Aquaculture 474 (2017) 95–100

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Fixation of bioactive compounds to the cuticle of *Artemia*

David Talens-Perales[a], Julia Marín-Navarro[a], Diego Garrido[b], Eduardo Almansa[b], Julio Polaina[a,*]

[a] Instituto de Agroquímica y Tecnología de Alimentos, CSIC, Paterna, Valencia, Spain
[b] Instituto Español de Oceanografía, Tenerife, Spain

ARTICLE INFO

Keywords:
Arthropods cuticle
β-galactosidase
Carbohydrate binding domain
Chimeric protein
Chitin
GFP

ABSTRACT

Artemia is extensively used in aquaculture to feed early stages of cultured marine species. A problem associated with this practice is that *Artemia* fails to supply some essential nutrients. As a possible solution, we have devised a procedure to make *Artemia* a vehicle for exogenous nutrients and other bioactive compounds. It consists of the construction of chimeric proteins composed of a chitin-binding domain, which binds to the cuticle of *Artemia*, and a carrier domain that conveys a functional property. As confirmatory examples, we describe the successful fixation to *Artemia*'s metanauplii of two hybrid proteins: a β-galactosidase from the thermophilic bacterium *Thermotoga maritima* and the jellyfish green fluorescent protein (GFP), both linked to the CBM2 chitin-binding domain from the hyperthermophilic archaeon *Pyrococcus furiosus*. Positive results of experiments carried out *ex vivo* and *in vivo* show the validity of this approach. The methodology used could become a general procedure for the attachment of different kinds of bioactive compounds, such as enzymes, hormones, antibiotics, *etc.*, to the cuticle of *Artemia* as well as other arthropods.

Statement of relevance: Our results overcome shortcomings of *Artemia* as a feedstock.

1. Introduction

Brine shrimp (*Artemia* sp.) is a remarkable living organism because of its role as an experimental model for different studies and its use as feedstuff in aquaculture (Podrabsky and Hand, 2015; Gajardo and Beardmore, 2012; Nunes et al., 2006). *Artemia* is the most frequent constituent of the diet of larval stages of all sorts of marine species, such as fish, crustaceans or cephalopods. The predatory nature of these species makes necessary the use of living preys instead of inert microdiet formulations. However, a serious limitation of *Artemia* based diet is its deficiency in some nutrients, for instance highly unsaturated fatty acids (HUFA) and vitamins among others (Navarro et al., 2014; Takeuchi, 2014). Larvae of marine species have a specific requirement of these compounds, which are essential for growth, embryonic and larval development and reproduction (Tocher, 2010; Monroig et al., 2013; Takeuchi, 2014; Reis et al., 2015). Therefore, considerable effort has been invested to enrich *Artemia* with essential nutrients, using different strategies (Coutteau and Sorgeloos, 1997; Dhert et al., 1998; Støttrup and McEvoy, 2003; Monroig et al., 2006). So far, these efforts have not yet provided the expected results. An example is the attempt to enrich *Artemia* with docosahexaenoic acid (22: 6n-3, DHA), a key fatty acid for larval development. The metabolism of the brine shrimp converts a fraction of ingested DHA into another fatty acid (eicosapentaenoic acid, 20: 5n-3, EPA) (Navarro et al., 1999). Non-converted DHA is stored with triglycerides (Bell et al., 2003; Guinot et al., 2013), which significantly reduces its digestibility since the larvae have a rudimentary digestive system with low levels of lipase and emulsifying substances (Olsen et al., 2014). Similarly, the carotenoid astaxanthin, a precursor of vitamin A with antioxidant properties, is converted into canthaxanthin (Davies et al., 1970).

Aforementioned results highlight the need to find effective ways to enrich the *Artemia* with HUFA, and likely other nutrients. In this study we propose a methodology to achieve this goal. As a general property of arthropods, the body of *Artemia* is covered by an exoskeleton that serves as a protective surface and barrier against pathogens. The main structure of the exoskeleton is the cuticle whose most abundant constituent is chitin, a polysaccharide composed crystalline units of *N*-acetyl-2-D-glucosamine (Horst and Freeman, 1993; Abatzopoulos et al., 2002). Chitin therefore represents a potential target at which adhering molecules or molecular complexes.

Carbohydrate binding modules (CBMs) are non-catalytic protein domains frequently present in enzymes involved in the hydrolysis of polysaccharides, like cellulases, amylases, xylanases, chitinases, *etc.*, whose function is the attachment of the catalytic machinery to the substrate (Gilbert et al., 2013; Guillén et al., 2010). The hypothesis of this work is that being chitin the major component of the exoskeleton of arthropods, a chitin binding module could be used to attach proteins and other bioactive compounds to the body of *Artemia*. The CBM that

* Corresponding author.
E-mail address: jpolaina@iata.csic.es (J. Polaina).

http://dx.doi.org/10.1016/j.aquaculture.2017.03.044
Received 12 September 2016; Received in revised form 21 March 2017; Accepted 25 March 2017
Available online 27 March 2017
0044-8486/

we selected for being used in this work is part of a chitinase from *Pyrococcus furiosus*, belonging to family 2 of carbohydrate binding domains in the CaZY database. Based on the mode of interaction with the substrate, it is a type A CBM characterized by possessing a flat, platform-like binding area rich in aromatic residues that interacts the planar structures of certain polysaccharides, such as cellulose or chitin (Boraston et al., 2004; Nakamura et al., 2008).

2. Materials and methods

2.1. Construction and production of hybrid proteins

Modular elements used for the construction of hybrid proteins were the β-galactosidase from *Thermotoga maritima* (TmLac) (Marín-Navarro et al., 2014), the chitinase binding module (CBM2) from *Pyrococcus furiosus* chitinase (Nakamura et al., 2008) and the green fluorescent protein (GFP) (Chalfie et al., 1994).

For the construction of hybrid TmLac-CBM2, the TmLac coding sequence excluding the stop codon was amplified by PCR using as template plasmid TmLac-pQE (Marín-Navarro et al., 2014) and primers JM771 (CACGAGCTCAAGAATATGCCCTACGAATGGG) and DT889 (AGCCGTCGACCCTCACGTAGATAGTTTTTCTCGTG). CBM2 from *Pyrococcus furiosus* (Nakamura et al., 2008) was amplified from a commercial preparation of genomic DNA of the archaea purchased from the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH (catalog# DSM3638) with primers 2DT23 (ATGAGTCGACGGCGCAACAACTACCCCTGTCCCAGTCTC) and 2DT24 (GGAGACAAGCTT AATTACTTGTCCGTTTATTTCTAGGGTTATTTCC). TmLac and CBM2 PCR products were digested with *Sac*I/*Sal*I or *Sal*I/*Hin*dIII, respectively and mixed with vector pQE80L (Qiagen) digested with *Sac*I/*Hin*dIII. The DNA fragments in the mixture were ligated and then used to transform *E. coli*. The resulting plasmid was named TmLac-CBM2-pQE.

GFP was amplified from plasmid pCAMBgfp (Sesma and Osbourn, 2004) with oligonucleotides 2DT53 (CCAGAGCTCAGCAAGGGCGAGGAGCTG) and 2DT52 (GTGCTGCAGTTACTTGTACAGCTCGTCCATGCC). The PCR product was digested with *Sac*I and *Pst*I and cloned in vector pQE-80L. The resulting plasmid was labelled GFP-pQE. In parallel, the GFP gene excluding the stop codon was amplified with oligonucleotides 2DT53 and 2DT51 (ATGAGTCGACCTTGTACAGCTCGTCCATGCC). The PCR product was digested with *Sac*I and *Sal*I and cloned in a disrupted version of plasmid TmLac-CBM2-pQE, in which the TmLac had been excised with the same restriction enzymes. The resulting plasmid was named GFP-CBM2-pQE.

E. coli Rosetta 2 strain (EMD Millipore) was used as the host for all gene constructs and for protein production. Crude cell extracts were prepared from induced transformants harboring the TmLac-pQE (Marín-Navarro et al., 2014), TmLac-CBM2-pQE, GFP-pQE or GFP-CBM2-pQE plasmids. Recombinant proteins were purified by heat shock treatment, 2 min at 85 °C (in the case of thermoresistant proteins TmLac-pQE and TmLac-CBM2-pQE) and nickel affinity chromatography using a 1 mL HisTrap FF Crude column (GE Healthcare) mounted in an ÅKTA-Purifier (GE Healthcare). Eluted protein was dialyzed against buffer (20 mM Tris–HCl, pH 7.5. 50 mM NaCl).

2.2. Artemia culture

Artemia cysts (Sep-Art AF, INVE Aquaculture, Dendermond, Belgium) were hatched in 45 L cylinder conical fiberglass tank with seawater (36.8 PSU), at 29 °C for 24 h, under 2000 lx illumination and vigorous aeration. Germinated cysts were grown for 4 days (to metanauplius stage) in a 500 L cylinder conical fiberglass tank with seawater, at 23 °C, at densities of 5 individuals per ml, fed with freeze-dried cells of *Isochrysis galbana* (easy algae®, Cádiz, Spain) added every day up to a density of 5×10^5 cells per ml, with mild aeration. Daily, 30% of water volume of the tank was renewed. Filtered seawater (1 μm) was used for growing and handling *Artemia*. For testing protein binding to the cuticle (*ex vivo* experiments) *Artemia* was harvested, washed gently with distilled water and kept in 70% ethanol.

2.3. Binding of TmLac to Artemia and assay of β-galactosidase activity

Metanauplii preserved in 70% ethanol were washed with water, and heated at 95 °C for 20 min to inactivate endogenous β-galactosidase activity that otherwise would interfere with the enzyme assays. Treated metanauplii were suspended in binding buffer (50 mM Tris, pH 8.5) at concentration of 75 mg/mL, and incubated with either TmLac or TmLac-CBM2 proteins at a 1 μM final concentration, during 4 h at 37 °C with gentle agitation. The metanauplii were collected by centrifugation, saving the supernatant to evaluate the amount of enzyme not bound, and washed five times in binding buffer before measuring the enzyme activity bound to the *Artemia*. TmLac β-galactosidase activity was assayed at 75 °C by two procedures, using either p-nitro phenyl β-D-galactopyranoside (pNPGal) or lactose as the substrate. The pNPGal hydrolysis assay was carried out in 50 mM phosphate, pH 6.5 buffer, with the substrate at a final concentration of 5 mM (Marín-Navarro et al., 2014). Alternatively, lactose was used at 5% w/v, in the same buffer. Reactions were stopped at 95 °C for 10 min. The lactase activity was determined by measuring the amount of glucose released with a glucose assay kit (Sigma). One unit of activity was defined as the amount of enzymes that releases 1 μmol of glucose per min.

2.4. Binding of GFP to Artemia and fluorescence microscopy

Metanauplii kept in ethanol were washed in water as before and suspended at a concentration of 20 mg/mL in binding buffer containing 1 mg/mL BSA and kept overnight at room temperature. Subsequently they were incubated in the same buffer with either GFP or GFP-CBM2 proteins at 1 μM concentration, during 4 h at 37 °C with gentle agitation. Afterwards, the metanauplii were collected by centrifugation and washed 3 times with the same buffer, keeping the last wash for several hours at room temperature. Finally, the specimens were mounted on a slide and examined in an Eclipse 90i fluorescence microscope (Nikon). For *in vivo* binding assays, metanauplii samples (*ca.* 100 individuals) were kept at room temperature (*ca.* 20 °C) for 16 h in 5 ml of sea water to which GFP-CBM2 protein was added at 1 μM concentration.

3. Results

3.1. Targeting of TmLac

The construction of plasmid TmLac-pQE, expressing the TmLac encoding gene has been described before (Marín-Navarro et al., 2014). The genetic construction made to produce enzyme TmLac-CBM2 and the modelled tertiary structure of the hybrid protein are represented in Fig. 1. A hybrid gene consisting of an in-frame fusion of the coding sequences of both protein modules, linked by a Gly-Ala dipeptide, was expressed in *E. coli*. The protein model is based on a previously reported model of TmLac (Talens-Perales et al., 2016) and the CBM2 crystallographic structure (Nakamura et al., 2008). Both versions of β-galactosidase, with and without the chitin binding module, at a concentration of 1 μM were used to treat a suspension of metanauplii at a concentration of 75 mg/mL. The initial amount of the enzyme and the leftover after incubation with *Artemia* were quantified using pNPGal as the substrate. Results presented in Fig. 2 (left panel) show that whereas no significant binding of TmLac was observed, about one-third of the added TmLac-CBM2 was linked to the metanauplii. This represents about 0.5 mg of enzyme per gram of *Artemia*. A more precise quantification of enzyme activity bound to metanauplii treated with either TmLac or TmLac-CBM2 was carried out using lactose as the substrate (Fig. 2, right panel).

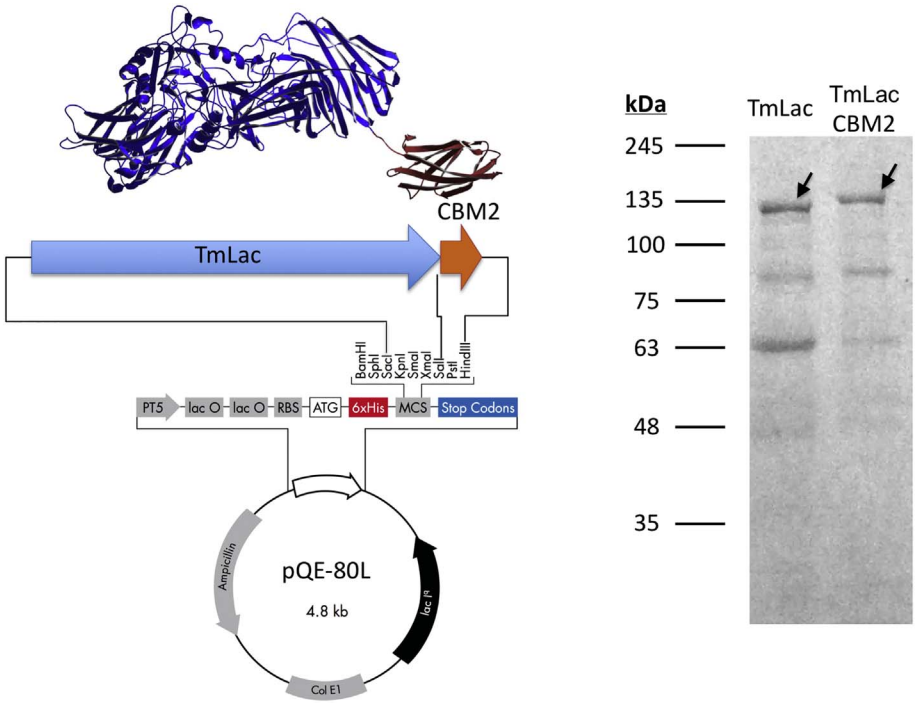

Fig. 1. Construction, production and purification of hybrid protein TmLac-CBM2. Left panel: cloning of TmLac-CBM2 coding sequence in *E. coli* and proposed structure of the hybrid protein. Right panel: SDS-PAGE with purified samples of TmLac (129 kDa) and hybrid TmLac-CBM2 (140 kDa) (pointed by arrows).

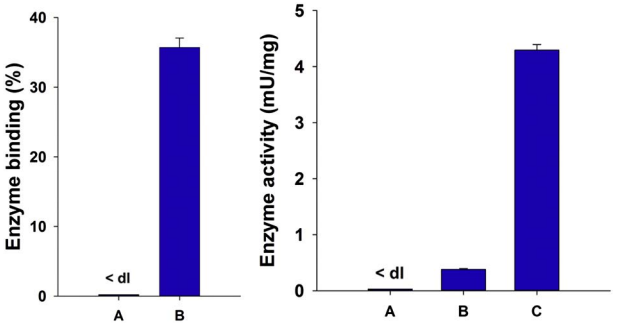

Fig. 2. Binding of β-galactosidase to *Artemia*. Left panel: enzyme bound to metanauplii, determined as % of initial activity. A, TmLac; B, TmLac-CBM. Right panel: enzyme activity (mU lactase) per mass (mg) of metanauplii resulting of three treatments. A, without enzyme; B, with TmLac; C, with TmLac-CBM2. < dl: under detection limit.

3.2. Targeting of GFP

A scheme similar to that used to produce TmLac-CMB2 was used for GFP and GFP-CBM2 (Fig. 3). The same Gly-Ala dipeptide was used to connect the two protein modules. A crystallographic structure of GFP (Jain and Ranganathan, 2004) was used to model the hybrid protein. Metanauplii treated with either GFP or GFP-CBM2 were examined under the microscope. Results presented in Fig. 4 show clear fluorescent labelling in the specimen treated with GFP-CBM2 (Fig. 4C). In contrast, treatment with GFP (Fig. 4B) yielded a low level of fluorescence, comparable to that of the untreated specimen (Fig. 4A). Most biological materials have to some degree inherent fluorescent emission, detectable when examined under fluorescent microscopy. Specifically, autofluorescence of crustacean cuticle has been reported (Michels, 2007). Interestingly, as it is clearly seen in (Fig. 4C), the fluorescent labelling resulting from GFP-CBM2 treatment occurs both on the body surface of the metanauplius and inside the body. This observation agrees well with the known structure of the peritrophic membrane made of chitin that covers the gut epithelium of arthropods (Eisemann and Binnington, 1994).

3.3. In vivo assays

Preliminary assays showed that the living metanauplii did not survive incubation, even for a short time, in the binding buffer (50 mM Tris, pH 8.5) used for *ex vivo* experiments. Therefore, the treatment was carried out in sea water to which the binding protein was added. Survival was not affected after 16 h of incubation. Fig. 5 shows the results of fluorescent microscopy observation of samples examined after the treatment.

4. Discussion

In this work we have tested the use of a chitin binding module as a vehicle for the attachment of proteins to the exoskeleton of arthropods. Since chitin is the main chemical component of the cuticle, our approach may seem quite obvious. However, factors concerning the structural and chemical complexity of the exoskeleton make the question less simple. For instance, the cuticle is covered by a lipid-rich external layer, the epicuticle, which could act as a barrier impeding the attachment of chitin-binding molecules. The presence of other chemical components, such as proteins, mineral salts, *etc.*, normally associated to the chitin, could also have a hindering effect (Horst and Freeman, 1993; Abatzopoulos et al., 2002). To overcome these problems we have used four days old metanauplii, whose active growth requires a high rate of chitin synthesis. We assumed that at this stage chitin must appear exposed, still free of other chemical components that shall accumulate at later stages of development to form the epicuticle. On the other hand, the early stages of *Artemia* (0–4 days old) are preferred as feedstuff in aquaculture. Therefore, implementing the binding of bioactive compounds at these early stages is also convenient for eventual applications. Initial experiments we carried out with *ex vivo* material for practical reasons. Protein binding treatments, washes, quantification, replicate experiments, *etc.*, required a considerable mass of *Artemia* that would be difficult to handle *in vivo*. Nevertheless, once the operational conditions were stablished we have been able to show that the procedure can be carried out in sea water with living *Artemia*.

Thermotoga maritima β-galactosidase proved to be useful as an

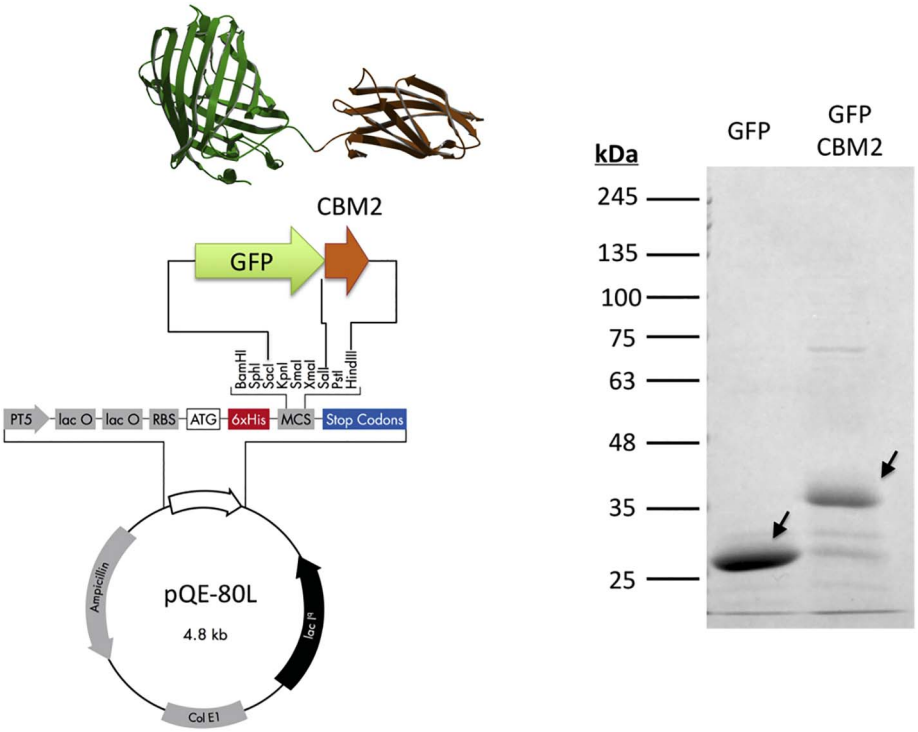

Fig. 3. Construction, production and purification of hybrid protein GFP-CBM2. Left panel: cloning of GFP-CBM2 coding sequence in *E. coli* and proposed structure of the hybrid protein. Right panel: SDS-PAGE with purified samples of GFP (28 kDa) and hybrid GFP-CBM2 (40 kDa) (pointed by arrows).

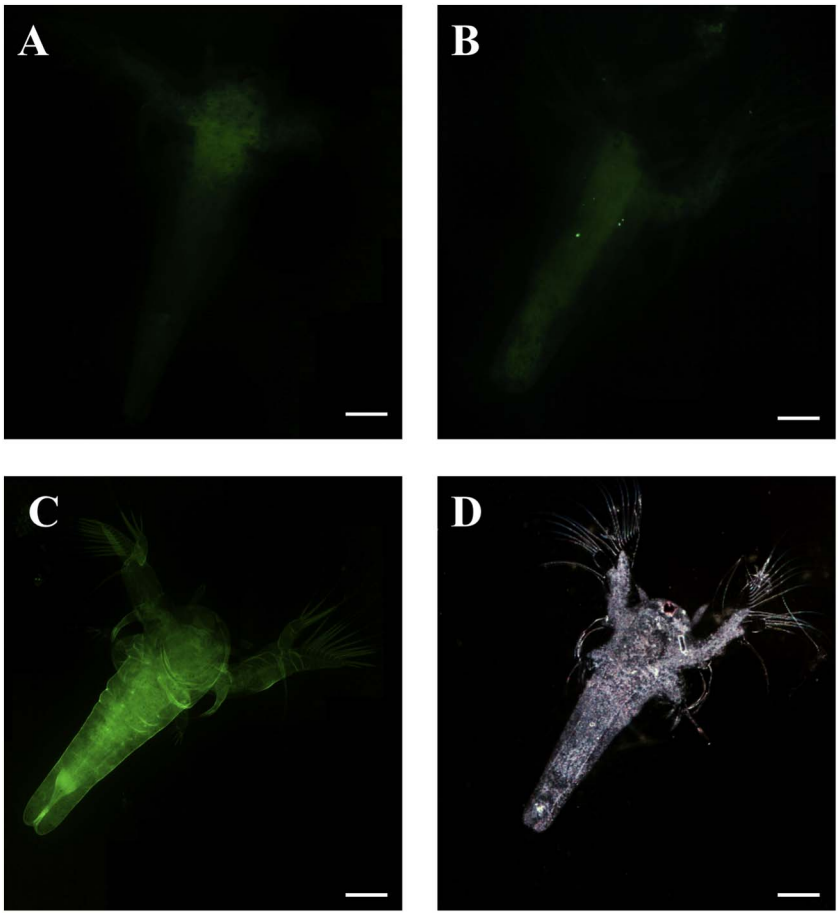

Fig. 4. Micrographs of *Artemia* metanauplii under fluorescent (A, B and C) or dark field (D) microscopy from *ex vivo* binding assays. Specimens shown in A and D were not subjected to any treatment. Specimens shown in B and C were treated with GFP and GFP-CBM2, respectively. The white bars correspond to 100 μm.

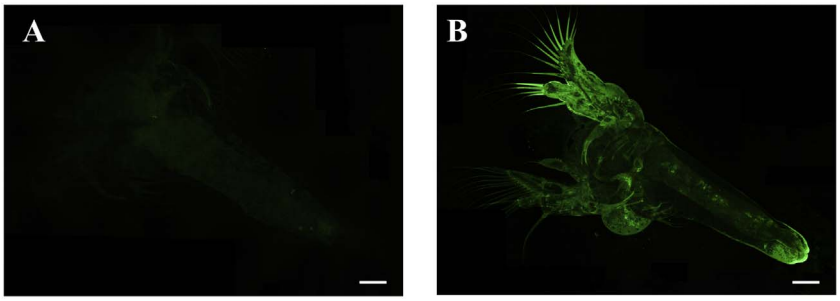

Fig. 5. Micrographs of living *Artemia* metanauplii under fluorescent microscopy from *in vivo* binding assays. The specimen shown in A was not subjected to any treatment. Specimen shown in B was incubated in sea water containing the GFP-CBM2 protein. The white bars correspond to 100 μm.

enzyme reporter to test the attachment of proteins to *Artemia*. TmLac shares high sequence similarity (*ca.* 40% amino acid identity) with *E. coli* β-galactosidase, commonly used as a reporter in gene cloning procedures (Talens-Perales et al., 2016). Moreover, TmLac has the property of being thermoresistant, which represents an advantage in many instances. Preliminary experiments carried out to assay the binding of Tm-Lac and TmLac-CBM2 to metanauplii, revealed that control samples that had not been treated with any enzyme gave considerable levels of β-galactosidase activity. This seems to be a logical consequence of the presumably high level of digestive enzymes required to assimilate phytoplankton components and other micro-organisms that constitute *Artemia*'s diet. To avoid this interference, endogenous β-galactosidase activity had to be eliminated previously to carry out the treatment with TmLac, which was achieved by heating the nauplii at 95 °C for 20 min. Our results show specific binding of protein, *ca.* 0.5 mg of metanauplii in the conditions tested. Moreover, the bound enzyme remained fully functional.

GFP is another extensively used reporter that confirmed the validity of CBM2-mediated targeting to *Artemia*. Additionally, the GFP-CBM2 construction allows direct visualization at the cellular level of those areas where binding has been produced, indicating the presence of exposed chitin. This could be used to follow the deployment and chemical modification of the chitin that takes place along the developmental stages of *Artemia* or other arthropods.

In summary, here we describe a procedure by which proteins with different structure and functional properties can be efficiently targeted to the body of *Artemia*. The methodology used could be adapted for the attachment of different kinds of bioactive compounds such as enzymes, hormones, immunomodulatory substances, and antibiotics.

5. Conclusions

This communication describes a procedure for the attachment of proteins to the cuticle of *Artemia.* The technique involves the construction of a hybrid protein in which the selected protein is fused to a chitin binding domain. As a proof of concept, two different proteins, an enzyme (β-galactosidase) and the green fluorescent protein, have been successfully targeted to the cuticle. These results support the idea that by using other proteins with the capability of binding lipids, hormones, vitamins, antibiotics, *etc.*, these compounds could be imprinted into the body of arthropods. Protein binding can be carried out in sea water using living *Artemia*, offering a good prospect for potential applications in aquaculture.

Acknowledgments

This work was supported by grants BIO2013-48779-C4-3-R and AGL2013-49101-C2-1-R from Spain's 'Secretaría de Estado de Investigación, Desarrollo e Innovación'. D T-P was supported by a FPU fellowship from 'Ministerio de Economía y Competitividad' (AP2012-0901) and D. Garrido by a fellowship from the Spanish Institute of Oceanography (BOE 03/11/2011).

References

Abatzopoulos, T.J., Beardmore, J.A., Clegg, J.S., Sorgeloos, P. (Eds.), 2002. *Artemia*: Basic and Applied Biology. Kluwer Academic Publishers, Dordrecht.
Bell, J.G., McEvoy, L.A., Estevez, A., Shields, R.J., Sargent, J.R., 2003. Optimising lipid nutrition in first-feeding flatfish larvae. Aquaculture 227, 211–220.
Boraston, A.B., Bolam, D.N., Gilbert, H.J., Davies, G.J., 2004. Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochem. J. 382, 769–781.
Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., Prasher, D.C., 1994. Green fluorescent protein as a marker for gene expression. Science 263, 802–855.
Coutteau, P., Sorgeloos, P., 1997. Manipulation of dietary lipids, fatty acids and vitamins in zooplankton cultures. Freshw. Biol. 38, 501–512.
Davies, B.H., Hsu, W.J., Chichester, C.O., 1970. The mechanism of the conversion of beta-carotene into canthaxanthin by the brine shrimp, *Artemia salina* L. (Crustacea: Branchiopoda). Comp. Biochem. Physiol. 33, 601–615.
Dhert, P., Divanach, P., Kentouri, M., Sorgeloos, P., 1998. Rearing techniques for difficult fish larvae. World Aquacult. 29, 48–55.
Eisemann, C.H., Binnington, K.C., 1994. The peritrophic membrane: its formation, structure, chemical composition and permeability in relation to vaccination against ectoparasitic arthropods. Int. J. Parasitol. 24, 15–26.
Gajardo, G.M., Beardmore, J.A., 2012. The brine shrimp *Artemia*: adapted to critical life conditions. Front. Physiol. 3, 185.
Gilbert, H.J., Knox, J.P., Boraston, A.B., 2013. Advances in understanding the molecular basis of plant cell wall polysaccharide recognition by carbohydrate-binding modules. Curr. Opin. Struct. Biol. 23, 669–677.
Guillén, D., Sánchez, S., Rodríguez-Sanoja, R., 2010. Carbohydrate-binding domains: multiplicity of biological roles. Appl. Microbiol. Biotechnol. 85, 1241–1249.
Guinot, D., Monroig, O., Hontoria, F., Amat, F., Varó, I., Navarro, J.C., 2013. Enriched on-grown *Artemia* metanauplii actively metabolise highly unsaturated fatty acid-rich phospholipids. Aquaculture 412-413, 173–178.
Horst, M.N., Freeman, J.A. (Eds.), 1993. The Crustacean Integument: Morphology and Biochemistry. CRC Press.
Jain, R.K., Ranganathan, R., 2004. Local complexity of amino acid interactions in a protein core. Proc. Natl. Acad. Sci. U. S. A. 101, 111–116.
Marín-Navarro, J., Talens-Perales, D., Oude-Vrielink, A., Cañada, F., Polaina, J., 2014. Immobilization of thermostable β-galactosidase on epoxy support and its use for lactose hydrolysis and galactooligosaccharides biosynthesis. World J. Microbiol. Biotechnol. 30, 989–998.
Michels, J., 2007. Confocal laser scanning microscopy: using cuticular autofluorescence for high resolution morphological imaging in small crustaceans. J. Microsc. 227 (Pt 1), 1–7.
Monroig, O., Navarro, J.C., Amat, F., González, P., Bermejo, A., Hontoria, F., 2006. Enrichment of *Artemia* in essential fatty acids with different types of liposomes and their use in the rearing of gilthead sea bream (*Sparus aurata*) larvae. Aquaculture 251, 491–508.
Monroig, O., Tocher, D.R., Navarro, J.C., 2013. Biosynthesis of polyunsaturated fatty acids in marine invertebrates: recent advances in molecular mechanisms. Mar. Drugs 11, 3998–4018.
Nakamura, T., Mine, S., Hagihara, Y., Ishikawa, K., Ikegami, T., Uegaki, K., 2008. Tertiary structure and carbohydrate recognition by the chitin-binding domain of a hyperthermophilic chitinase from *Pyrococcus furiosus*. J. Mol. Biol. 381, 670–680.
Navarro, J.C., Henderson, R.J., McEvoy, L.A., Bell, M.V., Amat, F., 1999. Lipid conversions during enrichment of *Artemia*. Aquaculture 174, 155–166.
Navarro, J.C., Monroig, O., Sykes, A.V., 2014. Nutrition as a key factor for cephalopod aquaculture. In: Iglesias, J., Fuentes, L., Villanueva, R. (Eds.), Cephalopod Culture. Springer, pp. 77–95.
Nunes, B.S., Carvalho, F.D., Guilhermino, L.M., Van Stappen, G., 2006. Use of the genus *Artemia* in ecotoxicity testing. Environ. Pollut. 144, 453–462.
Olsen, Y., Evjemo, J.O., Kjørsvik, E., Larssen, H., Li, K., Overrein, I., Rainuzzo, J., 2014. DHA content in dietary phospholipids affects DHA content in phospholipids of cod larvae and larval performance. Aquaculture 428-429, 203–214.
Podrabsky, J.E., Hand, S.C., 2015. Physiological strategies during animal diapause: lessons from brine shrimp and annual killifish. J. Exp. Biol. 218, 1897–1906.
Reis, D.B., García-Herrero, I., Riera, R., Felipe, B.C., Rodríguez, C., Sykes, A.V., Martín, M.V., Andrade, J.P., Almansa, E., 2015. An insight on *Octopus vulgaris* paralarvae lipid requirements under rearing conditions. Aquac. Nutr. 21, 797–806.
Sesma, A., Osbourn, A.E., 2004. The rice leaf blast pathogen undergoes developmental

processes typical of root-infecting fungi. Nature 431, 582–586.
Støttrup, J.G., McEvoy, L.A., 2003. Live Feeds in Marine Aquaculture. Blackwell Publ. Co., 350 Main St., Maldon, MA 02148-5018, pp. 318.
Takeuchi, T., 2014. Progress on larval and juvenile nutrition to improve the quality and health of seawater fish: a review. Fish. Sci. 80, 389–403.
Talens-Perales, D., Polaina, J., Marín-Navarro, J., 2016. Structural dissection of the active site of *Thermotoga marítima* β-galactosidase identifies key residues for transglycosylating activity. J. Agric. Food Chem. 64, 2917–2924.
Tocher, D.R., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquac. Res. 41, 717–732.