Comparative Biochemistry and Physiology, Part A 168 (2014) 96–109

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

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

# Bile enhances glucose uptake, reduces permeability, and modulates effects of lectins, trypsin inhibitors and saponins on intestinal tissue

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ARTICLE INFO

*Article history:*
Received 19 June 2013
Received in revised form 14 November 2013
Accepted 22 November 2013
Available online 1 December 2013

*Keywords:*
Soybean
Antinutrients
Atlantic salmon
Intestine
In vitro

ABSTRACT

Antinutritional factors (ANFs) can disrupt digestive and other intestinal functions. ANFs in soybean meal (SBM) are implicated in proliferative and inflammatory responses in the intestine of various (functionally) monogastric animals, including Atlantic salmon (*Salmo salar* L.). The goal of the current study was to investigate the effect of ex vivo exposure of mid and distal intestinal tissue of salmon to soybean saponins (SAP), lectin (LEC) and Kunitz' trypsin inhibitor (KTI), singly and in combination, on epithelial function, as assessed by measuring in vitro glucose uptake pathways along a glucose concentration gradient. As solubilization of SAP in the calcium-containing Ringer's solution was problematic but resolved with the addition of a physiological concentration of bile collected from the gall bladder of salmon, an evaluation of bile effects became an added element. Results indicated that bile increased baseline glucose absorption and possibly transport, and also had a protective effect on the epithelial barrier, at least partially due to taurocholate. Compared to controls, tissues exposed to LEC + bile, KTI + bile and LEC + KTI + bile exhibited increased glucose uptake at the higher glucose concentrations, apparently due to markedly increased tissue permeability. Addition of SAP, however, attenuated the response, possibly by binding bile components. SAP + bile, also in combination with LEC and/or KTI, as well as LEC, KTI and LEC + KTI without bile often reduced transcellular glucose uptake pathways, while maintaining low tissue permeability. SAP + LEC + KTI + bile, LEC and KTI caused the most marked reductions. The distal intestine was more affected, reflecting the restriction of in vivo SBM-induced inflammatory changes to this region.



## 1. Introduction

Antinutritional factors (antinutrients; ANFs) in plant crops can affect food/feed intake and digestive processes in the gastrointestinal tract. Some can have beneficial effects for humans and companion animals, including control of various lifestyle diseases. In the animal production industry, however, limitations in feed intake, nutrient digestibility and feed utilisation are not economically or environmentally desirable. Thus ANF content can place limitations on the types and amounts of plant ingredients, especially legumes, used in formulated feeds. For example, ANFs in standard qualities (full fat and extracted) of soybean meal (SBM) can cause proliferative or inflammatory conditions in the intestinal mucosa of monogastric or functionally monogastric mammals: mice and rats (Pusztai, 1989; Ge and Morgan, 1993; Govers et al., 1993), piglets and calves (Silva et al., 1986; Ratcliffe et al., 1989; Hancock et al., 1990), as well as cultured fish species Atlantic salmon, *Salmo salar* L. (Van den Ingh et al., 1991, 1996; Baeverfjord and Krogdahl, 1996), rainbow trout, *Oncorhynchus mykiss* (Rumsey et al., 1994; Yamamoto et al., 2007) and common carp, *Cyprinus carpio* L. (Urán et al., 2008). These conditions have been at least partially attributed to lectins (see review by Pusztai, 1989), trypsin inhibitors (Ge and Morgan, 1993; Breiteneder and Ebner, 2000; Yamanishi et al., 2003) and saponins (Gee and Johnson, 1988; Francis et al., 2002; Knudsen et al., 2007, 2008), or combinations of these (Alvarez and Torres-Pinedo, 1982; Iwashita et al., 2008a, 2008b, 2009; Chikwati et al., 2012). All may directly or indirectly affect cells, their membranes, or functional proteins inserted in the membranes. Lectins can bind to sugar moieties of membrane components and have antigenic properties (Tchernychev and Wilchek, 1996), Kunitz' trypsin inhibitors inhibit protease activities and can allegedly be allergenic (Breiteneder and

*Abbreviations:* ANF, antinutritional factor; AUC, area under the curve; dpm, disintegrations per minute; DI, distal intestine; GLUT2, high-capacity, low affinity, bidirectional, facilitative transport protein for hexoses; $K_t$, transporter affinity constant; KTI, soybean Kunitz' trypsin inhibitor; LEC, soybean lectin; MI, mid intestine; $P_{app}$, non-saturable component of uptake; SAP, soybean saponin; SBM, soybean meal; SGLT1, sodium/glucose-linked (co)transport protein of low-capacity and high affinity, stereospecific for D-glucose; TC, taurocholate; $V_{max}$, maximum rate of uptake.

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http://dx.doi.org/10.1016/j.cbpa.2013.11.010

Ebner, 2000; Yamanishi et al., 2003), while amphipathic saponins can disturb cell membranes and increase cellular/tissue permeability (Johnson et al., 1986; Gee et al., 1996; Knudsen et al., 2008), as well as have adjuvant properties (see review by Francis et al., 2002).

In piscivorous salmon, possibly the most sensitive model animal for ANF effects, the SBM-induced inflammation observed in the distal intestine has been characterised as sub-acute (Baeverfjord and Krogdahl, 1996) and appears to be T cell-mediated (Bakke-McKellep et al., 2007a; Marjara et al., 2012), thus indicating similarities to inflammatory bowel diseases in humans and other mammals. The enteropathy is accompanied by increased epithelial cell proliferation (Bakke-McKellep et al., 2007b) and dysfunction (Bakke-McKellep et al., 2000b; Nordrum et al., 2000; Krogdahl et al., 2003). Soybean saponins have been implicated as a causatory agent, but most likely the severity is amplified by other ANFs/antigens (Knudsen et al., 2007, 2008; Iwashita et al., 2008b, 2009; Chikwati et al., 2012). Due to the prohibitive cost of some of these ANFs in purified form, few in vivo trials have been conducted to conclusively verify their roles in the pathogenesis of the SBM-induced enteropathies. In lieu of this, ex vivo and in vitro methods may be useful in investigating tissue responses to ANFs, both singly as well as in combination. If ANFs modify function or cause damage to the intestinal epithelium, altered solute uptake can be an outcome. An established in vitro model for measuring intestinal solute uptake is the everted sleeve method (Karasov and Diamond, 1983), previously applied to numerous species of mammals and reptiles, as well as various fishes (Collie, 1985; Buddington et al., 1987; Bakke-McKellep et al., 2000a; Bakke et al., 2010). It has also been used to investigate pathophysiological responses to in vivo manifested tissue damage (Nordrum et al., 2000; Kristan and Hammond, 2001; Kristan, 2002). Assessing effects of ex vivo intestinal tissue exposure to ANFs or other possible modulators of intestinal tissue structure and function on in vitro solute uptake in whole tissue preparations has been conducted employing similar methods (Alvarez and Torres-Pinedo, 1982; Johnson et al., 1986; Gee et al., 1989, 1996; Önning et al., 1996).

Thus the aim of the study reported herein was initially to investigate the effects of short term ex vivo exposure of individual and combination soybean ANF preparations on mid and distal intestinal tissue from Atlantic salmon. Both regions have epithelial cells equipped with a brush border and therefore actively digest and absorb nutrients (Van den Ingh et al., 1991; Bakke-McKellep et al., 2000a, 2000b; Krogdahl et al., 2003). Saponins, lectin (agglutinin) and Kunitz' trypsin inhibitor were chosen due to their possible involvement in SBM-induced enteropathies. Kunitz' trypsin inhibitor was chosen rather than the Bowman–Birk inhibitor due to its alleged allergenic properties and reported heat stability in a soy flour matrix (DiPietro and Liener, 1989). In vitro glucose uptake mechanisms into the intestinal mucosa using the everted sleeve model system were assessed to gauge the tissue responses to ANF exposure. Glucose total absorption, as well as specific transcellular SGLT1-mediated transport, was measured along a glucose concentration gradient. Tissue permeability was also assessed.

## 2. Materials and methods

### 2.1. Experimental challenges

Soybean saponins dissolved poorly in the calcium-containing Ringer's solution used for the ex vivo tissue treatment (see Section 2.3.), but the presence of calcium was considered necessary for preserving tissue viability. The problem was resolved with the addition of bile to the Ringer's solution (for details see Section 2.3.). Therefore, bile effects on baseline glucose uptake pathways, as well as responses to ANF exposure in salmon intestinal tissue were also investigated.

### 2.2. Fish husbandry and tissue preparation

Atlantic salmon (a total of 120) were held and the experiment was conducted in accordance with the Norwegian Animal Welfare Act No. 73 of December 20th 1974 and the Regulation on Animal Experimentation of January 15th 1996, respectively. The fish had body masses ranging from 500 to 800 g and were held in a freshwater-containing tank with a surface area of 1 $m^2$ and water temperature was kept at 8–10 °C. Water level, recirculation and oxygen supply were adjusted to keep $O_2$ concentration above 7 mg/L as the number of fish decreased. Fish were fed a commercial, SBM-free salmon diet using automatic disc feeders at a feeding rate of approximately 1% of biomass per day.

Prior to sampling, each fish was fully anaesthetized by immersion in an anaesthetic bath containing 100 mg/L benzocaine and subsequently euthanized by cervical dislocation. The alimentary tract was excised and the two regions investigated were prepared, with the mid intestine (MI) defined as the region from the distal-most pyloric caecum to the start of the distal intestine, while the distal intestine (DI) is defined as the region from the mid intestine to the rectum and characterised by the transverse mucosal folds, larger diameter and darker colour. The serosal surfaces of both regions were cleared of mesenteric and adipose tissue, longitudinally opened, and freed from intestinal content. All handling of the intestinal segments and preparation of the tissue pieces was done in ice-cold, aerated (97% $O_2$, 3% $CO_2$) fish Ringer's solution, which contained (in mM) NaCl (117), KCl (5.8), $NaHCO_3$ (25), $NaH_2PO_4$ (1.2), $MgSO_4$ (1.2), and $CaCl_2$ (2.5). Osmolarity, as determined by freezing point depression, was 290 mOsmol/L, and pH was adjusted to 7.4 when aerated with the $O_2$:$CO_2$ mixture. All reagents were of high purification and obtained from Sigma-Aldrich Norway AS (Oslo, Norway).

To reduce the number of animals needed for the experiment, as well as prevent tissue damage that may incur during tissue manipulation (Starck et al., 2000), tissue pieces rather than everted sleeves of MI and DI measuring ca. 0.5 × 1 cm were secured by silk ligatures onto grooved stainless steel rods with the serosa against the rod, as previously validated by Nordrum et al. (2000). For each experimental treatment (see below), 6 tissues per intestinal region were used. Depending on the body size and hence the intestinal length of the fish, 9–12 pieces per intestinal region and fish were obtained. To ensure viability ex vivo, tissue preparation was performed as quickly as possible and the mounted tissues were kept in ice-cold, aerated fish Ringer's solution until further investigations commenced.

### 2.3. Preparation of pre-incubation solutions

See Table 1 for the ANFs and their combinations added to the pre-incubation solutions used for ex vivo exposure of the intestinal tissues, and Fig. 1 for a schematic experimental setup. The ANFs were dissolved in aerated Ringer's solution. The concentration of the various ANFs in these pre-incubation solutions was calculated from median levels reported in soybeans, and based on a SBM inclusion level of 30% in a diet, which has been established as a level that will cause SBM-induced enteropathy in nearly all Atlantic salmon studied (Van den Ingh et al., 1991; Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2003). Bile from Atlantic salmon was added to Ringer's solution to facilitate saponin (SAP) solubility, and was subsequently also added to investigate the effect of bile on 1) baseline in vitro glucose uptake pathways and 2) responses in tissues also exposed to lectin (LEC) and/or Kunitz' trypsin inhibitor (KTI). From a pooled sample of bile collected from 25 fish, the minimum amount of bile needed to dissolve the saponins was established to be 25 mL/L Ringer's solution, and this amount was subsequently added to the respective pre-incubation solutions. According to calculations based on reported bile composition (bile acid levels in bile; 23% reported by Bogevik et al., 2009; 12% reported by Kortner et al., submitted for publication) and bile salt levels reported in