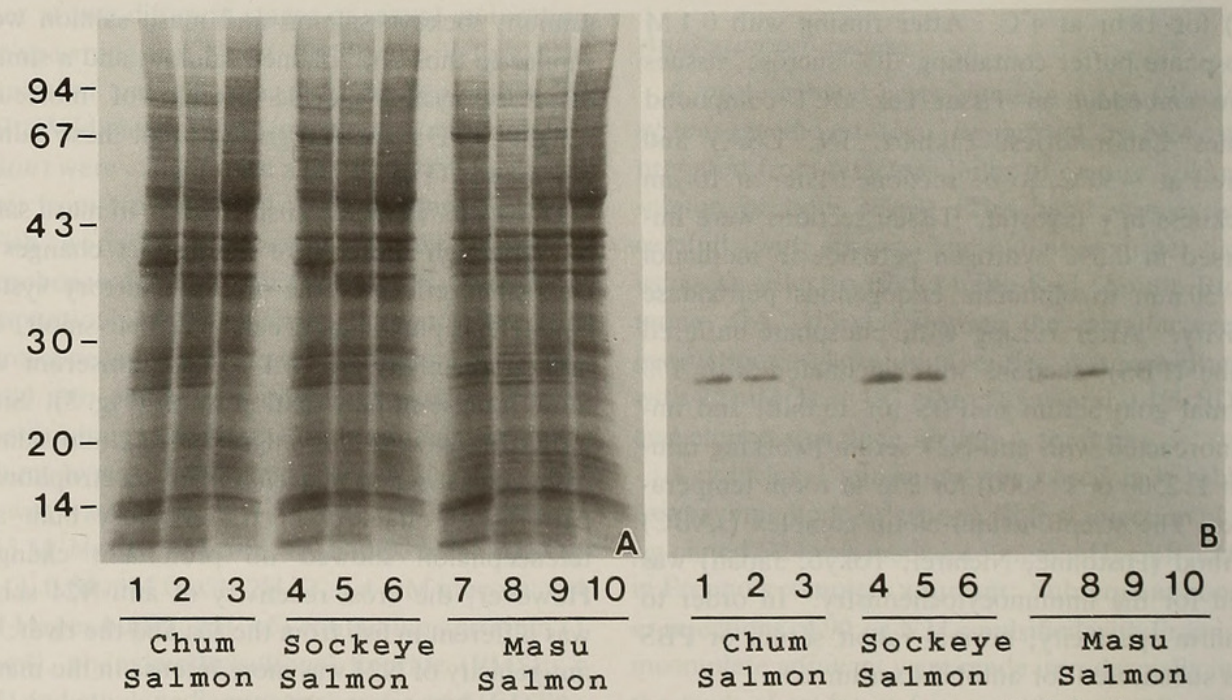

FIG. 2. SDS-PAGE (A) and Western blotting (B) of soluble extracts of the olfactory system (1, 4, 8, olfactory nerve; 2, 5, 9, olfactory bulb; 7, olfactory epithelium) and telencephalon (3, 6, 10) from chum salmon, sockeye salmon and masu salmon. Anti-N24 serum (1 : 2500) recognizes a band of molecular weight 24 kDa only in the olfactory system. Numbers at left are Mr standards $\times 10^3$.

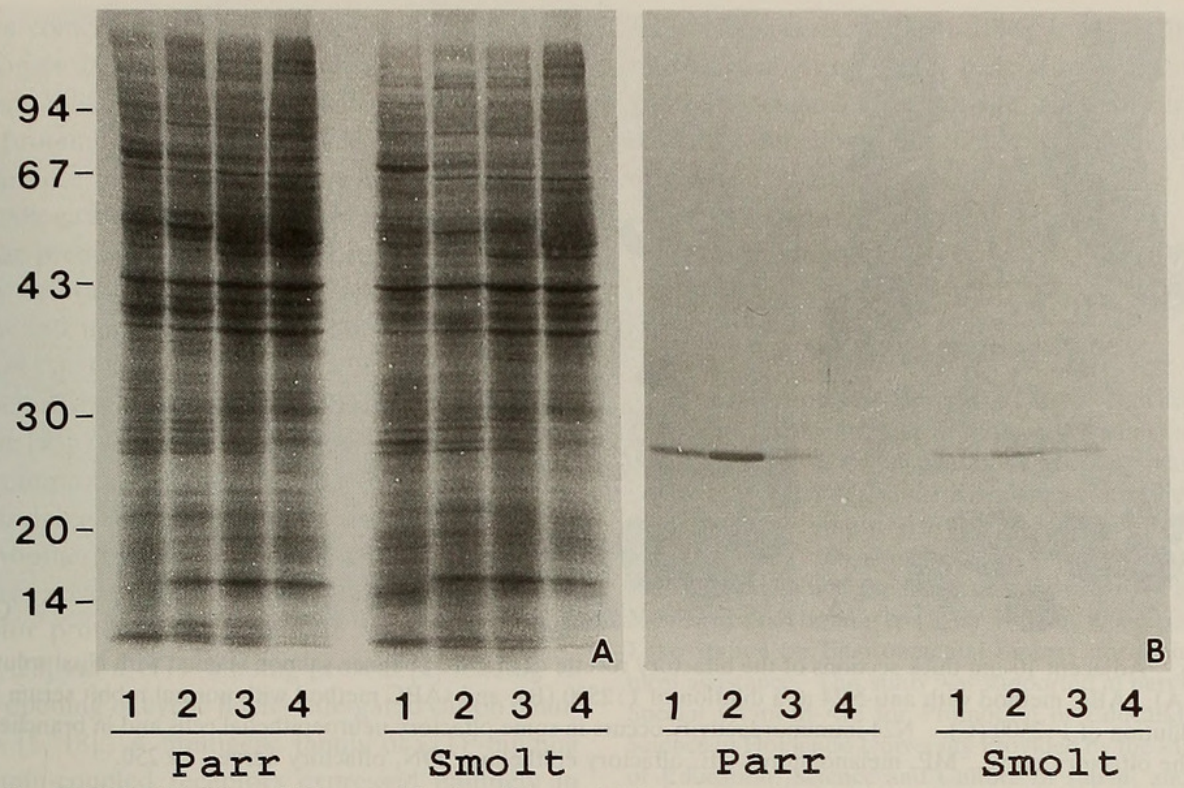

FIG. 3. SDS-PAGE (A) and Western blotting (B) of soluble extracts of the olfactory nerve system (1, olfactory epithelium; 2, olfactory nerve; 3, olfactory bulb) and telencephalon (4) in masu salmon during parr-smolt transformation. The immunoreactivity of anti-N24 serum (1:2500) is more intense in parr than in smolt. Numbers at left are Mr standards $\times 10^3$.

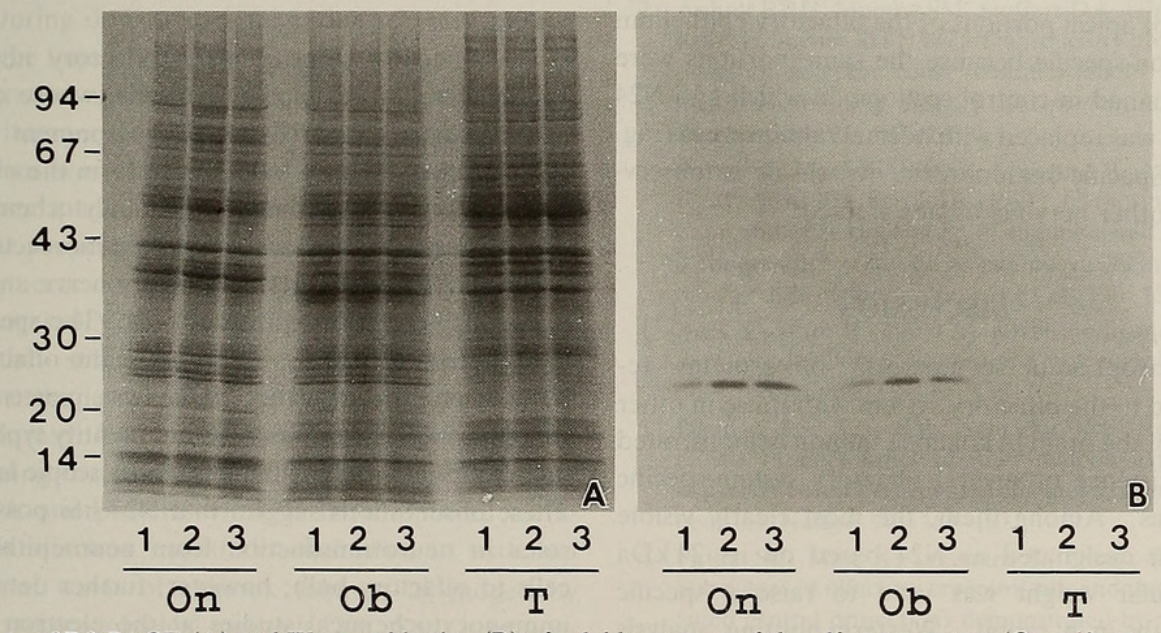

FIG. 4. SDS-PAGE (A) and Western blotting (B) of soluble extracts of the olfactory system (On, olfactory nerve; Ob, olfactory bulb) and telencephalon (T) in chum salmon during spawning migration (1, coastal sea; 2, pre-spawning fish in the maternal river; 3, spawning fish in the maternal river). The immunoreactivity of anti-N24 is more intense in fish from the maternal river than in fish from the coastal sea. Numbers at left are Mr standards $\times 10^3$.

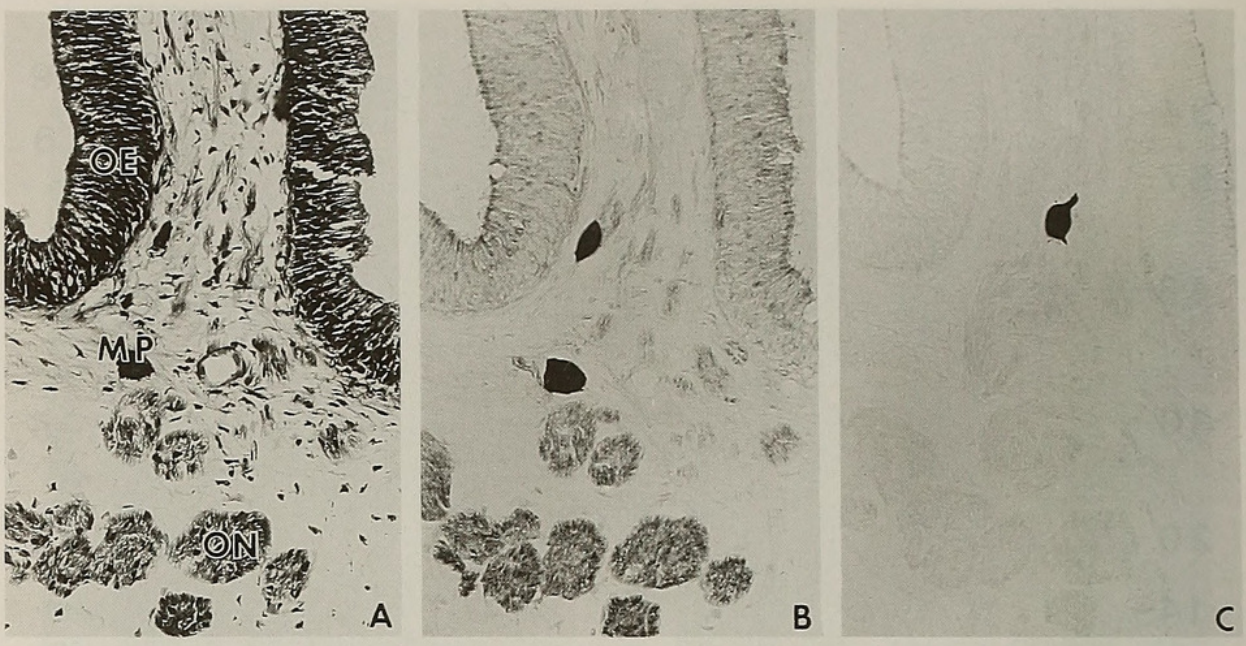

FIG. 5. Adjacent 10-μm thick sections of the olfactory rosette of mature kokanee salmon stained with Nissl solution (A), sABC method with anti-N24 at a dilution of 1:2500 (B), and sABC method with normal rabbit serum at a dilution of 1:2500 (C). N24 immunoreactivity occurs in some olfactory neuroepithelial cells and in branches of the olfactory nerve. MP, melanophore; OE, olfactory epithelium; ON, olfactory nerve. $\times$250.

nerve (Fig. 5A). The sABC method demonstrated that N24 immunoreactivity was localized exclusively in almost all branches of olfactory nerve and in some olfactory epithelial cells, presumably neuroepithelial cells (Fig. 5B). The immunoreactivity of apical portions of the olfactory epithelium was non-specific because the same portions were also stained in control sections, in which anti-N24 serum was replaced with normal rabbit serum (Fig. 5C). Specific immunoreactivity was never observed in other nervous tissues studied.

DISCUSSION

Electrophoretic comparison of proteins restricted to the olfactory system with those in other parts of the brain in kokanee salmon demonstrated the existence of several olfactory system-specific proteins. Among them, the most clearly visible protein designated as N24 based on its 24 kDa molecular weight was used to raise a specific antibody in a rabbit. Western boltting analysis revealed that the antiserum recognized only this 24 kDa band in the olfactory system, which was absent from other parts of the brain not only in kokanee salmon but also in sockeye salmon, masu salmon and chum salmon. However, no cross-reactivity of anti-N24 serum was observed in the olfactory system of carp (unpublished data). Therefore, all salmonids examined to date possess a 24 kDa protein (N24) specific to the olfactory system.

The olfactory sensory cells (olfactory neuroepithelial cells) are bipolar neurons, whose dendrites project to the external environment and axons to the olfactory bulb, which form the olfactory nerve. Preliminary immunocytochemical study showed that positive N24 immunoreactivity occurred exclusively in the olfactory nerve and in some olfactory neuroepithelial cells. The specific immunoreactivity was also found in the olfactory bulb where the olfactory nerve was penetrated (unpublished data). We could not identify types of neuroepithelial cells at the light microscopic level. These observations suggest that N24 has possible roles in neurotransduction from neuroepithelial cells to olfactory bulb; however, further detailed immunocytochemical studies at the electron microscopic level are needed to clarify this point.

A mammalian olfactory marker protein of molecular weight 20 kDa is localized in olfactory sensory neuron cell bodies and axons; its appear-

ance coincides with the establishment of sensory synapses in the olfactory bulb [7]. Although it seems unlikely that the mammalian olfactroy marker protein is identical to N24 because of the difference in their molecular weight, further immunological approaches are necessary. Carnosine is the predominant dipeptide present in the olfactory neurons and nerve in mammals, birds, reptilians and amphibians, but has not been reported in fishes [3]. Olfactomedin, a 57 kDa glycoprotein, is localized exclusively in frog olfactory neuroepithelium [23]. These olfactory-specific proteins may play important roles in chemoreception, however, their precise functions remain uncertain.

Another biochemical approach in higher vertebrates concerns the involvement of olfactory receptor proteins which activate adenylate cyclase [21, 22] *via* a GTP-binding protein [2] leading to the opening of cyclic nucleotide-gated cation channels [5, 18]. A multigene family of GTP-binding protein-coupled receptors expressed uniquely in olfactory tissues has recently been reported [4]. Immunocytochemical studies using monoclonal antibodies against rabbit olfactory bulb have demonstrated subclasses of olfactory nerve fibers and their projections to the olfactory bulb [6, 17]. These aspects need investigation in teleosts.

During downstream migration of masu salmon (at the time of imprinting) and upstream spawning migration of chum salmon (at the time of homing), the immunoreactivity of N24 in fish in the maternal stream was stronger than that in seawater fish. Either qualitative changes (including immunological properties) or quantitative changes of N24 may occur during migration. We found no evident changes in N24 or in other protein bands as judged by their staining properties with Coomassie blue. None the less, the change observed indicates that N24 may have a role in both imprinting and homing mechanisms in salmonids. Inhibitors of RNA and protein synthesis have reported to inhibit olfactory bulbar discrimination in maternal stream water of chinook salmon, as judged by electrophysiological methods [20]. So it is reasonable to consider that some specific proteins must be involved in the homing behavior of salmonids.

In conclusion, the present study identifies an olfactory system-specific protein (N24) in four salmonid species and describes some of its immunological properties. N24 may prove to be good molecular marker for the study of salmonid olfactory functions. Detailed biochemical and cytological analyses of N24 as well as the other olfactory system-specific proteins are now in progress in our laboratory.

ACKNOWLEDGMENTS

We thank Professor Howard A. Bern, University of California, for his interest in the present study and for critical reading of the manuscript. We also thank Dr Osamu Hiroi, Dr Masahide Kaeriyama, Hokkaido Salmon Hatchery, and Mr Hiroshi Kawamura, Hokkaido Fish Hatchery, for providing the chum salmon, the sockeye salmon and the masu salmon, respectively, and Mr Hiroyuki Haruna and Mrs Rirako Yamada, Toya Lake Station for Environmental Biology, for their technical assistance. This study was supported in part by the Special Grant-in-Aid for Promotion of Education and Science in Hokkaido University Provided by the Ministry of Education, Science and Culture of Japan, and by a reseach grant from the Akiyama Foundation to H.U.

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Shimizu, Munetaka et al. 1993. "Identification and Immunological Properties of an Olfactory System-Specific Protein in Kokanee Salmon : Oncorhynchus nerka." *Zoological science* 10, 287–294.

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