Fish & Shellfish Immunology (2009) **26**, 65–71

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Serum disposition of bovine lactoferrin after oral and anal administration and its proteolytic cleavage by gastric transit in rainbow trout (*Oncorhynchus mykiss* W.)

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Received 5 June 2007; revised 5 March 2008; accepted 8 March 2008
Available online 15 March 2008

KEYWORDS
Bovine lactoferrin;
Immunology;
Oral administration;
Proteolytic digestion;
Rainbow trout

Abstract Several studies have shown an immunomodulatory effect of orally administered bovine lactoferrin (LF) in fish, but the process of digestion was not characterized.

In the present study, we investigated the fate of bovine LF after oral and anal administration, and studied the appearance of intact LF in the bloodstream and its proteolytic attack during the gastric transit in rainbow trout (*Oncorhynchus mykiss*) held at 9 °C and 18 °C.

Data obtained showed the presence of intact bovine LF in the bloodstream only after anal administration in fish held at 18 °C and the presence of several peptides derived from bovine LF in the gastric content. Immunoblotting analysis showed that only a part of bovine LF-derived peptides reacted with the applied anti-bovine LF antibody. The concentration of intact bovine LF, after 30 min of administration, in the gastric content of fish reared at 18 °C, being extremely low, if any, led us to suspect that the immunoregulatory effect of dietary bovine LF shown in fish by several authors is not due to the intact form but to bioactive fragments, originated by the proteolytic attack during the gastric transit, as demonstrated in higher vertebrates.

Introduction

Lactoferrin (LF) is an 80 kDa iron-binding glycoprotein found in milk and other exocrine secretions and in specific granules of polymorphonuclear cells of higher vertebrates [1].

Although the presence of LF in teleosts is debated, Langston et al. [2] demonstrated the presence of an

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doi:10.1016/j.fsi.2008.03.005

iron-binding activity within blood leucocyte lysates from Atlantic salmon *Salmo salar* (L.) that is maintained even if the sample is exposed to acid pH, a distinctive feature of human LF [3]. Moreover, the presence of an LF-like factor is also suggested by Cecchini et al. [4]. In fact, the authors showed an enhancement of the iron-binding activity in supernatants of rainbow trout leucocytes after treatment with phorbol-12-myristate-13-acetate.

With respect to fishes, it is known that dietary bovine LF enhances the activity of the innate immune system [5–7], increases the tolerance to high temperature [8] and to air exposure and salinity stress [9], reduces the stress response of fish held under deteriorating conditions [10,11] and enhances the specific immune response and resistance of immunocompromised Asian catfish *Clarias batrachus* (L.) to *Aeromonas hydrophila* infection [12]. In contrast to the reported literature, Lygren et al. [13] did not obtain positive effects on growth, disease resistance and some immune parameters of Atlantic salmon fed with bovine LF-enriched diet for a period of 19 days.

To the authors' knowledge, whereas no information is available on serum disposition and on the process of digestion of LF after oral administration in fish, more knowledge is available about these aspects in mammals.

Antigenically intact LF was found in the urine of milk-fed pre-term human infants [14], while anti-LF immunoglobulins (Ig) A and IgG were detected in the intestinal fluid and serum of bovine LF-treated 10–15-week-old mice [15], showing that LF is transferred into blood from intestinal lumen. Moreover, Harada et al. [16] showed an intact LF-absorption by intestinal villi of orally bovine LF-treated neonatal piglets and its entero-hepatic circulation. The intact bovine LF was also detected in plasma from 6-week-old piglets, which cannot absorb macromolecules such as IgG [16].

With respect to the fate of orally administered LF, it is known that LF partially resists proteolytic attack both *in vitro* [17] and *in vivo* [18]. The pepsin hydrolysis of LF originates polypeptidic fractions. Among them, the peptide domain lactoferricin (LFcin) was identified by both *in vitro* [19] and *in vivo* [18] digestion, showing antimicrobial [19] and immunomodulating [20] activities much greater than that of an equimolar amount of LF.

With the preceding as background, the aim of this study was to examine the plasma disposition and the process of digestion of bovine LF after oral administration in rainbow trout. In order to evaluate the potential absorption of bovine LF by the gut, bovine LF was administered not only orally but also anally, to get round the proteolytic attack by digestive proteases. Moreover, the experiments were performed at two different environmental temperatures, at 18 °C, the normal summer temperature for rainbow trout, called immunologically ''permissive'' [21], and at 9 °C.

Materials and methods

Fish and experimental conditions

The study on plasma disposition and on digestion of bovine LF was carried out at two different temperatures, 9 °C (±0.5 °C) and 18 °C (±0.5 °C), applying the same experimental protocol, in order to examine the effect of different temperatures on evaluated parameters. Because natural temperatures were used, the experiment was repeated 6 months later on a similar rainbow trout population. Oxygen concentrations were kept at 90–100% of the saturation values at both temperatures and the applied photoperiods were natural.

For the evaluation of plasma disposition of bovine LF after oral and anal administration, two groups of 27 healthy rainbow trout of 200–250 g held in separate 5 m^3 rectangular tanks connected to a recirculation system were starved for 96 h and given bovine LF orally and anally, respectively. Regarding the oral administration, fish of the first group were anaesthetised with 2-phenoxyethanol (0.4 ml l^{-1}) and treated with 1 ml of bovine LF (Morinaga Nutritional Foods Deutschland GmbH) (100 mg ml^{-1}) in 0.01 M phosphate-buffered saline (PBS) pH 7.2, using a flexible tube attached to a syringe, as suggested by Harada et al. [16]. A group of 3 fish was orally treated with 1 ml of PBS only (negative control). With respect to the anal administration, 1 ml of Morinaga bovine LF (100 mg ml^{-1}) was intubated into the hindgut of fish of the second group using a flexible tube. The tube was inserted 60–5 mm into the gut via the anus in order to deposit the dose at the anterior end of the posterior intestine. Following this, 50 μl of Vaseline was intubated to prevent the leakage of LF from the intestine. A group of 3 fish was anally intubated with 1 ml of PBS only (negative control). After 30 min, 1, 2, 4, 8, 24, 48, 96 and 168 h of treatments, 3 fish for each sampling from both experimental groups were anaesthetised and blood was collected from the caudal artery using a 2.5 ml syringe. Blood was allowed to clot overnight at 4 °C and sera were obtained by centrifugation (3000 rpm, 10 min) and stored at −20 °C until the analysis of bovine LF concentration evaluated as described below.

For the evaluation of proteolytic cleavage of bovine LF by gastric transit, a group of 18 fish held at the same temperature in a separate tank of the same rearing system was orally intubated with 2 ml of Morinaga bovine LF (10 mg ml^{-1}) in PBS, applying the same LF concentration adopted by Kuwata et al. [18] for the evaluation of gastric digestion of LF in healthy human volunteers. A group of 3 fish was orally treated with 1 ml of PBS only (negative control). After 1, 5, 10, 15, 20 and 30 min of treatment, 3 fish for each sampling were anaesthetised and the gastric content was recovered using the flexible tube. To prevent further proteolysis, pepstatin A was added to a final concentration of 0.01% and the pH was adjusted to neutral using 1 M Trizma, as suggested by Kuwata et al. [18]. Supernatants of gastric contents were obtained by centrifugation (3000 rpm, 10 min) and stored at −20 °C until quantitative (bovine LF and total protein concentrations) and qualitative (electrophoresis and immunoblotting) analyses, as described below.

Sandwich-ELISA for bovine LF quantitation

Bovine LF content in sera and in gastric contents was assayed quantitatively by a double-antibody enzyme-linked immunosorbent assay (sandwich-ELISA). Optimal dilutions of antibodies and standard concentration were got ready in preliminary tests (data not shown).