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The effect of lectins on the attachment and invasion of *Enteromyxum scophthalmi* (Myxozoa) in turbot (*Psetta maxima* L.) intestinal epithelium *in vitro*

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ABSTRACT

The involvement of the lectin/carbohydrate interaction in the invasion of the turbot intestinal epithelium by *Enteromyxum scophthalmi* was studied *in vitro* using explants of turbot intestine and pre-treatment of parasite stages with the plant lectins of *Canavalia ensiformis* (Con A) and *Glycine max* (SBA). Both lectins inhibited the attachment and invasion of *E. scophthalmi* stages to the intestinal epithelium, though the inhibitory effect was higher for SBA than for Con A. Such results point to the involvement of *N*-acetylgalactosamine (GalNAc) and galactose (Gal) residues and also of mannose/glucose residues in the *E. scophthalmi*-intestinal epithelium interaction. The inhibitory effect of both lectins on the parasite adhesion and penetration points to the interest of further studies to confirm the presence of putative lectins recognising GalNAc-Gal and mannose/glucose residues in turbot intestine. The obtained results demonstrated also the adequacy of turbot intestinal explants as an *in vitro* model to study the interaction with *E. scophthalmi*.

1. Introduction

The phylum Myxozoa includes a large number of species most of them parasites of fish. Some myxozoans cause disease and impact upon wild and farmed fish populations. Enteromyxosis caused by *Enteromyxum scophthalmi* (Palenzuela et al., 2002) is among the most severe parasitic diseases in mariculture, producing serious loses in turbot *Psetta maxima* (L.) (Branson et al., 1999; Quiroga et al., 2006). *E. scophthalmi* is highly specific for the digestive tract, mainly for the intestine, the target organ.

Infection by pathogens is generally initiated by the specific recognition of host epithelia surfaces. Receptors present in the mucin layer can act as binding sites in the subsequent adhesion, which is essential for invasion. The lectin/glycoconjugate interactions, characterized by their high specificity, are known to play a significant role in the adhesion of bacteria and parasites and in their interaction with the host. In their infection strategy, microorganisms often use sugar-binding proteins, such as lectins and adhesins to recognise and bind to host glycoconjugates (Imberty and Varrot, 2008). The blocking or inhibition of microbial lectins by suitable carbohydrates or their analogous is the aim of anti-adhesion therapy for the prevention and treatment of infectious diseases (Sharon, 2006). In addition, many carbohydrate residues present on the surface of parasites are specifically recognised by host lectins (Jacobson and Doyle, 1996; Nyame et al., 2004; Hammerschmidt and Kurtz, 2005). These carbohydrate structures of parasites can be used as prototypes for the chemical or combined chemo-enzymatic synthesis of new compounds for diagnosis and vaccine development, or as inhibitors specifically designed to target glycan byosynthesis (Mendoça-Previato et al., 2005). Previous studies have demonstrated the binding of several plant lectins to carbohydrate residues present in *E. scophthalmi* stages (Redondo et al., 2008) and in the epithelial surface of turbot intestine, and a role of lectin/carbohydrate interaction in the turbot-*E. scophthalmi* relationship has been suggested (Redondo and Alvarez-Pellitero, 2009).

The unavailability of *in vitro* cultures of myxozoans, makes particularly difficult the studies on the interaction parasite/host. However, in the case of *E. scophthalmi*, the life cycle of the parasite can be experimentally maintained *in vivo* using effluent, cohabitation or oral infections (Redondo et al., 2002). In addition, turbot intestinal explants have been used *in vitro* to demonstrate the adhesion and penetration of this parasite into the intestinal epithelium (Redondo et al., 2004). In the present work, the involvement of the lectin/carbohydrate interaction in the invasion of the epithelium by *E. scophathalmi* was studied *in vitro* using explants of turbot intestine and pre-treatment of parasite stages with *Canavalia ensiformis* (Con A) and *Glycine max* (SBA), the two lectins showing the highest binding activity to *E. scophtalmi* (Redondo et al., 2008).

Abbreviations: Con A, *Canavalia ensiformis*; SBA, *Glycine max*; PBS, phosphate buffer saline; PSA, antibiotic/antimycotic mixture; FBS, foetal bovin serum; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; HBSS, Hanks' Balanced Salt Solution; Man, mannose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Gal, galactose; MBL, mannose-binding lectin.

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Table 1
Experimental conditions in the four trials of *in vitro* infection of *E. scophthalmi* in turbot intestine explants.

Conditions	Trial 1	Trial 2	Trial 3	Trial 4
Parasite stages processing	Washed once in PBS	Washed once in PBS	Washed trice in PBS	Washed trice in PBS + enzymatic treatment
Incubation medium	PBS	L-15	L-15	L-15
Stages/ml	10^6	10^6	2.5×10^5	2.5×10^5
Temperature (°C)	20	20	15	15
Incubation time (h)	2	2	3	3

2. Materials and methods

Four trials using different methods of parasite treatment and incubation were performed (see below and Table 1).

2.1. Intestine explants

Healthy turbot (*P. maxima*) were obtained from an *E. scophthalmi*-free farm. Fish were killed by overexposing to MS222 and bled from the caudal vein. After necropsy, portions of anterior or medium parts of intestine were collected, placed in sterile phosphate buffer saline (PBS) containing 2 × PSA antibiotic/antimycotic mixture (1 × PSA = 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 0.25 μg ml^{-1} amphotericin B), and cut in small pieces up to 10 × 6 mm. Small intestine pieces were placed (epithelial layer facing upwards) in each well of 24-well tissue culture plates (one piece per well), containing 800 μl of PBS (trial 1) or Leibovitz's L-15 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS) and 1 × PSA (trials 2, 3, 4). L-15 osmolarity was adjusted to 350 mOsm kg^{-1} by the addition of NaCl and pH to 7.2 with 20 mM HEPES.

All the experiments were carried out according to national (Royal Decrete RD1201/2005, for the protection of animals used in scientific experiments) and institutional regulations (CSIC, IATS Review Board) and the current European Union legislation on handling experimental animals.

2.2. Source of parasites

Live *E. scophthtalmi* stages were obtained from turbot experimentally infected at the Instituto de Acuicultura Torre de la Sal (IATS) facilities. Infected fish were initially obtained from spontaneous infections occurring in turbot farms of Galicia, North West Spain (Redondo et al., 2004; Quiroga et al., 2006). This infection was experimentally transmitted via effluent water from tanks containing diseased fish; *per os*, by feeding infected intestinal tissue; and via cohabitation of infected and uninfected fish (Redondo et al., 2002). Since the experimental infection model was obtained, the life cycle has been maintained *in vivo* at the IATS facilities by several series of cohabitation of infected and uninfected fish or by oral infection.

2.3. Isolation and processing of E. scophthalmi stages

Infected fish were killed as above, necropsied and processed as explained previously (Redondo et al., 2002). Briefly, the intestinal fluid was collected from infected fish using a syringe and deposited in 15-ml centrifuge tubes containing PBS supplemented with a 2 × PSA. A drop of the intestinal liquid was observed as a fresh smear at the microscope at 300× and the infection intensity was evaluated using the microscope at 300× magnification. Samples rich in parasite stages were centrifuged 10 min at 365g. The pellet was processed in a different way depending on the trial (see below and Table 1). In trials 1 and 2, the pellet was washed once in fresh PBS containing 2 × PSA. In trial 3, two further washes were done. In trial 4, a slight enzymatic treatment was applied after the third washing as follows: stages were incubated in PBS containing 0370 mg/ml EDTA (ethylenediaminetetraacetic acid) and 0145 mg/ml DTT (dithiothreitol) during 1 h at 18 °C under shaking, and washed in washing medium (HBSS pH 7.2, 5% FBS, 1 × PSA, 0.1 mg/ml DNase I). After filtration using a 40 μm cell stainer, the filtrate containing the parasites was resuspended in L-15 medium. In all cases, the stages in the final pellets were counted and their viability estimated using eosin dye-exclusion methods. The parasites present in the pellets belonged to stage 2 (a primary cell containing one or several secondary cells) and stage 3 (a primary cell containing one or several secondary cells, which in turn harboured one or more tertiary cells), according to Redondo et al. (2004). All the procedure was carried out using sterile material and aseptic techniques.

2.4. Parasite culture with intestine explants

In the four experiments, the isolated parasites were divided into three parts. Two of them were incubated with 50 μg/l Con A or SBA, recognising Man α-1>D-Glc α-1>GlcNAc α-1 and α,ß GalNAc>α,ßGal residues, respectively, during 30′, and the third one served as control, non-incubated with the lectin. Details on the parasite obtaining, temperature and incubation conditions can be found in Table 1. The lectin-incubated and control parasite suspensions (2.5×10^5–1×10^6 stages/ml) were added to the wells containing the intestinal explants. Control wells contained intestine pieces with no parasites added. After incubation at 15–18 °C, intestine portions were recovered at 2–3 h post-exposure (p.e.), fixed in 10% neutral buffered formalin and embedded in Technovit-7100 resin (Kulzer, Heraeus, Germany). Sections (2 μm) were stained with toluidine blue. The presence of parasite stages was evaluated in the histological sections at light microscope by counting the number of parasites that appeared attached or within the epithelia. For such purpose, serial sections of the epithelial tissue were obtained and sets of four sections per slide were mounted on successive slides. In order to assure the examination of different levels of the tissue and to avoid repetition of stages, the four sections of impair slides were examined. In each section, 32 observational fields of epithelium (460 × 460 μm surface) were seen at 300×. Three replicate series of counts were done per condition. In each replicate, four slides (16 sections, 108 mm^2 of epithelial surface) were examined, and the parasites attached or within the epithelium were counted.

2.5. Statistical analysis

Differences between the three conditions in each trial and between trials were analysed by One-way analysis of variance (ANOVA). When the test of normality or equal variance failed, a Kruskal–Wallis one-way ANOVA on Ranks followed by Tukey test or Holman–Sidak method was applied instead. All statistical analyses were performed using Sigma Stat Software (SPSS Inc., Chicago, IL, USA), and the minimum significance level was set at $P < 0.05$.

3. Results

The number of *E. scophthalmi* stages attached to or penetrated in the turbot intestinal explants was higher for control parasites