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Fixation of bioactive compounds to the cuticle of *Artemia*

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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

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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).

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