Journal of Shellfish Research, Vol. 19, No. 1, 129–132, 2000.

DELIVERY OF RIBOFLAVIN TO LARVAL AND ADULT PACIFIC OYSTERS, *CRASSOSTREA GIGAS* THUNBERG BY LIPID SPRAY BEADS

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ABSTRACT Lipid spray beads (SB) were prepared containing 13% w/w particulate riboflavin. Beads suspended in seawater lost 73% riboflavin after 24 h. Release of riboflavin from SB ingested by Pacific oyster (*Crassostrea gigas*) larvae was observed under epifluorescent light. Riboflavin concentrations in tissues of adult oysters fed on riboflavin-SB were significantly (SNK; $P < 0.05$) greater than those of oysters fed on seawater-filled SB. Concentrations of riboflavin in oysters exposed to dissolved riboflavin were not significantly greater than those of oysters fed on seawater-filled SB, indicating that elevated riboflavin concentrations in oysters fed on riboflavin-SB were attributable to breakdown of ingested beads rather than uptake of dissolved riboflavin leaked from SB into the culture medium. SB seem to be a promising means of delivering water-soluble nutrients to bivalve suspension feeders

KEY WORDS: Spray beads, lipid, riboflavin, oyster, larvae, *Crassostrea gigas*

INTRODUCTION

Little is known of the nutritional requirements of bivalve mollusks despite their obvious importance in aquaculture and natural ecosystems. The main reason for this lack of knowledge is that nutritionally satisfactory, defined artificial diets are not available. The development of microparticulate diets that both retain dietary ingredients when suspended in seawater and are digestible by bivalve mollusks has been a difficult goal to achieve. High surface area to volume ratios of microparticulate diets together with low molecular weights of essential nutrients, such as water-soluble vitamins, results in their rapid loss. For example, López-Alvarado et al. (1994) reported that > 80% amino acids were lost from microgel particles after only 2 minutes in aqueous suspension.

To address the problem of rapid loss of water-soluble nutrients from microparticulate feeds, Langdon and Siegfried (1984) developed lipid-walled microcapsules for the delivery of water-soluble vitamins to juvenile oysters (*Crassostrea virginica*). Later Buchal and Langdon (1998) and Langdon and Buchal (1998) developed lipid spray beads (SB) for the delivery of water-soluble nutrients and therapeutic substances to bivalves. Buchal and Langdon (1998) found that it was important to soften the walls of lipid-walled capsules and SB by adding 40% w/w fish oil to the tripalmitin walls of the particles in order to make them digestible by clams (*Tapes philippinarum*); however, softening the walls of SB in this way lowered 24-h retention efficiencies for encapsulated riboflavin from 97.9 to 85.1% (Buchal and Langdon 1998).

Seguineau et al. (1996) reported that the microalgal species *Isochrysis galbana, Pavlova lutheri*, and *Skeletonema costatum* contained high concentrations of riboflavin and thiamine; however, the concentrations of these two vitamins in scallop (*Pecten maximus*) larvae fed on a mixture of these algal species declined during growth and development. Seguineau et al. (1996) suggested that microencapsulated supplements of riboflavin and thiamine could be used to study the requirements of scallop larvae for these vitamins.

In this paper, we describe the results of feeding experiments in which larval and adult oysters (*Crassostrea gigas* Thunberg) were fed on SB containing particulate riboflavin to evaluate the potential usefulness of SB in delivering low-molecular weight, water-soluble nutrients to bivalve mollusks.

METHODOLOGY

Spray Beads

Preparation of Spray Beads Containing Riboflavin

Spray beads were prepared containing micronized, particulate riboflavin (Sigma) based on the method described by Buchal and Langdon (1998). Briefly, riboflavin crystals were ground to a fine powder (< 5-μm particles; McCrone micronizing mill, McCrone Scientific Ltd). Two grams of ground powder were mixed by sonication at 90 °C with 8 g of a lipid mixture made up with 4.8 g tripalmitin (Fluka Chemical Co.) and 3.2 g of menhaden oil (light cold pressed; Zapata Haynie Ltd.). The heated mixture was then forced under pressure (90 psi) through at atomizing nozzel (SUE-25B; Spraying Systems Ltd.) supplied with pressurized nitrogen at 10 psi. The beads were collected in a stainless steel cylinder cooled with liquid nitrogen then stored in the dark at −20 °C until use.

Determination of Encapsulation Efficiency

To determine encapsulation efficiencies, 10 g of SB were first dissolved in 3 mL chloroform and the vitamin core material extracted by addition of 3 mL distilled water with shaking. The aqueous supernatant was removed and the extraction repeated three times. Aqueous extractions were combined and the concentration of dissolved riboflavin determined spectrophotometrically (absorbance at 267 nm).

A subsample of 0.5 mL of chloroform was removed from the capsule extraction and transferred to a dry, tarred weighing boat. The chloroform was removed by heating for 24 h at 50 °C, and the boat was reweighed to determine the weight of extracted lipid.

Encapsulation efficiencies were expressed as the weight of encapsulated vitamin (mg) per 100 mg of lipid.

Retention of Riboflavin by SB Suspended in Seawater

Retention of riboflavin by SB was determined by measuring the proportion of initially encapsulated riboflavin remaining after 24 h suspension in seawater. To prepare SB for a leakage experiment, beads were first suspended in 2% polyvinyl alcohol with sonication. SB were then sieved using a 40-μm sieve, and beads under 40 μm were collected on a GF/C filter and rinsed with cold (5 °C) distilled water. The beads were then washed from the filter with cold distilled water and collected in a sealed vial and stored at 5 °C in the dark.

At the start of a leakage experiment, 75 to 100 mg of the sieved (< 40 μm) SB were suspended in 15 mL seawater (20 °C) by vigorous shaking. Immediately after suspension (t = 0), 1 mL of the bead suspension was taken and filtered onto a GF/C filter. The filtered SB were then washed with 1 mL of chilled (5 °C) seawater. The filtrate and washings were pooled and stored in the dark at 5 °C for analysis. Riboflavin concentrations were determined as described above. The remainder of the SB suspension was placed on a continuous agitator at 20 to 22 °C. Samples of suspended SB were taken over a period of 24 h to determine changes in the retention of riboflavin over time. Retention efficiency (RE) was defined as

$$RE = \frac{\text{riboflavin retained on the filter}}{\text{riboflavin on filter and in filtrate}} \times 100$$

Breakdown of SB and Release of Riboflavin by Larvae

Feeding experiments were conducted to determine if oyster larvae could ingest and breakdown SB, thereby releasing riboflavin into the digestive system. Broodstock oysters were spawned, and the resulting larvae were raised on a mixed diet of *Isochrysis galbana* (T-ISO) and *Chaetoceros calcitrans* (Breese and Malouf 1975). After 8 days of culture, larvae were sieved onto a 45-μm screen, divided into two groups and each resuspended in two liters of seawater at a density of 10 larvae per mL.

Riboflavin-SB at a concentration of 50 SB/μL were fed to one group of larvae with gentle aeration to maintain SB in suspension. After 1 hour, larvae were sieved from suspension using a 45-μm sieve, rinsed with seawater, then resuspended in two liters of filtered seawater and fed on T-ISO alone for 2 hours. The larvae were then sieved from the culture medium and preserved with 0.5% formaldehyde (final concentration made up in seawater, buffered at pH 8.0 with borax) for microscopic analysis. The second group of 8-day old larvae were fed on T-ISO alone for 2 hours, then sampled and preserved for microscopic analysis (as described above).

Sampled larvae were examined using an epifluorescent microscope (Leica DMRBE; excitation 355–425 nm, emission 525 nm) at ×400 magnification. Green and yellow fluorescence indicated the presence of dissolved and particulate riboflavin, respectively, while red fluorescence indicated the presence of chlorophyll derived from ingested algae.

Breakdown of SB and Uptake of Released Riboflavin by Adults

An experiment was carried out to determine if adult oysters could breakdown ingested SB and absorb released vitamin into the hemolymph and tissues. Three groups of six adult oysters were separately held in 20 L of seawater and fed for 6 hours on T-ISO at a concentration of 50 cells/μL in combination with one of the following additions:

1. 20 riboflavin-SB/μL (equivalent to a concentration of 1.15 mg riboflavin/L or a vitamin dose of 3.8 mg riboflavin/oyster);
2. 20 seawater-filled SB/μL; or
3. dissolved riboflavin at the same concentration provided in 1.

Two grams of riboflavin-SB were suspended in distilled water, and the suspension was then poured through a 20μm mesh sieve. SB smaller than 20 μm were collected and filtered onto a GF/C filter, rinsed, and resuspended in 10 mL of distilled water. Aliquots of 100 μL SB suspension were taken to determine riboflavin and bead concentrations. Riboflavin concentrations were determined as described above. SB concentrations were determined using a Coulter Counter (Model TA2). Seawater and food additions were replaced every 2 hours over a period of 6 hours. The cultures were gently aerated to maintain beads in suspension.

After 6 hours, oysters were removed and dissected. Hemolymph samples were taken with a syringe from both the heart and the sinus of the adductor muscle. Samples of stomach contents were removed with a Pasteur pipette inserted through the mouth. Tissue samples of mantle and adductor muscle were also removed. The samples were frozen at −20 °C for protein and riboflavin analysis.

Riboflavin and Protein Analysis

Hemolymph samples were centrifuged, and riboflavin concentrations of the supernatant fraction were determined by high-pressure liquid chromatography (HPLC) (Seguineau et al. 1996). Mantle and adductor muscle samples were ground in 0.2M HCl and 0.6N perchloric acid (PCA) and centrifuged. Supernatants were then removed, their volumes adjusted to 2 mL with distilled water and riboflavin concentrations determined by HPLC (Seguineau et al. 1996).

Protein concentrations of hemolymph samples were determined by the method of Bradford (1976), using bovine serum albumen as a standard. Treatment of adductor muscle and mantle samples with HCl and PCA for the extraction of riboflavin probably resulted in the precipitation of most proteins in these samples; therefore, Bradford assays indicated concentrations of PCA-soluble protein and peptides in muscle and mantle samples. Riboflavin concentrations were expressed in terms of ng riboflavin per mg protein in tissue samples or per mL of stomach extract.

Statistics

The rate of loss of riboflavin from SB suspended in seawater was analyzed by regression analysis. Riboflavin concentrations in oyster samples from the three experimental treatments were compared by analysis of variance (ANOVA) (Model III; Super ANOVA, Abacus Concepts). Concentrations were log-transformed to ensure homogeneity of variances, as determined by plots of residual values against fitted values. If ANOVA indicated a significant treatment effect on riboflavin concentration, individual treatments were compared by Student–Newman-Keuls (SNK) multiple range test (significance level $P < 0.05$).

RESULTS

Encapsulation and Retention Efficiencies

SB were found to have an encapsulation efficiency of 13% w/w (mg riboflavin per 100 mg lipid). Leakage experiments indicated

that almost half the encapsulated riboflavin was lost from SB during the first 3 hours of suspension in seawater, followed by a more gradual loss over the subsequent 21 hours (Fig. 1). Approximately 27% of the initially encapsulated riboflavin was retained after 24 hours of suspension, equivalent to 3.5 mg of riboflavin retained per 100 mg of lipid.

Regression analysis indicated that there was a significant (P = 0.0012) relationship between the log of the fraction of riboflavin retained and the duration [log time (h)] that SB were suspended in seawater (Fig. 1). The rate of loss of riboflavin could be expressed in terms of the equation:

Log fraction retained = $-0.116 - [0.333 \times \log \text{time (h)}]$ r^2 = 0.994

Breakdown of SB and Release of Riboflavin by Larvae

Larvae were able to ingest and breakdown SB, liberating encapsulated riboflavin into the digestive system. Free riboflavin was observed as a diffuse greenish fluorescence in the guts of larvae fed on SB, and riboflavin crystals present in intact or partially digested SB were evident as bright yellow points (Fig. 2). The digestive systems of larvae fed on algae alone fluoresced red because of the presence of chlorophyll from ingested algae but no yellow or green fluorescence was evident (Fig. 2).

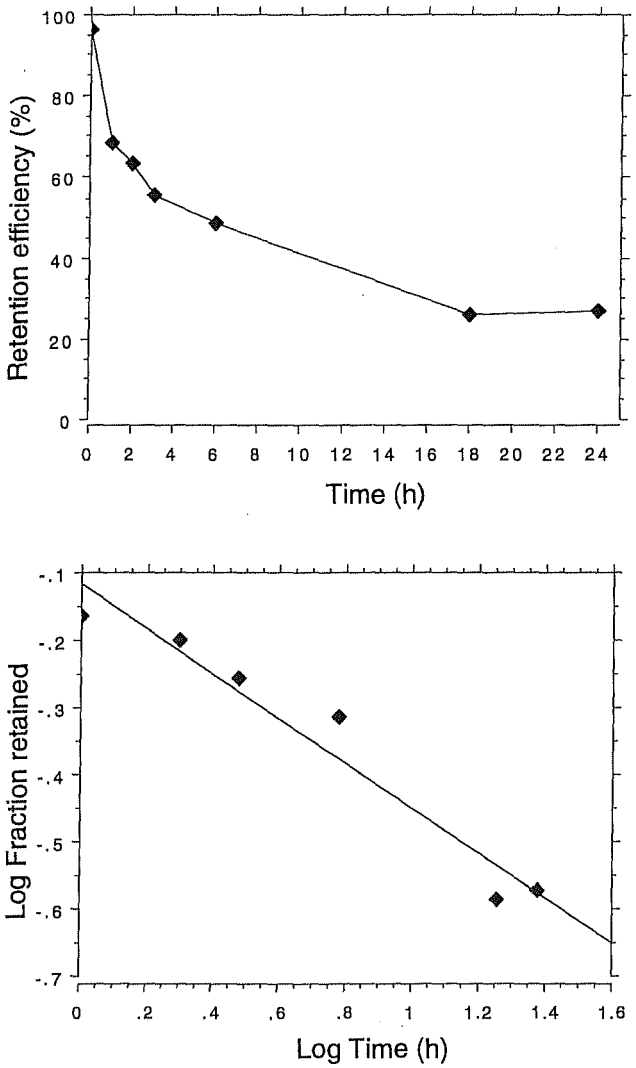

Figure 1. Retention of riboflavin by lipid spray beads suspended in seawater. Top: Change in percentage riboflavin retained by beads over a 24-h period Bottom: Relationship between log of fraction retained and log time duration of beads suspended in seawater. Log fraction retained = −0.116 −[0.333 × log time (h)]; r^2 = 0.994

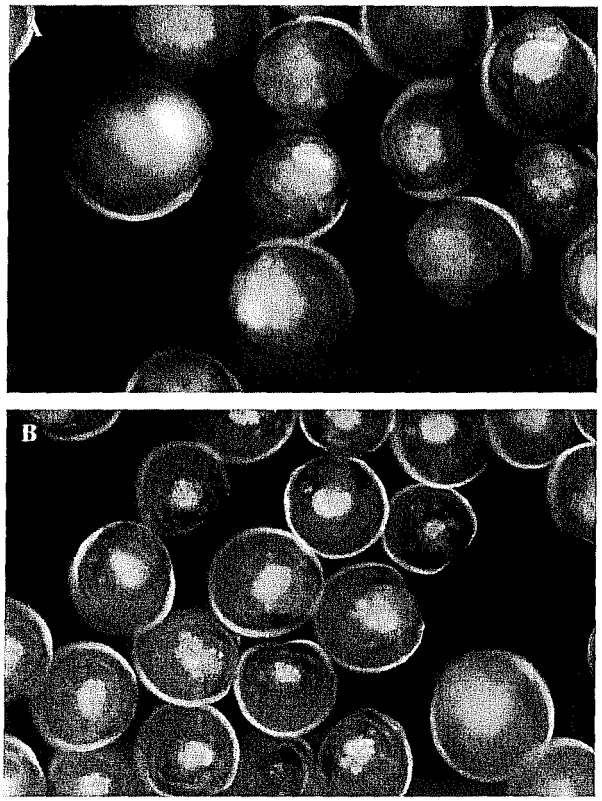

Figure. 2. Eight-day-old larvae of the Pacific oyster (*Crassostrea gigas*) viewed under epifluorescent light (excitation 355–425 nm, emission 525 nm) at ×400 magnification. Average larval shell length = 122 µm. Top: Larvae fed on riboflavin-containing lipid spray beads (50 beads/µL) for 1 hour, followed by a 2- hour period of feeding on T-ISO alone. Bottom: Larvae fed on *Isochrysis* sp. (T-ISO) alone for 2 hours.

Breakdown of SB and Uptake of Released Riboflavin by Adult Oysters

ANOVA of log-transformed riboflavin concentrations in oysters fed on riboflavin-SB were significantly greater (SNK; P < 0.05) than those of oysters either fed on seawater-filled SB or exposed to riboflavin dissolved in seawater (Table 1). The presence of significantly higher concentrations of riboflavin in the hemolymph, adductor muscle, and mantle of oysters fed on riboflavin-SB indicated that oysters were able to digest the lipid walls of SB and absorb released riboflavin. There were no significant differences in riboflavin concentrations in hemolymph sampled from either the heart or adductor muscle (ANOVA; P > 0.05).

Concentrations of riboflavin in the tissues of oysters exposed to dissolved riboflavin were not significantly (SNK; P > 0.05) different from those of oysters fed on seawater-filled SB, indicating a limited ability of adult oysters to take up dissolved riboflavin from seawater.

CONCLUSIONS

Feeding experiments indicated that both oyster larvae and adults were able to breakdown SB and release riboflavin. Free

TABLE 1.

Concentration of riboflavin in tissues of adult Pacific oysters exposed to either lipid spray beads (SB) containing 13% riboflavin at a concentration of 20 SB/μm, seawater-filled SB at a concentration of 20 SB/μL or dissolved riboflavin at the same concentration as that delivered by riboflavin-SB (1.15 mg/L).

Treatment	Stomach Contents (ng/mL)	Hemolymph		Mantle (ng/mg PCA-soluble protein)	Adductor muscle (ng/mg PCA-soluble protein)
		Heart (ng/mg protein)	Muscle (ng/mg protein)		
Seawater-filled SB	20 ± 4	47 ± 19	21 ± 10	198 ± 94	177 ± 115
Dissolved riboflavin	24 ± 4	84 ± 53	51 ± 32	112 ± 22	67 ± 20
Riboflavin-SB	7274 ± 1619	1844 ± 660	1165 ± 247	1633 ± 573	10636 ± 3808

Values are given as means (±1 SE, $n = 6$).

riboflavin was evident in the stomachs of larvae and elevated riboflavin concentrations were evident in the tissues of adult oysters after being fed on riboflavin-SB. Adult oysters exposed to concentrations of dissolved riboflavin, equivalent to those supplied by encapsulated riboflavin, did no show elevated tissue concentrations of riboflavin, indicating that uptake of dissolved riboflavin lost from SB was not a significant source for adult oysters.

About half of the riboflavin was lost during the preparation of SB, based on a comparison between the measured encapsulation efficiency of 13% and a maximum theoretical encapsulation efficiency of 25%. Further losses of riboflavin from SB occurred after suspending SB in seawater; for example, it can be estimated (based on Eq. 1) that 39% of encapsulated riboflavin would have been lost at the end of each 2-hour period of the adult feeding experiment.

In this study, retention of riboflavin by SB suspended in seawater for 24 h was only 27% compared with 85% reported by Buchal and Langdon (1998). This difference may have been attributable to higher encapsulation efficiencies of SB used in this study, because riboflavin-SB prepared by Buchal and Langdon (1998) had an encapsulation efficiency of 4.8% compared with an encapsulation efficiency of 13% for SB used in this study.

The effects of additions of riboflavin-SB on the growth and survival of oysters needs to be determined in future experiments. Because of the need to prepare SB with a high proportion (> 60%) of lipid wall material to ensure encapsulation of the core material, it is unlikely that SB will be useful in delivering bulk dietary ingredients, such as protein and carbohydrate, to oysters. However, SB may be useful in supplementing algal or artificial diets with water-soluble micronutrients, such as essential amino acids or water-soluble vitamins (Seguineau et al. 1996).

ACKNOWLEDGMENTS

This research was supported by a fellowship from the Organization for Economic Cooperation and Development (OECD), cooperative research program: Biological Resource Management for Sustainable Agricultural Systems. We also thank Jacques Panfili for his help in taking photographs of larvae fed on SB.

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