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Possible Involvement of Phosphatidylcholine in School Recognition in the Catfish, *Plotosus lineatus.*

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ABSTRACT—The catfish, *Plotosus lineatus*, forms a dense ball-shaped school soon after hatching. The involvement of a chemical cue(s) in this behavior was indicated from the observations that *P. lineatus* was attracted not only to seawater conditioned with the schoolmates (schoolmate seawater) but also to the skin mucus collected from the schoolmates. To determine the nature of the chemical cue, we first established a novel bioassay that monitored a characteristic 'turn behavior' toward an agar block containing skin mucus collected from the schoolmates. The bioassay-guided fractionations of skin mucus led to a final preparation wherein activity was contained in a single peak in high performance liquid chromatography on a polyamine column. The spectral data of the final preparation indicated that the purified material was a mixture of phosphatidylcholine molecular species, which was supported by the fact that the final active preparation lost the activity when treated with phospholipase A_2, indicating that the school recognition substance is degraded by phospholipase A_2. From these results, we proposed that the chemical cue to recognize the school in *P. lineatus* may be PC molecular species.

Key words: school recognition, chemical cue, bioassay-guided fractionation, phosphatidylcholine, *Plotosus lineatus*

INTRODUCTION

It is well known that kinship and familiarity play important roles in integrating social communications such as territoriality, courtship, dominance hierarchies, and group living (Hepper, 1991; Pfennig and Sherman, 1995). To classify conspecifics like 'how genetically related you are' or 'you are a family member', individuals must recognize any traits (*e.g.*, color, shape, sound, and odor etc.) of others (Sherman *et al.*, 1997). Chemically-mediated recognition (chemical communication) is the oldest method found in a wide range of organisms from bacteria to mammals (Bradbury and Vehrencamp, 1998). Numerous investigations have demonstrated the importance of chemical cues in a variety of behaviors depending on kinship or familiarity (Liley, 1982; Brown and Smith, 1994; Sun and Müller-Schwarze, 1998; Steck *et al.*, 1999; Bull *et al.*, 2000; Rasmussen and Krishnamurthy, 2000; Bull *et al.*, 2001; Porter *et al.*, 2001; Bloss *et al.*, 2002). There is no doubt that elucidating the nature of the chemical cue contributes to fuller understanding of recognition systems as well as chemoreception mechanisms.

Group living takes advantage of many adaptive functions, including anti-predator behavior, energy consumption, food location, migration, and cooperative reproduction (Bradbury and Vehrencamp, 1998). To enhance these functions, groups may be constructed on the basis of kinship or familiarity. There is considerable evidence that chemical cues are an important determinant of group membership and that conspecifics are recognized on the basis of distinctive chemical signals. For instance, social insects use cuticular hydrocarbons to recognize group members (Breed, 1998; Lahav *et al.*, 1999; Thomus *et al.*, 1999). In vertebrates, while the nature of the chemical signals (pheromones) has yet to be established, the major histocompatiblity complex (MHC) genes and the major urinary proteins (MUPs) have been implicated in pheromone production and thus a role in recognition of conspecfics (Olsén *et al.*, 1998, 2002; Hurst *et al.*, 2001 for MHC; Beynon *et al.*, 2002 for MUPs).

The catfish, *Plotosus lineatus*, forms a dense ball-shaped school soon after hatching as a clutch (Moriuchi and Dotsu, 1973), indicating that the school is initiated by sibling recognition. There have been a number of interesting reports on this phenomenon. If two different schools

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encountered and were mixed by chance, the original schools were re-formed (Kinoshita, 1975). However, genetic variation within the school increases as the fish grow (Iwasaki, 1995), suggesting that schoolmate mixing occurs. Sato (1937) and Kinoshita (1975) reported that visual cues play an important role in school formation. Chemical cues have also been implicated in school recognition (Kinoshita, 1975; Hayashi *et al.*, 1994). The evidence includes: i) *P. lineatus* is not only attracted to seawater conditioned with the schoolmates (schoolmate seawater) but also the skin mucus collected from the schoolmates; ii) *P. lineatus* could distinguish the odor of schoolmates from that of other fish (Kinoshita, 1975); and iii) anosmic fish, whose nasal cavities were plugged with dental cement, was not attracted to schoolmate seawater (Hayashi *et al.*, 1994), indicating *P. lineatus* recognize the schoolmates by olfaction. Hitherto, the chemical properties of the chemical cue (school recognition substance, SRS) have only been partially characterized: SRS is stable to heating (Kinoshita, 1975) and dialyzable through a cellulose membrane (Hayashi *et al.*, 1994).

The aim of this study is to determine the chemical nature of SRS in *P. lineatus*. To begin with, we established a novel behavioral assay to detect the activity of SRS based on characteristic 'turn behavior' demonstrated to an agar block containing the skin mucus of the schoolmates. Succeedingly, we attempted the separation of SRS by bioassay-guided fractionation of the skin mucus and the elucidation of the chemical nature of SRS by the nuclear magnetic resonance (NMR) and mass spectrometry. Furthermore, we examined the activity of the final active preparation treated with phospholipase A_2 (PLA_2).

MATERIALS AND METHODS

Collection and maintenance of the school

A school of about 450 juvenile fish (about 3 cm in total length) was collected by a landing net in Aburatsubo Inlet, Kanagawa, Japan (35° 09' 20" N, 139° 36' 49" E). The school was maintained in a 1000-l polyethylene tank with running natural seawater and fed *ad libitum* with krill, *Euphausia superba*, and Japanese sardine, *Engraulis japonica*, at the Misaki Marine Biological Station of the University of Tokyo.

Preparation of test agar blocks for behavioral assay

Skin mucus (mean volume ± s.e.=11.14±0.97 ml) was collected by rubbing the fish's body surface with Kimwipe® (Kimberly-Clark Corp., GA, USA) from all the schoolmates. A half portion of the mucus obtained was mixed with equivalent volume of agar solution [12 mg of agar (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 1 ml of distilled water], and the mixture was left to solidify into a block at room temperature. A control agar block was prepared with seawater instead of mucus. For dose-response experiments, a log dilution series of the mucus was prepared with seawater. To assay the fractions obtained during separation procedures (see **Separation of active substance by bioassay-guided fractionation**), they were dissolved in methanol (4% volume of agar block) and made up to the appropriate volumes with seawater and used to prepare the agar blocks. The control blocks were prepared with seawater containing the appropriate concentration of methanol.

Behavioral assay

An individual, chosen at random from the school, was introduced into a cream-colored rectangular plastic container (68×40×15 cm) filled to a depth of 3 cm with seawater and acclimated for 3 min. A treatment agar block (1 cm^3) was attached to one side of the container with metal wire (2×ϕ 0.1 cm) and the control agar block was similarly attached to the other side. The response of test fish to treatment and control agar blocks was video-recorded for 5 min (Fig. 1a). The positions of treatment and control agar blocks were switched every trial. The numbers of trials were given in figures. All samples were assayed blind.

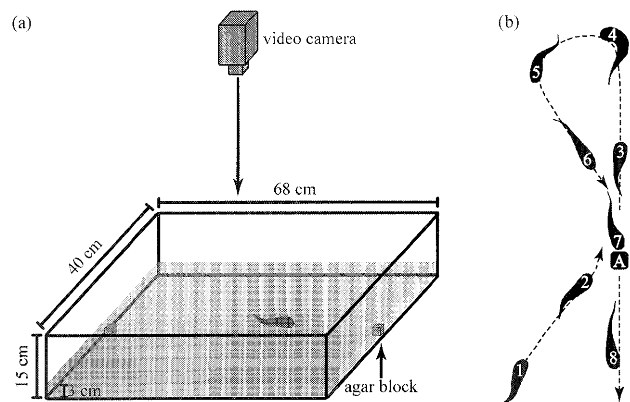

Fig. 1. Bioassay apparatus **(a)** and "turn behavior" of the catfish **(b)**. **(a)** Test agar blocks (gray cubes) were placed on the bottom of the container and fish behavior was recorded by a video camera fixed 60 cm above the tank. **(b)** Trace of fish behavior toward the agar block **(A)**. Sequential fish behavior is shown by numbering: Nos. 1 to 2, accessing the agar block; Nos. 3 to 7, turn behavior to the agar block; and No. 8, behavior after turn behavior.

Data analysis

P. lineatus showed characteristic behavior toward cue-containing agar blocks, which we called 'turn behavior'; fish returned to the agar block after first passing it by (Fig. 1b). Turn behavior was assessed by recording the behavior and analysed it during playback. We counted the number of passes of the agar block (see Fig. 1b Nos.1 and 2) and we counted the number of turns (see Fig. 1b Nos.3-7); a turn was counted only when the test fish returned to the agar block within 5 sec of passing it, except for the case of returning by the touching the sidewall after passing the agar block. From these data, the rate of turns (RT) was calculated using the following equation:

$$RT = (m / n) \times 100,$$

where m=number of turns and n=number of passes. For statistical analysis, RT was transformed to its arcsine. The transformation was performed using the equation below:

$$\text{Transformed value (TV, degree)} = \arcsin (RT / 100)^{1/2}.$$

When m was zero, the transformation was improved by replacing 0 / n with 1 / 4n (Zar, 1998). A one-sample *t*-test was used to compare TV between treatment and control agar blocks. Moreover, in order to compare the activity between each fraction, a response index (RI) was adopted. RI was calculated using the following equation:

$$RI = \{(RTT - RTC) / (RTT + RTC)\} \times 100,$$

where RTT is the RT obtained for the treatment agar block and RTC is that for the control agar block. To compare the RI of each sample, a two-sample *t*-test was used. Concentration response data were analysed using a one-way analysis of variance (one-way

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ANOVA). The statistical analysis was performed using SPSS 11.0 (SPSS Japan Inc., Tokyo, Japan).

Separation of active substance by bioassay-guided fractionation

The active substance evoking turn behavior was isolated from the skin mucus by bioassay-guided fractionation. The skin mucus, collected with Kimwipe® from all the schoolmates, was immediately extracted with ethanol. This ethanol extract was dried under vacuum and the residue was partitioned between chloroform and distilled water. Each fraction was dried and resuspended to the concentration corresponding to the skin mucus collected prior to bioassays. The active chloroform fraction was further partitioned between chloroform and 60% methanol. The chloroform layer was fractionated by centrifugal partition chromatography (CPC) (C. P. Chromatograph Model LLB-M Sanki Engineering Ltd., Kyoto, Japan) using the solvent system of *n*-heptane/dichloromethane/acetonitrile (10:3:7). Following operation in the ascending mode for 330 min at flow rate of 3 ml/min and rotor speed of 1,700 rpm, the descending mode was carried out for 120 min. Three fractions were obtained: AM, which was collected during ascending mode operation; DM1, which was eluted between 0–30 min in the descending mode; and DM2, which was eluted after 30 min in the descending mode. DM1 was separated further by high performance liquid chromatography (HPLC) on YMC Pack Polyamine II (250×20 mm I.D., Yamamura Chemical Laboratories Co., Kyoto, Japan). Elution was isocratic at 3.5 ml/min with acetonitrile/methanol/water (73:25:3). The eluates were monitored at 205 nm (UV) to obtain three fractions: BP, the fraction before the large peak; LP, the fraction of the large peak; and AP, the fraction after the large peak (Fig. 4a).

Spectral analysis of the final active preparation

The final active preparation (fraction LP) was analysed by nuclear magnetic resonance (NMR) spectroscopy on a JEOL JMN-A600 and fast atom bombardment (FAB) mass spectroscopy on a JEOL JMS-700T. NMR spectra were recorded in methanol-d_4 and chemical shifts were referenced to the signal: δ_H 3.30 ppm/δ_C 49.0 ppm. The chemical structure was assigned through one-dimensional ^1H NMR and two-dimensional correlation spectroscopy (COSY), proton-carbon hetero nuclear single quantum coherence

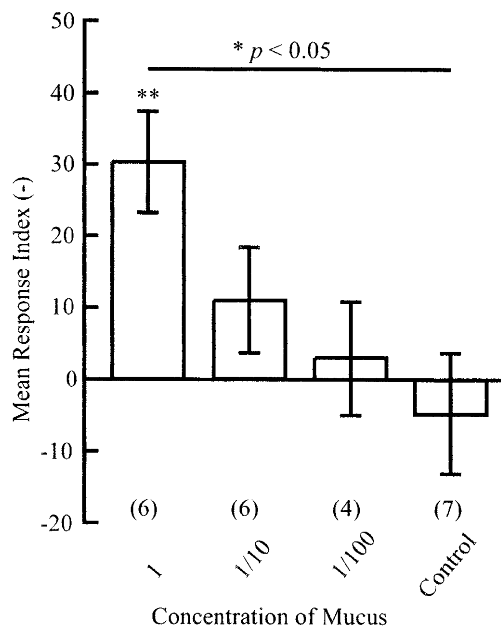

Fig. 2. Response (mean RI ± s.e.) of fish to the mucus and its dilution series. Horizontal bar and asterisks represent significant differences (*; p<0.05, **; p<0.005) based on one-way ANOVA or one-sample t-test. The numbers of trials are shown in parentheses.

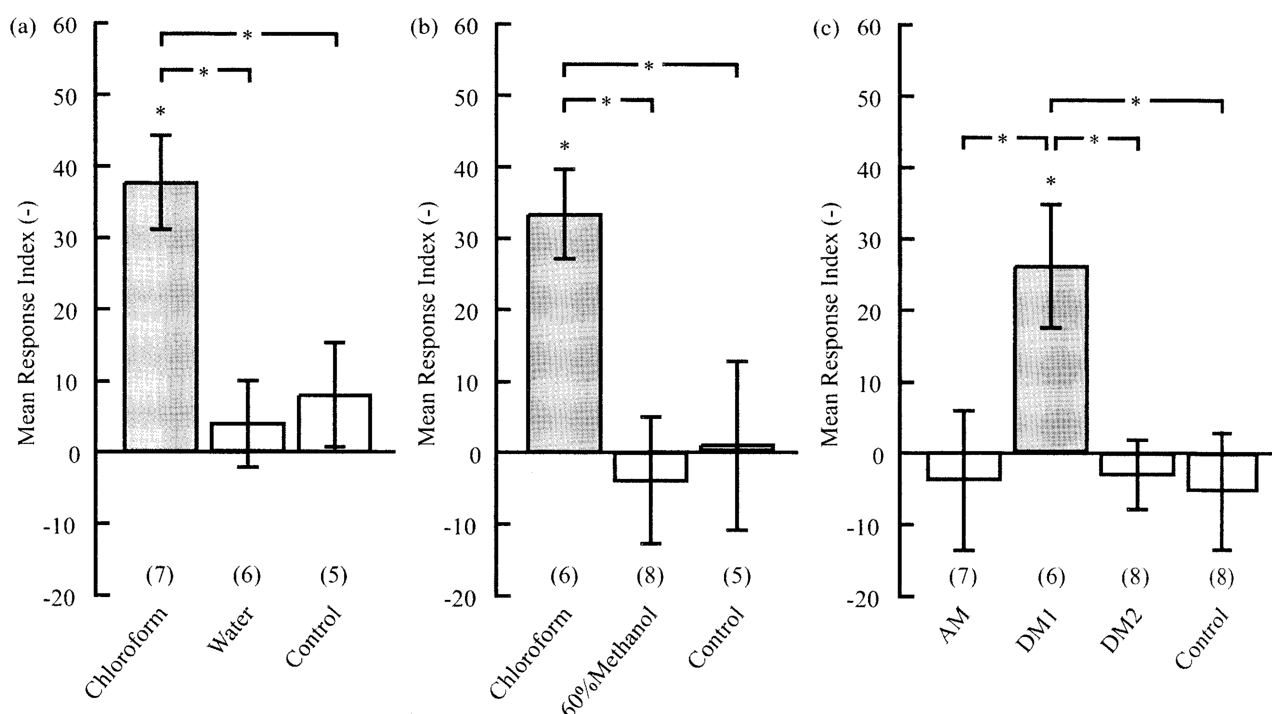

Fig. 3. Response (mean RI ± s.e.) of fish to the fractions separated from mucus. **(a)**; the activity of the fractions from first-step separation, **(b)**; second-step separation, and **(c)**; third-step separation. Grey bars mean the active fractions, which were subjected to the next fractionations. The numbers of trials are shown in parentheses. Asterisks indicate significant difference, one-sample t-test and two-sample t-test, p<0.05.

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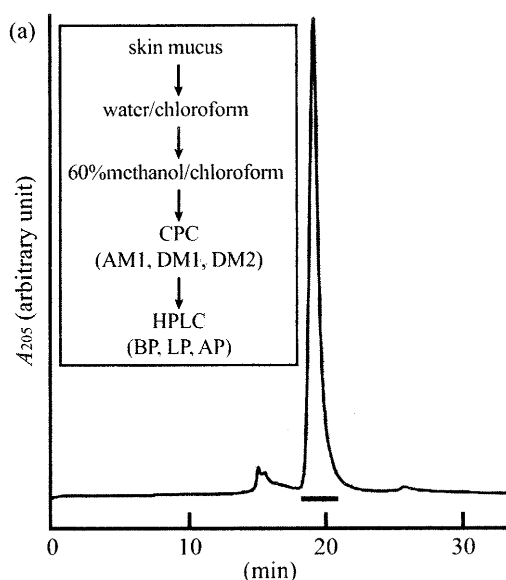
spectroscopy (HSQC), and hetero nuclear multiple bond coherence spectroscopy (HMBC) experiments. FAB mass spectra were recorded using *m*-nitrobenzyl alcohol as a matrix.



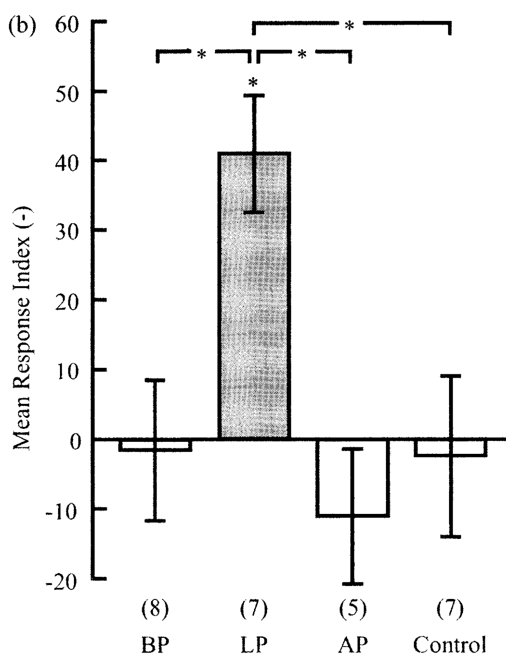


Fig. 4. Final purification by HPLC and the activity of the fractions obtained. **(a)** HPLC profile on YMC Pack Polyamine II. Large sharp peak was observed (the horizontal bar at around 20 min of elution time). Inset box represents the flow chart of the separation from the skin mucus to the final preparations by bioassay guidance. BP, LP, and AP are abbreviations for the fractions before the large peak, for the large peak, and after the large peak, respectively. **(b)** Response (mean RI ± s.e.) of fish to the fractions obtained from HPLC. The numbers of trials are shown in parentheses. Asterisks indicate significant difference, one-sample *t*-test and two-sample *t*-test, $p<0.05$.

Enzymatic Digestion of Fraction LP

Fraction LP (1–2 mg) was dissolved in 195 μl of diethyl ether/ ethanol (95:5, v/v) to which was added 5 μl of PLA_2 (bee venom, Sigma, 0.2 units/μl) suspended in buffer (220 mM NaCl/ 20 mM $CaCl_2$/1 mM EDTA/100 mM Tris HCl, pH 8.9). The mixture was incubated at 37°C for 1hr with gently stirring, and the reaction was stopped by addition of 200 μl of 4 M guanidine hydrochloride solution (GuHCl; Sigma) (LP +PLA_2). Digestion of phosphatidylcholine (PC) was confirmed by silica gel thin layer chromatography, which was developed with chloroform/methanol/water (70:30:5, v/v/v) and detected with anisaldehyde sulfate. To examine the effects of enzyme and GuHCl on the activity, the enzyme solution was added to the LP solution and immediately denatured by addition of the GuHCl solution (LP+PLA_2+ GuHCl), while the GuHCl solution was added to the LP solution (LP+GuHCl). Each sample was dried in a stream of nitrogen; this was used to prepare the agar block (0.15 mg fraction LP/ml agar block).

RESULTS

Establishment of the bioassay

In a preliminary experiment, we observed that the fish made a characteristic 'turn' behavior toward a urethane sponge containing the schoolmate seawater. After exploring various assay systems, we finally developed the present assay system using agar blocks; that is, the agar block containing the skin mucus elicited turn behavior (Fig. 1b). The fish showed more turn activity toward the mucus than toward seawater. Comparison of the turn behavior with the transformed value (TV) revealed a significant difference between the mucus and seawater (mean TVs were 39.0 for the mucus and 28.2 for the seawater; one-sample *t*-test, $p<0.005$). In contrast, under a no-choice condition, where agar block containing seawater was tested, there was no significant difference (mean TVs were 35.3 and 37.2; $p>0.1$). Furthermore, the dose-response experiments showed that lower concentrations of the mucus exhibited a progressively lower activity; statistical analysis between the doses using RI demonstrated that the activity of the mucus was dose-dependent (one-way ANOVA, $F_{3,19}=3.57$, $p<0.05$) (Fig. 2). Thus, this assay system was found to be reliable for the evaluation of turn behavior elicited by skin mucus in the catfish *P. lineatus*. In other words, school recognition activity can be detected by this bioassay.

Separation of the active substance from the skin mucus

The active substance was separated from the skin mucus by bioassay-guided fractionation (Fig. 4a inset). The ethanol extract of the mucus, collected from all the schoolmates, was initially partitioned between chloroform and water. In order to assess the activity of each fraction, we compared TV between the fraction and its blank control. The chloroform fraction (0.83 mg/ml agar block) was significantly more active than the control (mean TVs were 44.9 for the chloroform fr. and 29.0 for the control, $p<0.01$), while the water fraction and methanol (control) did not show significant differences (mean TVs were 41.9 for the water fr. and 39.5 for the control, $p>0.1$; 37.2 and 34.2 for the controls,

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$p>0.1$). Moreover, we compared the activity between each fraction using RI. The RI of the chloroform fraction exhibited significant activity against that of the water fraction ($p<0.01$) (Fig. 3a).

The active chloroform fraction was partitioned between chloroform and 60% methanol. The chloroform fraction (0.47 mg/ml agar block) was significantly more active than the control (mean TVs were 37.2 for the chloroform fr. and 25.2 for the control, $p<0.01$), whereas there were no significant differences between the 60% methanol fraction and the methanol (mean TVs were 30.1 and 31.8, $p>0.1$ for 60% methanol fr.; mean TVs were 29.9 and 28.8 for the methanol, respectively). The RI of the chloroform fraction showed significant difference from those of the 60% methanol fraction and methanol ($p<0.05$) (Fig. 3b).

The chloroform fraction was further fractionated by CPC to obtain three fractions; AM, DM1, and DM2. There was a significant difference in TV between DM1 (0.21 mg/ml agar block) and the control (mean TVs were 42.3 for DM1 and 31.1 for the control, $p<0.05$), while the other fractions were not different from the control ($p>0.1$ for AM and DM 2). DM1 showed more active than the other fractions ($p<0.05$) (Fig. 3c).

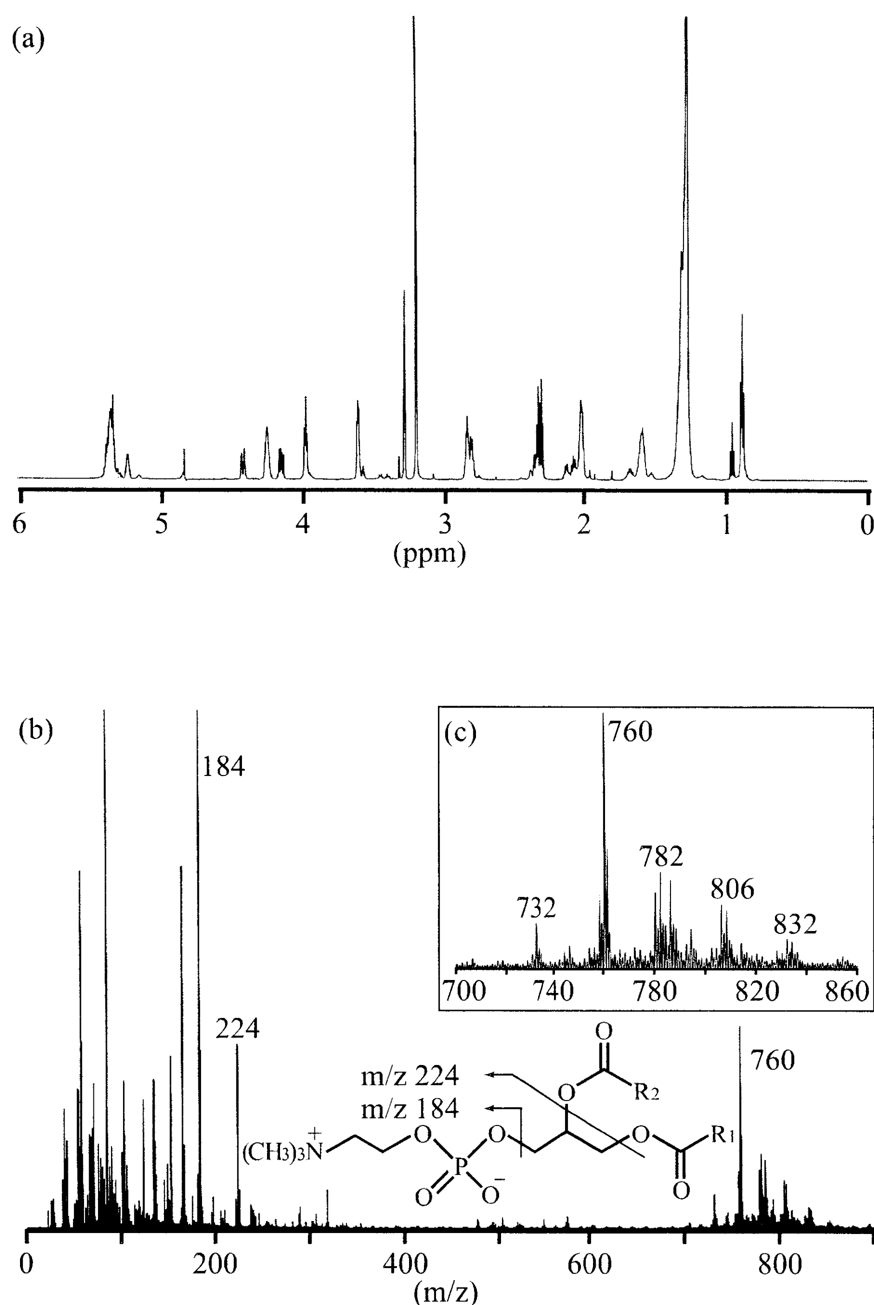

Fig. 5. ^{1}H NMR **(a)** and FAB mass spectra **(b** and **c)** of the fraction LP obtained from HPLC. Arrows in **(b)** indicate the typical fragmentation patterns. R_1 and R_2 in **(b)** mean fatty acid chains. **(c)** The enlarged spectrum of **(b)**.

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DM1 was finally purified by HPLC on a polyamine column to afford three fractions (BP, LP, and AP) (Fig. 4a). The fraction obtained as a large single peak (LP) (0.15 mg/ml agar block) was significantly more active than the control (mean TVs were 40.9 for LP and 25.3 for the control, $p<0.005$); while the other fractions and methanol did not show significant differences ($p>0.1$). Comparisons of RI revealed that LP showed more active than the other fractions ($p<0.05$) (Fig. 4b).

Characterization of the substance in the active fraction obtained from HPLC

The ^{1}H NMR spectrum of LP exhibited intense methylene (δ_H 1.20–1.40) and terminal methyl signals (δ_H 0.80–1.00), indicating the presence of long methylene chains. Signals for methylenes adjacent to carboxyl group (δ_H 2.26–2.38), *N*-methyl group (δ_H 3.2), and disubstituted olefinic strucutre (δ_H 5.32–5.40) were also observed (Fig. 5a). Analysis of the ^{13}C NMR, COSY, HSQC, and HMBC spectra led to the identification of two acyl, a glycerol, and a choline groups (data not shown). These features were reminiscent of PC. The FAB mass spectrum (positive mode) (Fig. 5b) further supported that the purified substance is a mixture of PC molecular species.

Effect of enzymatic treatment of fraction LP

The PLA_2-digested fraction LP (LP+PLA_2) was not active, whereas other controls (LP+PLA_2+GuHCl, LP+GuHCl) showed activity (one-way ANOVA, $F_{5,50}=4.23$, $p<0.01$) (Fig. 6), indicating that SRS was degraded by PLA_2.

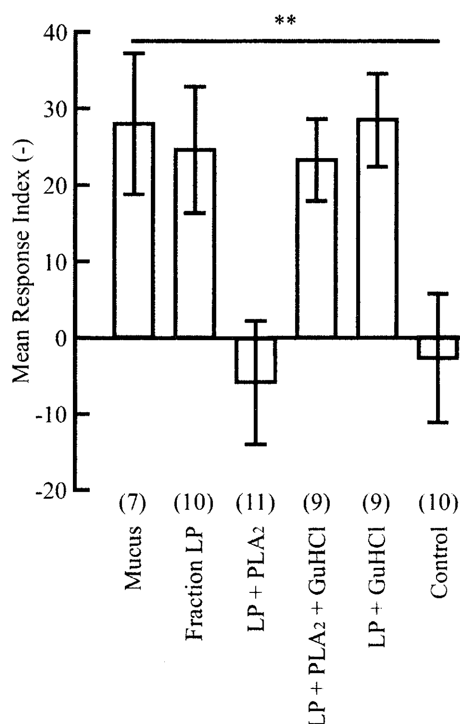

Fig. 6. Response (mean RI ± s.e.) of fish to the fractions; LP, final active preparation; LP+PLA_2, LP treated with PLA_2; LP+PLA_2+GuHCl, control for added protein effect; and LP+GuHCl, control for the denaturing step. The numbers of trials are shown in parentheses. Double asterisk indicates significant difference, one-way ANOVA, $p<0.01$.

DISCUSSION

There have been many assay systems developed to detect characteristic behaviors elicited by signal substances in various fish species (Todd *et al.*, 1967; Courtenay *et al.*, 1997; Bjerselius *et al.*, 2000; Valentinčič *et al.*, 2000; Courtenay *et al.*, 2001; Olsén *et al.*, 2002). Most of these are based on the fish's ability to discriminate chemical cues. Two-choice assay systems are most often used for this type of research. In fact, Kinoshita (1975) and Hayashi *et al.* (1994) employed two-choice bioassay with running-water (Y-maze assay system) to detect the school recognition activity of *P. lineatus*. Since this type of bioassay requires large amounts of samples, they are not suitable for isolating chemical cues from limited amounts of biological specimens. We, therefore, attempted to develop a more appropriate bioassay, resulting in the agar block assay described above. It should be noted that only one individual of *P. lineatus* is used for each assay, because they are attracted to each other by means of chemical and visual cues (Kinoshita, 1975). Agar blocks containing the skin mucus elicited 'turn behavior' in fish; typically the fish showed the turn after passing the agar block (Fig. 1b). When turn behavior was employed as a criterion of the bioassay, the mucus was significantly more active than the control. Moreover, the dose-response experiment indicated that the bioassay was capable of detecting differences in turn activity (Fig. 2), whereby enabling a comparison of fractions for activity.

The bioassay has allowed a candidate of SRS to be purified from skin mucus. All active fractions retained their initial activity throughout the fractionation steps (Figs. 3a–c and 4b); no other fractions showed activity. There is, therefore, little doubt that SRS was concentrated in the fraction LP.

Possible SRS was shown to be phosphatidylcholine (PC) by analysis of NMR and mass spectral data (Fig. 5a-c). Furthermore, FAB mass data indicated that PC was a mixture of more than ten PC molecular species; many peaks corresponding to PC molecular species were observed at around *m/z* 760 (Fig. 5c). Furthermore, the fraction LP lost the activity when treated with PLA_2 (Fig. 6), thus strongly indicating that PCs are SRS. It should be noted that egg yolk and soybean PCs did not elicit turn behavior in the catfish (data not shown). Moreover, PC was detected in schoolmate seawater (data not shown), indicating that *P. lineatus* releases PC into seawater.

During the spawning season, from June to July, a spawning pair of *P. lineatus* digs a nest on a sandy bottom under rocks in shallow water where 300-700 eggs are laid. After hatching, the juveniles stay on the bottom and start schooling within a week (Moriuchi and Dotsu, 1973). Perhaps the imprinting of the schoolmate-specific odor, in this

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case PC molecular species, occurs during the early developmental stage, which may be predicted from social behaviors in salmonid fishes (Winberg and Olsén, 1992; Courtenay *et al.*, 2001), tadpoles (Waldman, 1991), and hamsters (Todrank *et al.*, 1998). In fact, *P. lineatus* return to the original school even when two schools happen to be mixed (Kinoshita, 1975). These arguments bring us to the hypothesis that the composition of PC molecular species is different from school to school, allowing *P. lineatus* to discriminate their own school from others. A similar phenomenon has been reported for the nestmate recognition in bees (Breed, 1998) and in ants (Lahav *et al.*, 1999; Thomus *et al.*, 1999), where chemical cues are the mixture of cuticular hydrocarbons. It has been reported, however, that *P. lineatus* forgets the schoolmate odor within a short period and learn an other odor (Kinoshita, 1975), thus indicating a plastic sensitivity to schoolmate odor. Moreover, a considerable genetic variation was found within the school of grown *P. lineatus* (Iwasaki, 1994), which may indicate that the discrimination between schools is imperfect. In order to test this hypothesis, the composition of PC molecular species obtained from various schools needs to be examined. Of course, a single PC molecular species may be involved in the school recognition of *P. lineatus*.

Although there are considerable evidences for the involvement of chemical cues in the recognition of the group members based on kinship and/or familiarity, little is known about the chemical nature of the cues involved. Therefore, our investigation will shed light not only on recognition systems but also on dynamics of interactions between groups.

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