Optimization of the protein fraction of microcapsules used in feeding of marine fish larvae using *in vitro* digestibility techniques

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Abstract

The development of artificial feeds for marine fish larvae is demanding and, taking into account the difficulties and costs associated with the rearing of the larvae of some fish, preliminary evaluation of feed ingredients using *in vitro* techniques may be an alternative to *in vivo* assays. Some proteins used in microfeeds for marine fish were tested *in vitro* for examination of their effect on the proteases of seabream larvae. Casein and cuttlefish meal did not affect protease activity of crude larval extracts, whereas ovalbumin produced a 60% inhibition. The use of a pH-stat for the assessment of the degree of hydrolysis (DH) of proteins confirmed the low nutritive value of ovalbumin, since a much lower value was obtained for microcapsules prepared using this protein source when compared with those prepared using casein or cuttlefish meal (3.2 vs. 7.3 and 7.6, respectively). Products resulting from such hydrolysis were analysed using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Image analysis of such gels led to the formulation of an index named the coefficient of protein degradation (CPD) which expressed the extent to which the main protein fractions were hydrolysed by larval proteases in a given time. A significant correlation ($r^2 = 0.98$, $P < 0.05$) was obtained between DH and CPD values measured for either protein sources or microcapsules. The combination of protease inhibition assays with measurements of DH and CPD is proposed as a preliminary evaluation of protein ingredients used in the formulation of artificial feeds for larval fish.

KEY WORDS: *in vitro* digestibility, larval diets, marine fish, microcapsules, proteases

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Introduction

In recent years, attention has been focused on the development of artificial diets for larval stages of marine fish (Tandler & Kolkovski 1991; Jones *et al.* 1993; Yúfera *et al.* 1996). Bioavailability of essential nutrients may be limited by a relative inefficiency of the digestive processes, but endogenous production of digestive enzymes by larval sea bass and bream is reported to be adequate for digestion of protein and carbohydrates (Cahu & Zambonino 1995; Moyano *et al.* 1996). Hence, the formulation of microfeeds for the larvae of these species should combine selection of the most suitable ingredients and a manufacturing technology providing high bioavailability of nutrients. *In vivo* assessment of nutrient digestibility in fish larvae is extremely difficult owing to their small size so *in vitro* approaches may offer an alternative.

In the present work, *in vitro* methods were examined to assess interactions between the digestive enzymes of seabream larvae and protein sources that may be used in the preparation of microcapsules. Tests were carried out to measure the potential inhibitory effect of protein sources on digestive protease activity, assess the ability of larval proteases to hydrolyse different proteins, and evaluate hydrolysis, using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) to study the protein fractions released during digestion.

Materials and Methods

Preparation of microcapsules and protein solutions

Microcapsules (MCs) were manufactured as described by Yúfera *et al*. (1996), by interfacial polymerization resulting from the action of a cross-linking agent on different proteins. Proteins used were ovalbumin (Sigma A-5253; Sigma Chemical Co., St. Louis, MO, USA), haemoglobin (Sigma H-2625), Hammerstein soluble casein (ICN Biomedicals 101289; ICN Pharmaceuticals Inc., Costamesa, CA, USA) and cuttlefish meal, provided by the laboratory. Each of these protein sources was included in the dietary formulation at 500 g kg^{-1}. The remaining ingredients were 170 g kg^{-1} fish protein, 112.5 g kg^{-1} fish oil, 105 g kg^{-1} dextrin, 50 g kg^{-1} vitamin complex, 50 g kg^{-1} mineral complex and 12.5 g kg^{-1} lecithin.

Solutions of protein sources and MCs prepared by homogenization (100 mg mL^{-1} in distilled water) followed by centrifugation (15000 g, 10 min) were used in the assays.

Preparation of larval extracts

Sea bream *Sparus aurata* larvae that had been fed on rotifers following the routine described by Yúfera *et al*. (1996), were sampled at 8 days old. Larvae were sampled after 2 h of food deprivation, rinsed in distilled water and freeze-dried. Enzyme extracts were prepared by homogenization of the whole larvae (35 mg mL^{-1} in distilled water) followed by centrifugation (16.000 g, 30 min, 4°C). Supernatants were used immediately in the protein digestion assays.

Determination of protease activity in crude larval extracts

Total protease activity of extracts was measured as described by Walter (1984), using casein (10 g L^{-1}) in 50 mM Tris-HCl buffer at pH 8.5 as the substrate. The mixtures were incubated for 2 h at 25°C, the reaction was stopped by addition of 0.5 mL trichloroacetic acid (TCA) (120 g L^{-1}), and the absorbance of the soluble TCA peptides was recorded at 280 nm. One unit of enzyme activity was defined as 1 μg of tyrosine released per minute, using the extinction coefficient for tyrosine = 0.005 mL μg^{-1} cm^{-1}. All measurements were carried out in triplicate. It was assumed that the protease activity measured was mostly due to digestive proteases. The concentration of soluble protein in larval extracts and in protein and MC solutions was determined according to Bradford (1976), using a standard microassay procedure.

Effect of different protein sources on protease activity

The inhibitory effect of the crude proteins and MC preparations on larval proteases was tested by measuring the reduction in protease activity of extracts using a modification of the method described by García-Carreño (1996). The method is based on the measurement of residual protease activity remaining after preincubation with different protein solutions. Two sets of each sample (20 μL) were used: one set was incubated in 500 μL HCl Tris buffer (50 mM, pH 9.0, 60 min at room temperature) with the protein solutions (20 μL), and the second was incubated with an equivalent amount of distilled water to serve as a control. Then, 100 μL of a casein solution (110 g L^{-1} in distilled water) was added and incubation was continued for a further 120 min. The reaction was stopped by addition of 500 μL TCA (120 g L^{-1}). Blanks were prepared for each set using the same ingredients but TCA was added prior to the casein. Protease inhibition was assessed as the reduction in protease activity relative to that of the controls.

The effects of variable amounts of ovalbumin or cuttlefish meal-based MCs on larval protease activity were tested using different concentrations of MCs with enzyme to substrate (E:S) ratio ranging from 1.5 to 0.5 U mg^{-1} protein. This range was established assuming the physiological E:S ratio of 0.8 U mg^{-1} calculated from the maximum intake of MCs by 8-day-old larvae (6.25 μg per individual; Yúfera *et al*. 1995) and protease activity estimated for larvae of that size (5 mU per larva; Moyano *et al*. 1996).

Determination of the degree of hydrolysis (DH) of proteins and protein fractions in MCs using pH-stat

Protein hydrolysis was assessed according to Dimes & Haard (1994) using the pH-stat method; 5 mL protein or MC solution (containing 8 mg mL^{-1} protein), were adjusted to pH 8.0 with 0.1 M NaOH and equilibrated to 25°C for 10 min in a 10-mL jacketed reaction vessel. Larval enzyme extract was then added to give an E:S ratio of 0.8 U mg^{-1}. The mixture was continuously agitated by a magnetic stirrer and the course of the reaction was automatically recorded and plotted using a pH-stat titrator (718 Stat Titrino, Methrom Ltd, Herisau, Switzerland). The DH was calculated after 90 min of reaction, on the basis of the hydrolysis equivalent (h) derived from the volume of alkali (0.01 M NaOH) required to maintain the pH of the reaction mixture at 8.0:

$$h = (B \times 1/ \times M_b)/(M \times (S/100)),$$

where B = ml of NaOH consumed $\alpha = 10^{pH - pK}/1 + 10^{pH - pK}$, M_b = molarity of the titrant, M = mass (g)

of reaction mixture and S = protein concentration in reaction mixture in mg mL^{-1}. DH was calculated from h as follows:

$$DH(\%) = (h/h_{tot}) \times 100,$$

where h_{tot} is the total number of peptide bonds in the protein. For pure proteins h_{tot} was estimated from the molecular weight of amino acid residues, which for most feed proteins is 120. Each determination was performed in triplicate.

Sequential analysis of protein hydrolysis products

Simultaneously with the measurement of DH, the reaction mixture was sampled (10 μL) at different given times to evaluate the progressive hydrolysis of proteins resulting from the activity of larval proteases. Samples were frozen at –20°C until analysed by SDS–PAGE according to Laemmli (1970) using 120 g kg^{-1} acrylamide and $8 \times 10 \times 0.075$ cm gels. Samples were thawed, diluted (1:1) in buffer (0.125 M Tris-HCl, pH 6.8 + 100 mL L^{-1} glycerol + 0.2 mg mL^{-1} bromophenol blue + 1.5% SDS) and boiled for 5 min. Each sample contained 35–40 μg protein and protein standards were also run (low molecular weight markers (LMWM) from Amersham Pharmacia-Biotech, Björkgatan, Uppsala, Sweden). Electrophoresis was run at 100 V for 45 min at 5°C. Gels were then fixed in 120 g L^{-1} TCA and stained with 1 mg mL^{-1} Coomassie blue (BBC R-250, Sigma) in methanol–acetic acid–water (50:20:50). Destaining was carried out in methanol–acetic acid–water (35:10:55) and the molecular mass (kDa) of hydrolysed proteins calculated from a linear plot of $\log_{10} M_r$ of protein standards (MWM) and relative mobility (R_f).

Changes resulting from protein hydrolysis were assessed after density scanning the gels for image analysis (Diversity One™; PDI, Huntington Station, NY, USA). Hydrolysis was assessed as a coefficient of protein degradation (CPD), as follows:

$$CPD = \sum_{i=1}^{n} \left[\frac{A_i(t=0) - A_i(t=90\ \text{min})}{A_i(t=0)} \times 100 \right] \frac{A_i(t=0)}{\sum_{i=1}^{n} A_i(t=0)}$$

where i = major protein bands identified from 1 to n, A_i = absorbance of the protein band i and t = total time of reaction.

Statistical methods

Data were analysed using specific software (CSS-Statistica®). Analysis of variance and the LSD test were used to compare DH. Simple regression analysis was performed to determine correlations between DH and CPD.

Table 1 Inhibition of protease activity measured in extracts obtained from 8-day-old seabream larvae following incubation with solutions of different microcapsules (MCs). The protein content of MCs was 50% of total dry weight; MCs AT and AB, contained bovine trypsinogen and a probiotic (BIOFEED-PRO), respectively

Microcapsule identification	Protein	Inhibition degree of protease activity (± SD)
A	Ovalbumin	54.9 ± 2.4
AT	Ovalbumin	58.0 ± 0.4
AB	Ovalbumin	62.2 ± 4.3
HB	Haemoglobin	13.3 ± 7.6
C	Casein	7.2 ± 6.7
CM	Cuttlefish meal	4.3 ± 2.1
CCM	Casein/cuttlefish meal (50/50)	n.d.
COM	Commercial; for shrimp	1.9 ± 1.0
R	Rotifers	n.d.[1]

[1] n.d. = not detected.

Results

Inhibition of larval enzymes by proteins used in the elaboration of MCs

Inhibition of larval protease activity (67.0 ± 5.4%) was induced by ovalbumin and thermal processing with either dry heat to 80°C (65.6 ± 0.3%) or steam (63.5 ± 0.5%) to destroy a possible thermolabile inhibitor, did not result in any reduction. Inhibition produced by other proteins was small (cuttlefish meal 9.5 ± 3.0%) or even undetectable (haemoglobin, soluble casein, fish meal). Assays performed with other chemical compounds used in the manufacturing of MCs showed little effect of such substances on larval protease activity (trimesoyl chloride 2.1 ± 1.4%, succinyl chloride 5.7 ± 1.4%, cyclohexane 4.0 ± 0.5%). Inhibition assays carried out using MCs prepared using the various proteins confirmed these results (Table 1). Addition of exogenous enzymes (bovine trypsinogen, BIOFEED; Novo Nordisk A.S. Bagsvaerd, Sweden) to ovalbumin MCs did not result in any increase in the activity of larval proteases.

Inhibition obtained with modified E:S ratios (ovalbumin or cuttlefish meal) is shown in Fig. 1. Simulation of an E:S ratio obtained after maximum intake of ovalbumin MCs by 8-day-old larvae (0.8 U mg^{-1}) resulted in a 40% inhibition of protease activity. In contrast, when cuttlefish meal-based MCs were tested, protease inhibition was low.

In vitro *digestibility of protein sources and MCs assessed by hydrolysis in a pH-stat*

Protein hydrolysis measured for the different MCs is shown in Table 2. The lowest values of DH were obtained for

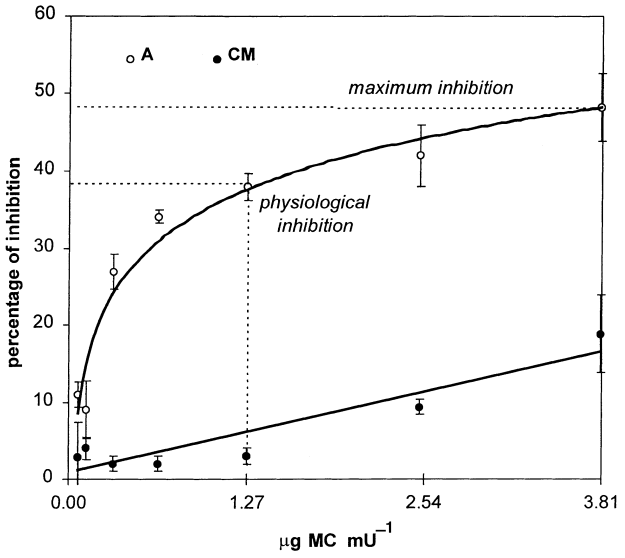

Figure 1 Inhibition of protease activity of larval extracts by ovalbumin (A) and cuttlefish meal (CM), measured under different enzyme:substrate (E:S) ratios. Maximum inhibition and that corresponding to the E:S ratio obtained after a normal intake (physiological) are indicated.

capsules containing ovalbumin (A and COM). DH was intermediate (6.3–7.6) for MCs prepared using casein (C), cuttlefish meal (CM) or a combination of both (CCM), and the highest DH (> 9.0) was obtained for MCs prepared using rotifers (R) as the major protein source.

Sequential analysis of the products of protein hydrolysis

SDS–PAGE was used to assess the progress of protein hydrolysis when either proteins or MCs were incubated in the presence of larval enzyme extracts. Protein bands at 25.7 and 33.1 kDa present in crude casein and casein MCs disappeared after only 20 min of digestion, resulting in high CPD values of 92.2 and 67.6%, respectively (Fig. 2). Cuttlefish meal or MCs including this protein were hydrolysed less rapidly, giving CPD values of 45.4% and 57.0%, respectively (Fig. 3). Hydrolysis measured in an MC prepared using a combination of casein and cuttlefish was greater than that of

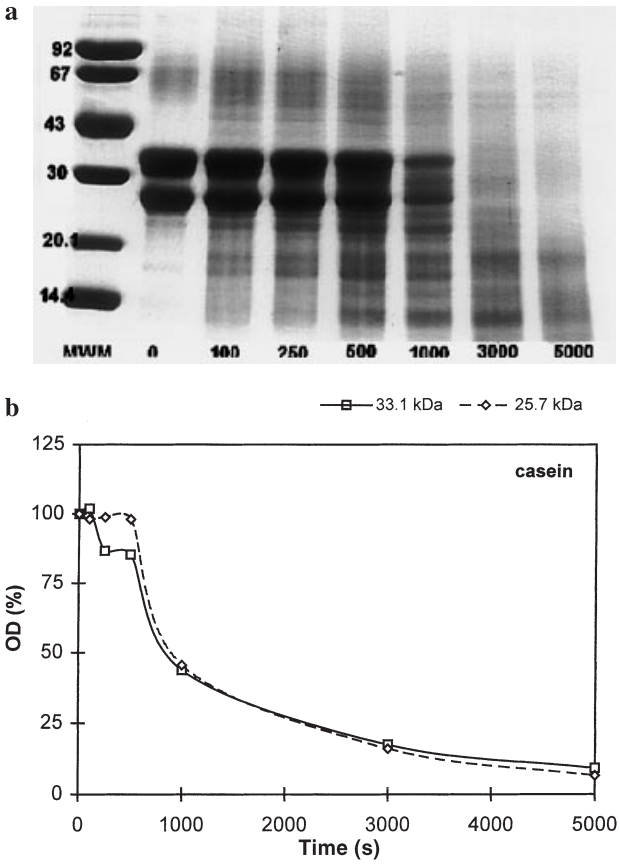

Figure 2 (a) SDS–PAGE profile obtained at different time intervals (in seconds) after digestion of casein using larval crude extracts. Molecular weight markers (MWM; 5 μg): phosphorylase b (92 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), SBTI (20.1 kDa) and α-lactalbumin (14.4 kDa). (b) Changes in optical density of the main protein bands with time of reaction.

MCs prepared using that protein sources alone, the CPD being 71.2.

When either commercial or laboratory-made MCs containing ovalbumin were tested, the main protein bands (131, 79.2, 43, 35.9 kDa) persisted over time. For the former, CPD was lower than that of other MCs (12%), and for the latter, no hydrolysis was measured after 90 min.

Table 2 Degree of hydrolysis (DH) of microcapsules measured after 90 min of digestion using pH-stat. Notation of microcapsules is as in Table 1

	Microcapsule type					
	R	CCM	C	CM	A	COM
DH	9.24 ± 0.52a	7.65 ± 0.35b	7.26 ± 0.67b	6.30 ± 0.75b	3.21 ± 0.43c	3.49 ± 0.59c

Values not sharing a common superscript are significantly different ($P < 0.05$).

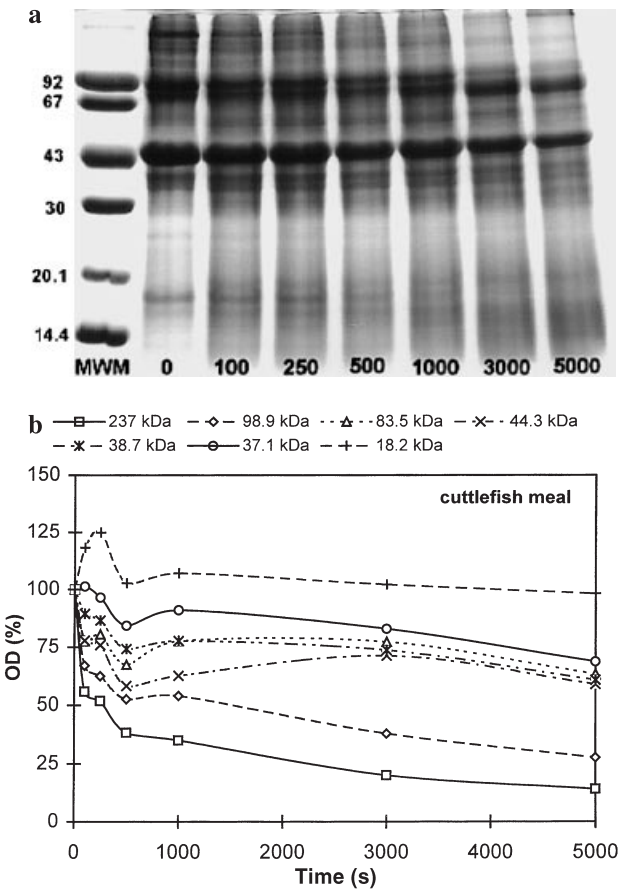

Figure 3 (a) SDS–PAGE profile obtained at different time intervals (in seconds) after digestion of cuttlefish meal protein using larval crude extracts. (b) Changes in optical density of the main protein bands with time of reaction.

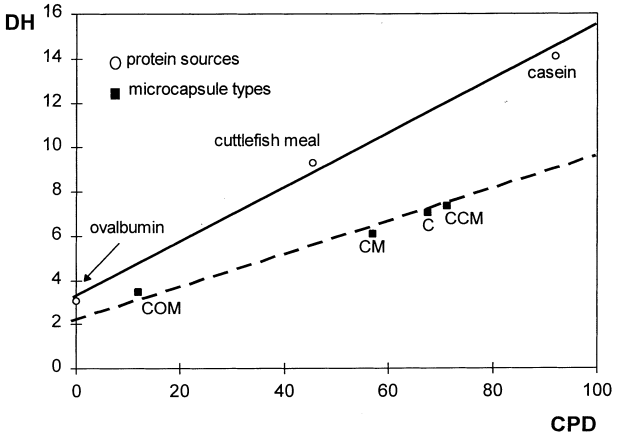

Figure 4 Correlation between protein degradation obtained using pH-stat (DH) and image analysis of gels (CPD). Equation for crude proteins is $y = 0.12x + 3.35$, $r^2 = 0.97$; equation for microcapsules is $y = 0.06x + 2.68$, $r^2 = 0.99$.

There was a highly significant correlation ($r^2 = 0.98$) between CPD and DH values for both protein sources and MCs (Fig. 4).

Discussion

Microcapsules have been developed for feeding larval marine fish (Walford *et al.* 1991; Holt 1993; Jones *et al.* 1993; Yúfera *et al.* 1996). Although such feeds have been accepted, poor growth has often resulted, possibly because of the poorly developed digestive capacity of the larvae. Larvae of some species (e.g. sea bass or bream) appear to have high protease and amylase activities at an early stage of development (Cahu & Zambonino 1995; Moyano *et al.* 1996) and it has been hypothesized that the limited utilization of artificial feeds may be related to partial inhibition of enzyme activities by some dietary ingredients. *In vitro* assays may provide information about these effects; such assays have proven to be useful for evaluation of proteins used in feeds for adult fish (Grabner 1985; Dimes *et al.* 1994) or shrimp (Lan & Pan 1993; Ezquerra 1997) and Oskicilzik & Chu (1996) showed that incubation of microcapsules with pure enzymes or larval seabass extracts gave an indication of their utilization *in vivo*.

The present study demonstrated that ovalbumin significantly reduced (60%) the activity of digestive proteases present in 8-day-old seabream larvae. Similar results were obtained when commercially produced microcapsules containing ovalbumin were tested using shrimp proteases (Alarcón *et al.* 1997). This may explain why poor results are obtained when such feed is used as the sole protein source in commercial shrimp cultures. In contrast, cuttlefish meal did not affect larval protease activity, which makes it good candidate for the preparation of MCs. Proteases did not appear to be inhibited by other chemicals (emulsifiers, polymerizing agents) used in the preparation of the microcapsules.

The construction of inhibition curves allowed a more detailed assessment of the effects that ovalbumin and cuttlefish meal had on protease activity (Fig. 1). The inhibition curves for ovalbumin showed that, even when relative concentrations of enzyme to MC were below those expected for a 'normal' intake, there was a reduction in larval protease activity by 20–40%. In contrast, cuttlefish meal-based MCs did not inhibit larval proteases within the same range. These results would point to the inadequacy of ovalbumin and the suitability of cuttlefish meal as ingredients in artificial feeds for seabream larvae.

The measurement of DH can be used as an estimation of bioavailability of proteins (Eggum *et al.* 1989; McDonough *et al.* 1990; Swaisgood & Catignani 1991). The results

obtained in this study confirmed the low bioavailability of protein in ovalbumin-based MCs, the DH for ovalbumin being lower than those obtained with MCs prepared using other proteins (Table 2). The DH of protein in casein-based MCs implies that this protein source should be suitable for the preparation of feeds for seabream larvae, in agreement with the favourable results obtained when casein has been used in feeds for larval prawns (Teshima *et al*. 1982; Koshio *et al*. 1989).

Sequential sampling of the reaction mixture, performed simultaneously with DH measurements, followed by visualization of protein fractions using SDS–PAGE, allowed evaluation of protein hydrolysis produced by the larval proteases. Disappearance of bands characterizing a protein source, together with the appearance of new bands corresponding to protein fractions, was considered a good indicator of the bioavailability of a protein. Using this method, it was possible to confirm the low bioavailability of ovalbumin, which remained undigested during the assay period. Protein hydrolysis proceeded to variable extents (from 30 to 70%) for MCs prepared using casein, cuttlefish meal, or a combination of both, and the CPD was significantly correlated with DH. Different regressions were obtained when native proteins and MCs were considered (Fig. 4), probably because of the modification of protein structure during the process of microcapsulation. The existence of such correlations suggests that either parameter (DH or CPD) may be used as a predictor of protein bioavailability *in vivo*. DH gives information about the degradability of the protein as a whole, whereas CPD makes it possible to provide a more detailed analysis of the course of digestion. The latter may be especially useful when evaluating feeds formulated to contain different protein sources.

The extent to which the *in vitro* assessments reflect *in vivo* phenomena still needs to be elucidated. Nevertheless, the information obtained from *in vitro* assays may provide an explanation for why ovalbumin-based MCs gave poor results *in vivo* (Yúfera *et al*. 1995). Further, *in vitro* assessment could result in improved MC formulation. For example, a casein/cuttlefish meal-based MC has been fed to sea bream larvae from day 5 to day 20 and survival rates equivalent to those of larvae fed on zooplankton were obtained (Yúfera *et al*. 1998).

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