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Differences in IgY gut absorption in gastric rainbow trout (*Oncorhynchus mykiss*) and agastric common carp (*Cyprinus carpio*) assessed *in vivo* and *in vitro*

Anja Winkelbach [a,b,c,*], Dorothee Günzel [d], Carsten Schulz [b,c], Sven Wuertz [a]

[a] Department of Aquaculture and Ecophysiology, Leibniz Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 310, 12587 Berlin, Germany
[b] Gesellschaft für Marine Aquakultur, Hafentörn 3, 25761 Büsum, Germany
[c] Institute of Animal Breeding and Husbandry, Christian-Albrechts-Universität zu Kiel, Olshausenstraße 40, 24098 Kiel, Germany
[d] Institute of Clinical Physiology, Charité-Universitätsmedizin Berlin, 12203 Berlin, Germany

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ABSTRACT

Oral IgY antibodies offer promising potential for passive immunization strategies. To evaluate barriers for successful IgY plasma recovery after oral application *in vivo*, gastric rainbow trout and agastric common carp were comparatively assessed. A positive control that received a low dose of unspecific IgY antibodies by intraperitoneal injection (0.0076 mg IgY g BW^{-1} d^{-1}; BW = body mass) was compared with an oral administration of 75 and 150 fold in rainbow trout (corresponding to 0.57 and 1.14 mg g BW^{-1}) and in carp (0.57 mg g BW^{-1}). Dietary antibodies were delivered with the antacid sodium bicarbonate and three different putative uptake enhancers (Tween 20, vitamin E TPGS, sodium deoxycholate). IgY concentrations in the plasma were determined 1 d (rainbow trout) or 5 d after last feeding (both species). Irrespective of the enhancer used, ELISA revealed IgY absorption after feeding in carp, whereas IgY concentration in rainbow trout remained below the detection threshold. Intraperitoneal injections revealed IgY in plasma of both species. *In vitro* Ussing chamber experiments with posterior intestine tissue of carp and trout were carried out to determine whether species-specific differences in IgY translocation were due to acidic stomach passage or species-specific differences in transepithelial IgY passage. Significantly higher IgY translocation was measured in carp at high application dosage compared to all other groups, indicating that species-specific differences in IgY uptake after oral administration are not only related to peptic IgY degradation in the stomach, but also likely a result of differences in IgY transcytosis in the posterior intestine.

1. Introduction

IgY technology has several attractive features explored for the production of specific antibodies. Yields of up to 300 mg specific IgY per hen can be obtained monthly, which is nearly 18 times more than those from rabbits (Gottstein and Hemmeler, 1985). Furthermore, costs for animal care are substantially lower and antibody titers in hen's eggs are far more stable than those in blood (Carlander et al., 2000; Xu et al., 2011). Undoubtedly, this cost-efficiency as well as animal welfare concerns promote IgY technology on a commercial scale. Over the 20th century, several diagnostic and therapeutic applications have been documented in human and veterinary medicine, but advances in aquaculture have only been reported rather recently. Here, prophylaxis and treatment of common fish diseases are the primary focus (Gutierrez et al., 1993; Hatta et al., 1994; Lee et al., 2000; Arasteh et al., 2004; Xu et al., 2011; Baloch et al., 2014), but passive immunization targeting growth promotion and improved feed utilization is an emerging research domain (Lakeh et al., 2011; Lakeh et al, 2014). In fish farming, intraperitoneal injection is the main form of IgY application for disease prevention, treatment or growth promotion. Still, oral delivery is undoubtedly more cost-effective as well as less invasive and thus considered the most viable application form, not only for antibodies but also for any peptides and protein therapeutics. Up to now, oral delivery has hardly been evaluated in fish and industry is lacking a simple and efficient method for oral drug application (Rombout et al., 2011) overcoming immunological, physical and chemical barriers during passage through the intestinal tract (McLean and Craig, 2003).

In teleost fish, the absorption of biologically active macromolecules across the intestinal wall has been demonstrated (Rombout et al., 1985; Le Bail et al., 1989; McLean and Donaldson, 1990; Duan and Hirano, 1991) and protein absorption predominantly takes place in the posterior intestine (Fujino et al., 1987; Moriyama et al., 1993). In contrast to mammals, absorption is independent of the life stage and has been proven for larvae as well as for adults (Georgopoulou et al., 1985; Georgopoulou et al., 1986; McLean et al., 1999). Furthermore, passage through the epithelial lining may be increased if vaccines are supplemented with mucosal modifiers or mucolytic agents (McLean

* Corresponding author. Tel.: +49 4834 96539914; fax: +49 4834 96539999.
E-mail address: winkelbach@gma-buesum.de (A. Winkelbach).

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et al., 1999; McLean and Craig, 2003), which disintegrate cellular epithelium or increase transcytosis (Zilles and Tillmann, 2010). Still, gastric digestion may inactivate proteins prior to reaching the absorptive region (Gudding et al., 1999).

Protein hydrolyzation is consequently an important obstacle for the effective oral administration of therapeutic proteins (Moriyama et al., 1993). Coating may protect active compounds from denaturation and digestion and may increase stability particularly towards acidic degradation in the stomach (Kovacs-Nolan and Mine, 2005; Li et al., 2009; Lee et al., 2012). Nevertheless, additional costs may threaten economic feasibility on a commercial scale. Antacids and protease inhibitors may provide comparable protection of bioactive peptides (McLean et al., 1999), but are considered cheaper alternatives. McLean et al. (1990) and Arasteh et al. (2004) reported successful application of sodium bicarbonate ($NaHCO_3$) as an antacid combined with penetration enhancers for oral immunization of rainbow trout and coho salmon (*Oncorhynchus kisutch*). Unfortunately, the experimental setup did not allow differentiation of the single compounds used nor of the actual mode of action. As antacid, $NaHCO_3$ has a buffering capacity which neutralizes low pH in the stomach, thereby counteracting acidic proteolysis. The use of $NaHCO_3$ could offer an extremely cost effective alternative to expensive coating methods and is considered less problematic as food supplement. Within the group of penetration enhancers, sodium deoxycholate (SDC) originally isolated from the bile has been described as a mucolytic agent and membrane solubilizer (Womack et al., 1983). Improved drug transport through the intestinal wall has been shown in many species such as chinook salmon *Oncorhynchus tshawytscha* (Schep et al., 1997), common carp (Hertz et al., 1991) and rainbow trout (Arasteh et al., 2004). Similarly, Tween 20 (T20) is a licensed food additive and emulsifier (E 432), declared safe for human consumption and animal feed within the European Union (EFEMA, 2009). In African catfish (*Clarias gariepinus*), vitamin E tocopherol polyethylene glycol 1000 succinate (VitE) has been administered orally and increased *Vibrio anguillarum* (serotype O2) antigen uptake (Vervarcke et al., 2004).

In the present study, we evaluated optimization strategies targeting 1) low stomach pH and 2) enhanced uptake by use of mucosal modifiers. Comparing gastric trout and agastric carp in a feeding trial, intestinal passage of IgY was determined after oral administration. In rat, multiple applications of horseradish peroxidase (HRP) lead to an increased endocytotic HRP uptake and transcytosis, suggesting that specific receptor-mediated transport mechanisms may be involved (Berin et al., 1997). Thus, single and multiple (5×) applications were assessed separately to evaluate different uptake enhancers and quantify build-up of IgY during multiple applications. The effect of the antacid $NaHCO_3$ and mucosal modifiers – T20, VitE and SDC – was compared based on the plasma concentration of IgY. Furthermore, uptake *via* the intestinal wall was determined in an *in vitro* approach, applying IgY on the mucosal side and determining passage through the intestinal wall on the basolateral side by IgY specific ELISA.

2. Materials and methods

2.1. In vivo experiment, setup and sampling

In two experimental trials, IgY uptake upon oral administration was assessed in the plasma of juvenile rainbow trout (132.4 ± 30.1 g; mean ± SD) and juvenile common carp (116.4 ± 8.6 g; mean ± SD). Fish that received an effective intraperitoneal (IP) injection of IgY (0.0076 mg IgY g BW^{-1} d^{-1}, Lakeh et al., 2011) served as positive control. Oral ratios corresponded to 75 and 150 fold of this control dosage in rainbow trout and 75 fold in carp. Diets were supplemented with different enhancers, either stabilizing IgY at low pH ($NaHCO_3$) or accelerating the absorption (Table 1). Furthermore, a group fed the basic diet without any IgY was accessed as negative control. During acclimatization for 14 d in the experimental system, fish were fed with the basic control diet at 1.5% BW d^{-1}. Then, fishes were randomly distributed to 18 (trout) or 6 (carp) 50 L aquaria (daily water exchange 1250 L kg^{-1} feed, water turnover rate 4 h^{-1}), attached to a mechanical sponge filter, a moving bed biofilter and a disinfection unit (UV-light). Each group comprised 5 individuals. Fish were fed individually to assure that the assigned amount of diet (designated dose of IgY) was consumed. The photoperiod was adjusted to 12 h light 12 h dark. Water parameters were determined daily (trout: 11.8 ± 0.4 °C, 9.0 ± 0.3 mg L^{-1} O_2, 0.05 ± 0.06 mg L^{-1} NH_4^+–N and 0.15 ± 0.05 mg L^{-1} NO_2^-–N; carp: 23.7 ± 0.1 °C, 7.8 ± 0.1 mg L^{-1} O_2, 0.1 ± 0.2 mg L^{-1} NH_4^+–N and 0.5 ± 0.3 mg L^{-1} NO_2^-–N). During the experiment, animals were fed once per day at 1.5% BW d^{-1} by hand to ensure that all pellets were ingested, thereby providing the designated IgY dosages. Trout fish were fed for 1 d as well as for 5 d. The subsequent study with carp was carried out over five feeding days considering the results of the first trial. For the IP injection (positive control group), fish were anesthetized with tricaine methanesulfonate (MS 222, 50 ppm) in a water bath. All fish were sampled 24 h after the final IgY application. Blood was drawn from the caudal vein with a heparinized syringe, blood samples were centrifuged (3 min; 4 °C; 1000 *g*) to separate plasma and plasma samples were stored at −20 °C.

2.2. IgY isolation

Eggs bought from a local supermarket were used for IgY isolation according to Pauly et al. (2011): Egg yolk was separated from egg white and yolk skin and mixed with PBS (0.01 M, pH 7.6; 1:2 egg/PBS v/v). 3.5% PEG 6000 (total volume) was added, and the suspension was vortexed and mixed on a horizontal mixer for 10 min (Stuart roller mixer SRT1, Bibby Scientific Limited, UK). After centrifugation (4000 *g* at 4 °C, 30 min), supernatant was filtered (Macherey-Nagel paper filter, porosity < 12 μm, MN 615 1/4; Ø 150 mm) and precipitated lipids were discarded. PEG 6000 was added (8.5% of total volume) to precipitate the protein fraction, stirred and centrifuged (4000 *g* at 4 °C, 30 min). Finally, the supernatant was discarded and the precipitated protein fraction resuspended in PBS. Suspensions were pooled and IgY content of the solution was measured in duplicate by enzyme-linked immunosorbent assay (Chicken IgY (IgG) ELISA, Alpha Diagnostic International, USA) as described (Subsection 2.4). Dissolved antibodies were stored at −20 °C until usage for the experimental trials.

2.3. Diet preparation

Experimental diets were prepared ultimately prior to the trial. Commercial carp feed (Aller Classic, 4.5 mm; AllerAqua A/S, Denmark) and trout feed (Performa 45/20, 2 mm; AllerAqua A/S, Denmark) were supplemented with IgY. In brief, pellets were grounded with a mortar and mixed with IgY solution and enhancer according to Table 1. $NaHCO_3$ and SDC were added according to the effective dose reported without any adverse effects to the intestinal cell lining (Arasteh et al., 2004). T20 was supplemented according to the acceptable daily intake (ADI) for human nutrition (EFEMA, 2009). VitE content was adjusted to the same amount as T20. Re-pelleting >was carried out by hand using a pastry press and subsequent drying at room temperature. Experimental feeds were formulated with 38 g and 76 g IgY kg^{-1} diet. IgY content was confirmed by specific IgY ELISA. Therefore, 1 g of pellets was homogenized in 10 mL PBS with an Ultra Turrax (MICRA D-9, ART Prozess-& Labortechnik GmbH & Co. KG, Germany). After incubation at 3 °C for 24 h, the suspension was filtered (Macherey-Nagel paper filter, porosity < 12 μm, MN 615 1/4; Ø 150 mm) and supernatant was used to determine IgY content in the pellets as described (Subsection 2.4). Pellets were stored at 3 °C and used for the trial within 3 weeks.