Journal of Chemical Ecology, Vol. 18, No. 11, 1992

AMINO ACID PROFILES AND LIPOSOMES: THEIR ROLE AS CHEMOSENSORY INFORMATION CARRIERS IN THE MARINE ENVIRONMENT

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(Received March 4, 1992; accepted July 13, 1992)

Abstract—Yellowfin tuna enhance their hunting success in the vast pelagic environment by using their sense of smell to detect intact (uninjured) prey that are beyond visual range. However, the olfactory cues that tuna use would normally face huge and rapid dilution in the open ocean. We demonstrate that these prey odors are complexed within biologically derived lipid structures that probably delay the dilution of the amino acids to subthreshold concentrations and provide persistent arousal and search cues for the tuna. This may be the first demonstration of an extracorporeal biological function for liposomes. Tuna may also form "chemical search images" to maximize feeding efficiency. We demonstrate that the amino acid profiles of various prey species are consistent over time and between schools, which makes the formation of search images feasible.

Key Words—yellowfin tuna, *Thunnus albacares*, amino acid profiles, liposomes, olfaction, search image, lipid complexes.

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0098-0331/92/1100-2107$06.50/0

INTRODUCTION

Yellowfin tuna, *Thunnus albacares*, feed primarily on schooling prey that are patchily distributed in a huge ecosystem and that are consequently difficult to detect even in clear pelagic waters. Captive tuna respond to the odors of intact prey organisms (Atema et al., 1980), and, in the wild, arousal to food beyond visual range is probably mediated by olfactory detection of excretory and mucal slough-off compounds produced by the schools of prey. Aqueous rinses of the surfaces of tuna prey organisms contain complexes of lipids and amino acids, the latter being chemosensory feeding stimuli for a wide range of fish species including tuna (Bardach and Villars, 1974; Atema, 1980; Atema et al., 1980; Hara, 1982; Olsén et al., 1986).

Maximum energetic advantage (i.e., energy expended in hunting versus caloric value of the food consumed) may be obtained by use of a "chemical search image" shaped by recent feeding success on locally or seasonally abundant food types, and there is evidence that tuna respond preferentially to the odors of recently consumed prey species (Atema et al., 1980). However, for a chemical search image to be effective, the amino acid profile of a particular prey type must remain constant over time and be qualitatively similar between different schools of the same species. Furthermore, to provide arousal and, possibly, chemotactic cues, the prey odors must stay above detection thresholds even in the face of the immense dilutions possible in the open ocean. Detection thresholds for yellowfin tuna are around 5×10^{-6} g/liter for squid rinse (Yost, 1988) and estimated as 1×10^{-11} M for single amino acids (Atema et al., 1980). However, this estimate was extrapolated from minimum detectable amino acid concentrations in complex prey stimuli and is in the range of background concentrations found in ocean water (Carr and Derby, 1986).

There were two principal objectives of the current study: first, determine whether the amino acid profiles of tuna prey species were consistent over time and between schools, and second, because our previous attempts to separate prey rinses into discrete molecular weight fractions were consistently confounded by the presence of small-molecular-weight compounds in the supposedly pure large-molecular-weight fractions, investigate the possibility that amino acids were being entrained in the lipid components of the prey rinses.

METHODS AND MATERIALS

Rinses of the following prey were analyzed: (1) Hawaiian mackerel—"opelu" (*Decapturus macarellus*), (2) Hawaiian anchovy—"nehu" (*Stolephorus purpureus*), (3) bigeye scad—"akule" (*Selar crumenopthalmus*), and squid (*Loligo sp.*). Specimens were collected live from fishermen, from the wild, or from a local wholesale fish auction. In all cases, contact with possible contam-

inants was nonexistent or minimal. For the fish species, rinses were obtained through gentle washing of fresh dead whole prey in an agitating bath of either fresh- or saltwater for about 20 min. Washing was always terminated before any rupturing of the gills occurred. Squid rinses were obtained in the same way from previously frozen specimens. The rinses were filtered, and analyses were performed on both the fresh and lyophilized product.

Amino acid content of the rinses was determined by ion-exchange chromatography using a Beckman autoanalyzer with ninhydrin postcolumn derivatization. The lipophilic components of the prey rinses were Soxhlet-extracted using dry methylene chloride or chloroform. These extracts were then analyzed with high-performance thin-layer chromatography (HPTLC). The quantities of the various lypophilic compounds were ascertained using enzymatic methods of analysis (Anon., 1987).

Opelu rinses were prepared for electron and fluorescence microscopy by filtering the prey rinse through a Whatman No. 1 paper, followed by centrifugation at 5000 rpm for 20 min. For fluorescence microscopy, 2-ml aliquots of rinse were mixed with equal volumes of the derivitizing agent *o*-phthalaldehyde (Pickering Labs., Mountain View, California), which is specific for primary amine-bearing compounds (Chen et al., 1979) and which was prepared by dissolving 80 mg in 10 ml of 100 mM borate buffer, pH 9.4, and adding 100 μl of β-mercaptoethanol. One drop of derivatized prey rinse was viewed under UV illumination (340 nm) and observed through a 4-nm narrow band-pass filter. The image was captured with a video attachment interfaced with an IBM XT computer. Samples of opelu rinse for electron microscopy were stained with either phosphotungstic acid or uranyl acetate, both 2% in seawater. To ensure that the lipid vesicles were not an artifact of the centrifugation procedure, a sample of crude, filtered opelu rinse was scanned for the presence of liposomes.

Fluorescence spectroscopy was conducted on fresh opelu rinse diluted 1:20 with filtered seawater and excited with UV light at 280 nm. The intrinsic fluorescent properties of tryptophan (Trp) allowed us to utilize this amino acid as a probe to determine the location of amino acids occurring in the prey rinses. Fluorescence-quenching studies were conducted by adding increasing concentrations of potassium iodide to the prey rinse and measuring the degree of quenching with a multifrequency phase fluorometer (Alcala et al., 1985). Prey rinses were also analyzed with ^{1}H NMR techniques in which the emission spectra of Trp in opelu rinse was compared with the spectra of free Trp in deuterated seawater.

Behavioral bioassays, which quantify the responses of captive tuna to the presentation of dilute chemical stimuli, were conducted as described by Atema et al. (1980). A small school of tuna (three to five animals) was tested daily and their responses quantified by an observer who did not know the nature of the stimulus. Responses were ranked on a scale of 0 to 9, with 0 being no

response and 9 a very strong behavioral response. The fish were fed to satiation on the day before testing to minimize variations in response intensity due to hunger motivation. The stimuli were presented in a quasirandomized sequence with trials separated by about 1 hr. Responses to the whole squid rinse served as a measure of the tuna's responsiveness on each day and allowed evaluation of the relative efficacy of the test stimuli.

Four types of stimuli were used: (1) pure lipids produced from soybean lecithin (0.9 g/500 ml starting concentration); (2) reconstituted squid amino acid profile (0.5 g/500 ml); (3) reconstituted amino acid profile (0.5 g) plus soybean liposomes (0.9 g/500 ml); and (4) a control stimulus of lyophilized whole squid rinse (0.5 g/500 ml). The lipids and lipid–amino acid mixtures were sonicated for approx. 15 min to promote the formation of lipid complexes in the form of liposomes (Naoi et al., 1977; Klein et al., 1971).

RESULTS

The amino acid profiles proved to be species specific and consistent across replicate samples of each species taken over a period in excess of two years (Figure 1). In addition to these physiological amino acids (i.e., those occurring naturally in proteins), taurine was also found in the rinses of all prey species and was incorporated into the artificial amino acid mixtures. Microscopic examination using ultraviolet (UV) illumination of fresh rinses combined with the derivatizing reagent *o*-phthalaldehyde revealed that fluorescence was localized within the lipid structures and absent from the background medium, thereby verifying that amino acids were sequestered in the lipid domain.

The emission spectra of prey rinses excited at 280 nm demonstrated a Trp fluorescence that was blue-shifted by up to 30 nm compared with the emission maximum of 355 nm observed for Trp in pure seawater, indicating that Trp was complexed within the lipid structures. Quenching of this Trp fluorescence with potassium iodide resulted in Stern-Volmer plots (Lehrer, 1971), which indicate that the Trp associated with the liposomes was substantially shielded relative to Trp free in solution (Figure 2). The fluorescence lifetime of Trp in liposomes was also found to be heterogeneous and lengthened (average = 3.6 nsec) compared to Trp free in solution (average = 2.9 nsec), which supports the conclusion that Trp is associated with lipid matrices in the prey rinses.

Further indication of lipid–amino acid complexation was acquired from ^{1}H NMR examination of the rinses. Spectra of Trp in the presence of liposomes formed both with lipid extracts from the prey fish rinses and from standard lipid mixtures showed chemical shift and line broadening when compared with spectra of free Trp in deuterated seawater.

Examination of rinses under the electron microscope revealed classic mul-

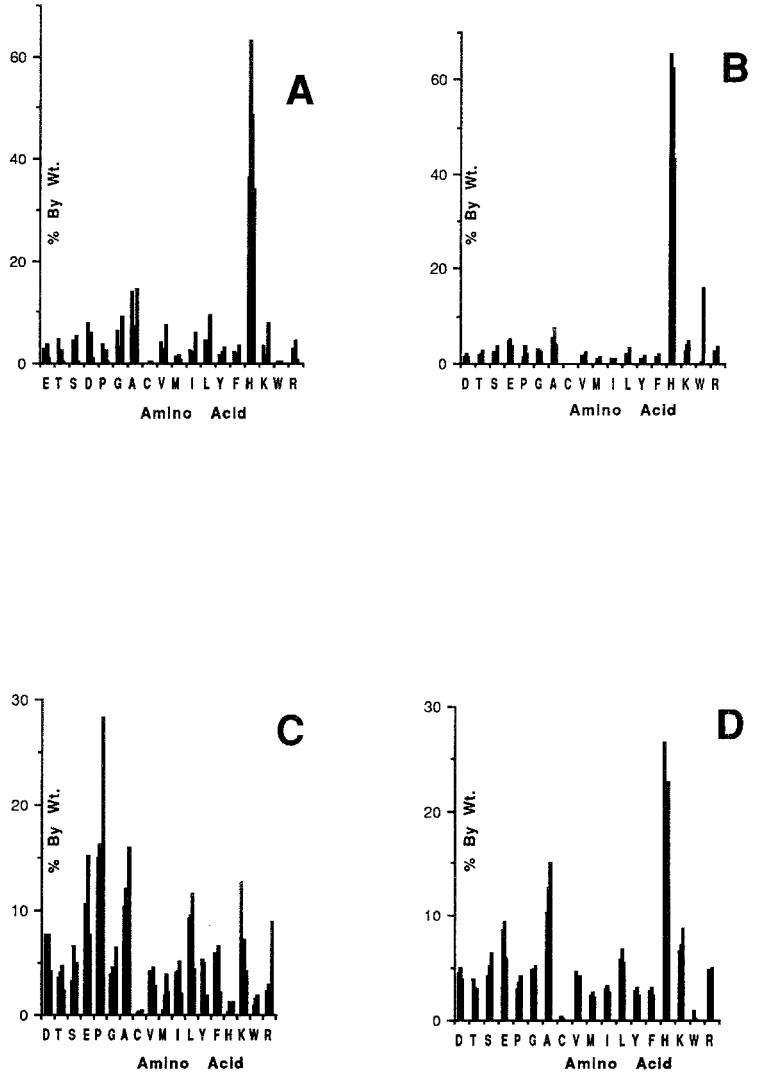

FIG. 1. Amino acid profiles of the aqueous rinses of four tuna prey species. (A) Opelu: profiles of rinses from four schools acquired over a period of two years. (B) Akule: profiles of rinses from three samples collected over two years. (C) Squid: four profiles of rinses sampled over two years. (D) Nehu: profiles of samples from three separate schools of fish taken from Kaneohe Bay, Oahu, Hawaii, over a period of approximately three years. Amino acids are as follows: A, alanine; C, cysteine; D, apartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; P, proline; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

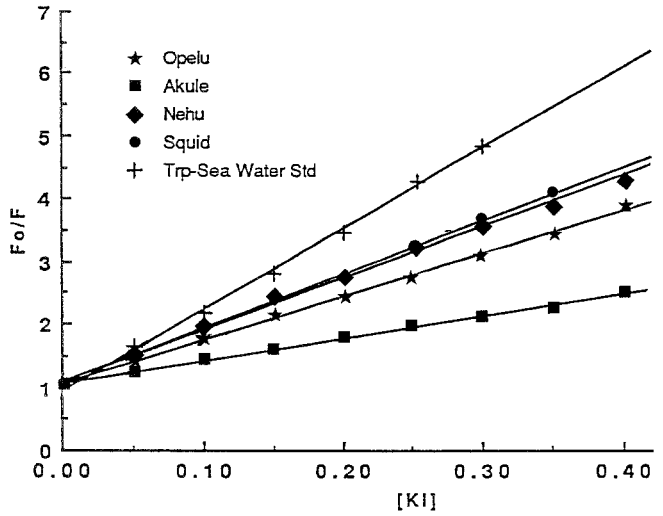

FIG. 2. Stern-Volmer iodide quenching plots of the rinses of four tuna prey species and plain sea water. F_0 = fluorescence intensity of Trp in the rinse in the absence of quencher; F = Trp fluorescence intensity with quencher. The flatter slope of F_0/F for the prey rinses relative to Trp free in solution indicates that Trp is occluded to varying degrees within lipid matrices in the rinses of each species.

tilamellar onion-skin structures, which are usually associated with the formation of liposomes (Figure 3). These structures were observed in both the centrifuged and crude opelu rinses.

HPTLC analysis of the extracts revealed the presence, in all species, of cholesteryl esters, cholesterol, free fatty acids, glycero-fatty acid triesters, and phospholipids.

The behavioral response intensities of captive tuna to the various stimuli are shown in Table 1. Kolmogorov-Smirnov chi-square approximation analysis of the behavioral data indicated that response intensities to the reconstituted squid amino acid profile and to the same profile complexed with soybean lipids were not significantly different. They were both significantly better stimuli than the lipids alone, but evoked significantly weaker responses than the squid odor control. The average response to the amino acid–liposome mixture was 62% as strong as responses to the unaltered squid odor.

DISCUSSION

The specificity and constancy of the amino acid profiles among schools of the same species, in some cases over periods in excess of two years, supports the concept of chemical search images as a viable foraging technique for tuna.

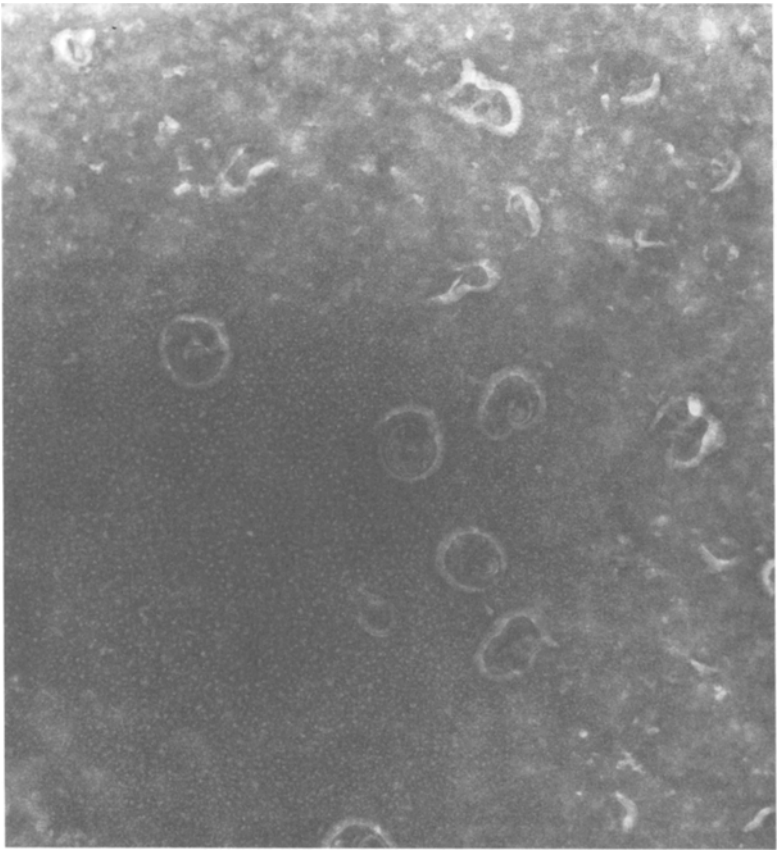

FIG. 3. Transmission electron micrograph of opelu seawater rinse at 127,500×. Lipid vesicles display the classic onion-skin structure of liposomes.

TABLE 1. BEHAVIORAL RESPONSE INTENSITIES TO OLFACTORY STIMULI

Stimulus type	Average response intensity (SD)	Number of trials
Soy bean liposomes	0.8 (0.8)	9
Amino acid mixture	3.0 (1.6)	7
Soy liposomes plus amino acid mixture	4.4 (0.5)	9
Whole squid (control)	7.1 (1.0)	11

These chemical search images may heighten appetitive feeding behavior in response to the odors of previously consumed prey (Atema et al., 1980) and thereby improve foraging efficiency. Even without a learned response to a particular prey species, the predictable nature of the prey odors could facilitate innate responses to specific food types. The qualitatively similar profiles of opelu and akule perhaps reflect the fact that they are both in the family Carangidae.

Amino acids appear to be routinely bound into a variety of biogenic lipid structures. The presence of tryptophan fluorescence in the liposomes, and its absence in the background medium, suggests that a large portion of amino acids emanating from prey are incorporated into lipid complexes. The HPTLC results indicate that lipid compounds suitable for liposome formation are present in the prey rinses. The rate of efflux of a particular amino acid from lipid vesicles is determined by the size and complexity of the lipid structure, the type of lipids from which the liposome is constructed, pH, and temperature (Klein et al., 1971; Naoi et al., 1977). Therefore, the time course of amino acid elution from liposomes in the open ocean will be variable, but it is probable that the lipid complexation significantly delays the dilution of prey odors to subthreshold levels.

The similar behavioral response intensities to the free amino acids and to the amino acid–liposome mixture indicate that lipid complexation does not significantly alter the biological potency of prey amino acid profiles. The reconstituted amino acid–lipid mixtures were not as effective as the whole rinse, but this is consistent with results from tuna and other species, which indicate that, although amino acids are important components of prey odors, there are other compounds present in the natural stimuli that contribute to their total effectiveness (Carr and Derby, 1986; Atema et al., 1980; Holland, 1978; Carr and Chaney, 1976). Diminution of maximum stimulus strength is probably less important to a passing school of tuna than the prolonged suprathreshold dwell time induced by lipid complexation of the amino acids. This would improve the chances of the tuna detecting the presence of food beyond visual range and possibly provide orientation cues for subsequent food search behavior.

Even though the amino acid profiles were temporally and spatially consistent and may permit olfactory recognition of the type of prey, lipid complexation may modify the profile detected by the predator because different amino acids elute from liposomes at different rates (Klein et al., 1971). Thus, the perceived prey profiles may well be constant, but somewhat modified, from those determined by our methods of analysis. This could explain why amino acid profiles constructed from ninhydrin-based analyses do not match the biological potency of the original natural stimulus. We believe this is the first demonstration of biogenic liposomes mediating interspecific interactions and that lipid structures may have a widespread role in the semiochemistry of aquatic chemoreception.

Acknowledgments—This work was supported by the Sea Grant College of the University of Hawaii and by the State of Hawaii Department of Planning and Economic Development. We thank the National Marine Fisheries Service, Honolulu Laboratory, for access to their facilities. The fluorescence lifetime experiment and analysis of these data were performed at the laboratory for Fluorescence Dynamics at the University of Illinois at Urbana-Champaign. H.I.M.B./S.O.E.S.T. Contribution No. 3018.

REFERENCES

ALCALA, J.R., GRATTON, E., and JAMESON, D.M., 1985. A multifrequency phase fluorometer using the harmonic content of a mode-locked laser. *Anal. Instrum.* 14:225–250.

ANON. 1987. Biochemical analysis and food analysis, pp. 118–119, *in* Biochemica. Boehringer Mannheim, Indianapolis, Indiana.

ATEMA, J. 1980. Chemical sense, chemical signals and feeding behavior in fishes, pp. 57–101, *in* J.E. Bardach, J.J. Magnuson, R.C. May and J.M. Reinhart (eds.). Fish behavior and its use in the capture and culture of fishes. International Center for Living Aquatic Resources Management. Manila, Philippines.

ATEMA, J., HOLLAND, K.N., and IKEHARA, W. 1980. Olfactory responses of yellowfin tuna (*Thunnus albacares*) to prey odors: Chemical search image. *J. Chem. Ecol.* 6:457–465.

BARDACH, J.E., and VILLARS, T. 1974. The chemical senses of fishes, pp. 49–57, *in* P.T. Grant and A.M. Mackie (eds.). Chemoreception in Aquatic Organisms. Academic Press, New York.

CARR, W.E.S., and CHANEY, T.B. 1976. Chemical stimulation of feeding in the pinfish Lagodon rhomboides: Characterization of stimulatory substances extracted from shrimp. *Comp. Biochem. Physiol.* 45A:437–441.

CARR, W.E.S., and DERBY, C.D. 1986. Chemically stimulated feeding behavior in marine animals: The importance of chemical mixtures and the involvement of mixture interactions. *J. Chem. Ecol.* 12:987–1009.

CHEN, R.F., SCOTT, C., and TREPMAN, E. 1979. Fluorescence properties of *o*-phthaldialdehyde derivatives of amino acids. *Biochim. Biophys. Acta* 576:440–455.

HARA, T.J. 1982. Structure–activity relationships of amino acids as olfactory stimuli, pp. 135–158, *in* T.J. Hara (ed.). Chemoreception in Fishes. Elsevier Scientific Publishing, Amsterdam.

HOLLAND, K.N. 1978. Chemosensory orientation to food by an Hawaiian goatfish (*Parupeneus porphyreus*, Mullidae). *J. Chem. Ecol.* 4:173–178.

KLEIN, R.A., MOORE, M.J., and SMITH, M.W. 1971. Selective diffusion of neutral amino acids across lipid bilayers. *Biochim. Biophys. Acta* 233:420–433.

LEHRER, S.S. 1971. Solute perturbation of protein fluorescence. The quenching of the tryptophyl fluorescence model compounds and of lysozyme by iodide ion. *Biochemistry* 10:3254–3263.

NAOI, M., NAOI, M., SHIMIZU, T., MALVIYA, A.N., and YAGI, K. 1977. Permeability of amino acids into liposomes. *Biochim. Biophys. Acta* 471:305–310.

OLSÉN, K.H., KARLSSON, L., and HELANDER, A. 1986. Food search behavior in arctic char, *Salvinus alpinus* (L.), induced by food extracts and amino acids. *J. Chem. Ecol.* 10:1987–1998.

YOST, R.M. 1988. Yellowfin tuna (*Thunnus albacares*) olfactory threshold: Behaviorally determined sensitivity to a prey odor. MS thesis. Zoology Department, University of Hawaii at Manoa, Honolulu, Hawaii.