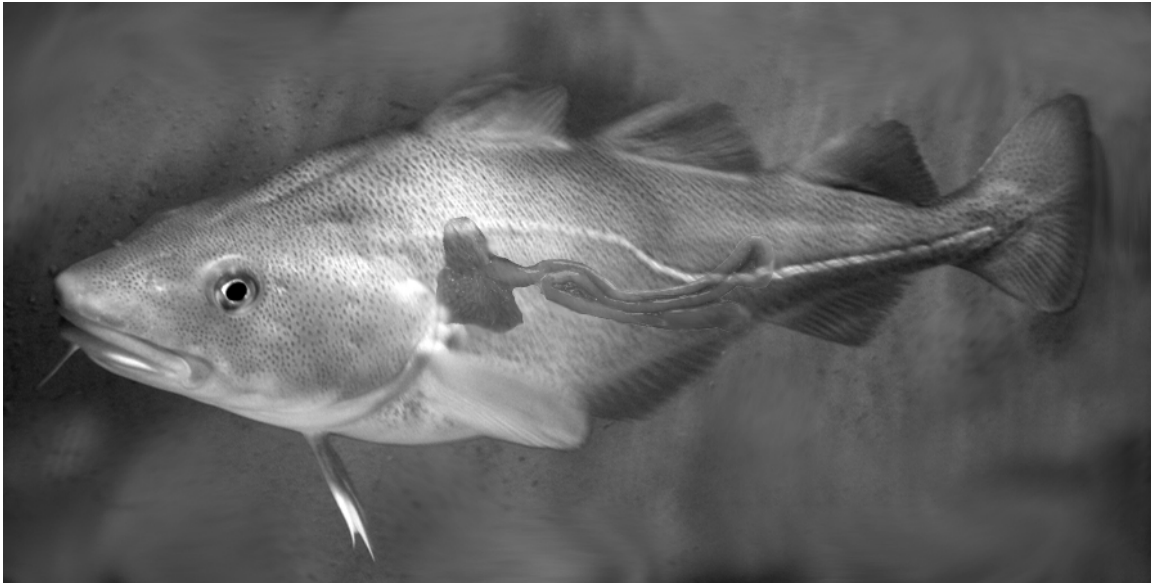# MASTER THESIS

UNIVERSITY OF BERGEN, NORWAY

Dietary inclusion of peptides and the effect on the regional
expression of the oligo-peptide transporter PepT1 (Slc15a1)
in the intestine of juvenile Atlantic cod (*Gadus morhua*)

Thesis submitted in partial fulfillment of the Masters degree in fish biology

By

Snorre Bakke

Department of Biology

June 2008

Abstract

PepT1 (Peptide Transporter 1, SLC15A1) is a proton-coupled peptide transporter responsible for uptake of short peptides and peptide-like drugs from the intestinal lumen into enterocytes. A PepT1-type transporter has recently been sequenced for Atlantic cod and found to be expressed along the whole post-gastric intestine including pyloric caeca. In the present thesis the effect of dietary inclusion of peptides and amino acids on expression of PepT1 mRNA in different regions of juvenile Atlantic cod intestine was investigated. Five groups of cod weighing 10-15 grams were fed for 46 days with a diet containing approximately 42% crude protein, either as fishmeal (FM diet) or 30 % of the fish meal substituted by either whole fish hydrolysate (FH diet), retenate after ultra filtration of fish hydrolysate (UFR diet), retenate after nano filtration of fish hydrolysate (NFR diet) or a mix of free amino acids (FAA diet). After 14 days (T1) and at the end of the experiment (46 days; T3), three fish were sampled from each group and the intestine divided into five segments, namely pyloric caeca (S1) and the remainder of the intestine from the pyloric caeca to the anus divided into four equally long segments (S2 to S5). Total RNA isolated from each segment was subjected to a two-step quantitative RT-PCR using SYBR green and Atlantic cod elongation factor 1 alpha as reference gene.

For all diets PepT1 was found to be expressed in all segments, suggesting that for juvenile Atlantic cod the whole intestine is involved in peptide absorption. The different groups of fish showed a similar PepT1 mRNA expression in all segments, but differences in regional expression were found. At T3 the regional expression profile showed statistical differences in PepT1 expression between segments in the FAA and UFR fed fish (diets included amino acids or larger sized peptides). Both groups had a higher expression in S2 and S3 (mid intestine) then pyloric caeca (S1) and S5. A similar trend was also observed for the UFR at T1, while the FAA fed fish had a low and stable expression in all segments at T1. No significant differences in PepT1 expression were found between segments in the FM, FH or the NFR fed group for any time. These results suggest that for diets where 30% of the fish meal has been replaced by fish hydrolysate containing short peptides (FH and NFR) the involvement of the pyloric caeca in peptide absorption is similar to the remainder of the intestine, while diets with no or low amounts of short chained peptides included (FAA and UFR) have a lower involvement of pyloric caeca in peptide absorption compared to mid intestine. A general higher expression of PepT1 was found at T3 then at T1 in all segments except S4, but no interaction between time and diet was found, indicating that some developmental or growth related changes in PepT1 expression occurs during this life stage of Atlantic cod. These results suggest that dietary peptides of various chain lengths as well as free amino acids affect the regional expression of PepT1 mRNA in the intestine of juvenile Atlantic cod. The signaling pathways responsible for PepT1 regulation still remains to be described.

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Preface and acknowledgements

The research group Developmental Biology of Fish at the Department of Biology, University of Bergen, Norway, has through an inter-institutional collaboration (NettMett) with the Department of Medicine and NOFIMA focused on the effect of peptides on growth and health in fish and humans. In relation to this collaboration an experiment was conducted to study the effect of dietary inclusion of fish-hydrolysate for juvenile Atlantic cod. The present thesis was an integrated part of this feeding trial and focused on the activity of the oligopeptide transporter PepT1 in different regions of the post-gastric intestine and how its expression was affected by the dietary inclusion of peptides. The selected methods to measure PepT1 activity was quantitative reverse trascriptase polymerase chain reaction (Q-RT-PCR) for detection at gene expression level and transport studies with isolated brush border membrane vesicles (BBMV). In order to learn the use of BBMV, I spent seven weeks from mid January to the beginning of March 2007 at the Department of Biological and Environmental Sciences and Technologies, University of Salento, Lecce, Italy, and as part of this training a range of analysis was conducted on European eel (*Anguilla anguilla)* intestine. Although we failed to establish the BBMV protocol for Atlantic cod intestine for the main experiment described in this thesis, the data on eel formed the basis for a paper that is now being published in the journal Aquaculture Nutrition, and is attached in this thesis as Appendix A. The main findings in this thesis is therefore based on PepT1 gene expression, though the method and central observations from BBMV isolation from Atlantic cod will also be addressed. The feeding experiment was conducted from mid May to early August 2007 followed by laboratory work from early September 2007 to late January 2008. The experiment was financed through funding by Research Council of Norway (BILAT Grant #175021 PepTalk, Grant #165203 FishProteomics) and NettMett from HelseVest and University of Bergen.

The completion of this thesis would not have been possible without the help of many people, so I would therefore like to express my gratitude to the following persons:

My main supervisor Professor Ivar Rønnestad for ideas and constructive feedback. And for introducing me to the field of fish nutrition and peptide transport.

My co-supervisor Dr Ann-Elise Olderbakk Jordal for feedback, methodical training and providing me with new insights into the world of molecular biology and qPCR

My co-supervisor Professor Tiziano Verri for feedback and for making my stay in Lecce and the University of Salento a very pleasant and memorable experience. I would also like to thank him together with Dr Antonio Danieli and the other people at the laboratory at the University of Salento for the insightful methodical training on preparation and use of BBMV, and for the cooperation during the preparation of the article describing this method (Appendix A).

A special thanks to Dr Pedro Gómez Requeni for technical and statistical help and for a fruitful cooperation during the whole experimental and thesis process.

I would also like to thank Dr. Anders Aksnes for provision and chemical analysis of experimental diets, Dr Einar Heegaard for statistical suggestions and comments and Anne-Grethe Gamst Moen, Bjørn Sveinsbø, Frank Midtøy, Katerina Kousoulaki, Valentina Tronci and Cècile Jolly for technical and experimental help.

Finally I would like to thank my better half Mariann for patience, understanding and moral support.

Front page picture credited National Oceanic & Atmospheric Administration's (NOAA) and modified with permission.

1. Introduction

1.1 Background

Due to uncertainty concerning the ecological status of wild Atlantic cod (*Gadus morhua*) (Brander 2006), the efforts to establish this species in aquaculture has been intensified. In the last years there has been a rapid increase in the total number of farmed cod in Norway, from a live stock of about 180 thousand in 1999 to more than 15 million fish in 2006 (Norwegian Directorate of Fisheries 2007). One of the areas that are of major interest to the fish farmers is optimizing the growth of the fish, so an essential step is therefore to produce feed with optimal nutrient composition to minimize the food to growth ratio. Growth is mainly accretion of protein (Houlihan 1988) and estimates have shown that 47 – 60 % of the diet should contain protein for optimal growth efficiency of Atlantic cod fed a fish meal diet (Lied and Rosenlund 1983; Rosenlund et al. 2004). The use of fish meal based on harvest from wild stocks in the production of a high quality food product has raised the question about fish farming as a sustainable industry (Naylor 2000; Pauly et al. 2002). Because of this and the recent shortage and increased cost of fish meal world-wide, the feed producers have started an intensive search for substitutes. Various sources of plant proteins have received the most attention and Atlantic cod seems to be efficient at utilizing protein from vegetable sources (for recent studies see Albrektsen et al. 2006; Hansen et al. 2006; Refstie et al. 2006a; Refstie et al. 2006b; Hansen et al. 2007a; Hansen et al. 2007b; Olsen et al. 2007). However, a negative effect on both feed utilization and growth has been found when the level of vegetable protein exceed 50 % (Hansen et al. 2007a; Olsen et al. 2007) and at even lower levels if the indispensable amino acid requirements are not met (von der Decken and Lied 1993). One of the reasons for inefficient utilization of vegetable sources in fish is the presence of anti-nutritional factors like protease inhibitors, antivitamins, phytic acid, lectins, allergens and phytoestrogens (reviewed by Francis et al. 2001). However, in a study on Atlantic cod where 24% of the protein was substituted with either dietary soybean meal (SBM) or SBM bioprocessed to remove these anti-nutrients, no significant difference was found in utilization of the two diets (Refstie et al. 2006a; Refstie et al. 2006b). On the other hand, both soy diets gave lower feed efficiency and protein retention than a pure fish meal diet,

thus indicating non-optimal feed utilization when vegetable protein is included. Another reason for inefficient utilization of vegetable dietary sources could be that plant materials are missing some components that are necessary for optimal growth and development. Aksnes (2005) compared the amount of taurine, anserine, carnosine, nucleotides and free amino acids in different raw materials and found no or small amounts of these compounds in vegetable sources compared to material from animal origin. Indeed, some of these components (amino and nucleic acids) have been found to stimulate growth when included in feed for marine fish (Burrells et al. 2001a; Burrells et al. 2001b; Kim et al. 2005), and suggests that some proportion of the diet should contain material from marine origin in order to sustain optimal growth. A major by-product from the fish industry is fish silage, which during enzymatic breakdown releases fractions of hydrolyzed proteins that can be utilized in the production of fish protein hydrolysate (Liaset et al. 2000). Fish hydrolysate was found to contain high levels of all the compounds investigated in the study by Aksnes (2005), and its use as a substitution for fish meal has been investigated. In a study on Atlantic cod, Aksnes et al.(2006b) found that replacement of about 30% of the fish meal with fish hydrolysate gave no significant differences in growth or feed efficiency compared to a full fish meal diet. Successful substitution of fish meal with fish hydrolysate has also been found for other species like rainbow trout (*Oncorhynchus mykiss*)(Aksnes et al. 2006c), Atlantic salmon (*Salmo salar*) (Berge and Storebakken 1996; Refstie et al. 2004; Hevroy et al. 2005) and sea bass larvae (*Dicentrarchus labrax*) (Cahu et al. 1999). In a study on Atlantic salmon Espe et al. (1999) found that the absorption of amino acids were more efficient when up to 30 % of the fish meal was replaced by protein concentrate from fish silage. However, moderation also seems to be important when substituting fish meal with hydrolyzed proteins because too high inclusion levels appears to have a negative effect on growth (Espe et al. 1999; Hevroy et al. 2005). Thus, as suggested by Refstie et al. (2004) a more efficient digestion and absorption might be obtained by a balanced and controlled inclusion of alternative sources. Although numerous studies have been focusing on the effect of fish meal substitution on growth and digestion, no known studies have been conducted on Atlantic cod on how the dietary composition affect the intestinal absorption of protein *in vitro*. The activity and capacities of nutrient transporters in the intestine of fish has been found

to be affected by the dietary composition (Buddington et al. 1987), however details on the regulatory mechanisms is scarce. So in order to replace fish meal in diets for a carnivorous teleost like Atlantic cod it is therefore important to have thorough knowledge on how the chemical composition of the diets affects the digestive and absorptive process.

1.2 Protein digestion

The digestion of food is a complex process involving mechanical and enzymatic degradation, from food intake to nutrient absorption in the intestine (for a review on these mechanisms in fish see Rust 2002). In contrast to higher vertebrates where mechanical breakdown of the food starts in the mouth, most fish have limited ability to chew so the first step of degradation is in the stomach. The increased volume of the stomach stimulates the secretion of hydrochloric acid, water and the digestive enzyme pepsin into the lumen. Muscular contractions together with ingested water and secretions from the stomach helps break down and mix the food into a homogenized mass called chyme. Pepsin is the first enzyme involved in the proteolysis of ingested protein, and is secreted from oxynticopeptic cells as its inactive precursor pepsinogen. The low pH in the stomach activates pepsinogen and starts an autocatalytic process that will generate active pepsin which then can hydrolyze dietary protein into large polypeptides (Ganapathy et al. 2006). In addition the acidic conditions will also directly contribute to the degradation of protein (Rust 2002). As the chyme enters the intestine more alkaline conditions (caused by the presence of bicarbonate) cause neutralization of the acidic digest (Rust 2002). Protein (and lipid) in the chyme stimulate the release of the hormone cholecystokinin which further stimulate the secretion of pancreatic enzymes or their inactive precursors into the intestinal lumen (Buddington and Krogdahl 2004). The active forms of these digestive enzymes are trypsin, chymotrypsin, elastase and carboxypeptidase, which through their proteolytic activity break down polypeptides in the chyme to shorter peptides. The final step of protein hydrolysis is completed by brush border membrane bound peptidases which break down the protein to absorbable amino acids and short peptides (two to three amino acids long) (Ganapathy et al. 2006). Even though amino acids are absorbed, a major proportion of protein is believed to be transported over the brush border membrane as di- and tripeptides (Adibi 1997). Studies on rainbow trout

(Boge et al. 1981; Dabrowski et al. 2003) and African tilapia (*Oreochromis mossambicus*) (Reshkin and Ahearn 1991) indicate that this is also the case in fish. The transport of these di- and tripeptides is mediated by a low affinity high capacity transporter called oligopeptide transporter 1 (PepT1) (Daniel 2004).

1.3 PepT1

PepT1 or solute carrier family 15, member nr 1 (Slc15a1) is a member of the superfamily POT (proton oligopeptide transporters) (Paulsen and Skurray 1994). After the first reported cloning of PepT1 was done in rabbit intestine (Fei et al. 1994), numerous studies have been conducted and the transporter has now been found in a number of animals and in different organs (reviewed by Meredith and Boyd (2000)). Recently the gene coding for a PepT1-type transporter in Atlantic cod was sequenced (Rønnestad et al. 2007), adding much needed information to this transporter in fish where the only published sequence available is from zebrafish (*Danio rerio*) (Verri et al. 2003). The gene coding for Atlantic cod PepT1 (assigned GeneBank accession number AY921634) was found to be 2838 bp long with an open reading frame of 2190 bp coding for a protein with 729 amino acids. Comparative analysis of the amino acid sequence showed that the transporter had 58 – 63 % similarity with other characterized PepT1 sequences, with highest identity and grouping phylogeneticaly to zebrafish PepT1. The conformation of the protein in the apical membrane of the enterocytes was found to follow the general PepT1 model (Fei et al. 1994; Verri et al. 2003; Meredith and Price 2006) with 12 transmembrane domains and a large extracellular loop between domain 9 and 10. However, within the extra cellular loop of Atlantic cod Pept1 a sequence of 8-12 amino acids with unknown function was found that was not observed in any of the other PepT1 sequences, including zebrafish (Rønnestad et al. 2007).

Investigation of the tissue distribution in Atlantic cod showed no expression of the transporter in heart, gill, eye or liver, very low expression in ovary and higher expression in spleen, kidney and intestine (Rønnestad et al. 2007). This distribution is similar to the results obtained in zebrafish (Verri et al. 2003), with the exception of ovary where no expression of PepT1 was found in zebrafish. Intestinal expression of PepT1 has also been

found in Asian weatherloach (*Misgurnus anguillicaudatus*) (Gonçalves et al. 2007). Also in this study no expression of PepT1 was found in gill. However, contrary to cod and zebrafish there was a fairly high expression of PepT1 in liver and heart but with no detectable expression in kidney, indicating that differences in tissue distribution exist among species of fish. In the study on Atlantic cod (Rønnestad et al. 2007), investigation into the regional expression of PepT1 along the digestive tract showed no expression in the stomach, high signals in pylorus caeca, proximal intestine and mid intestine but with a weaker signal in the most distal part of the intestine. Amberg et al. (2008) studied the spatial expression of PepT1 in the digestive tract of developing Atlantic cod larvae, and found that PepT1 mRNA was present in the whole intestine before onset of exogenous feeding. These findings indicate that PepT1 is important in protein uptake in all life stages of Atlantic cod. In both fish (Verri et al. 2003; Rønnestad et al. 2007) and mammals (Freeman et al. 1995; Sai et al. 1996) histological studies have showed that PepT1 mRNA is expressed in the villus of the intestinal epithelium, while the protein is mainly located from the mid part to the tip of the villus, anchored in the apical membrane of the absorptive cells microvilli (Freeman et al. 1995; Sai et al. 1996; Ogihara et al. 1999).

The transport of peptides via PepT1 into the enterocyte is regulated by a trans-membrane electrical potential and a inward proton gradient across the membrane (Ganapathy and Leibach 1983; Sai et al. 1996). The enterocyte inside negative membrane potential is generated by Na^+-K^+-ATPase and a potassium channel in the basolateral membrane and the H^+ gradient by a Na^+-H^+-exchanger in the apical membrane (Ganapathy et al. 2006). The presence of the H^+ gradient is essential because the peptides are co-transported together with the hydrogen ions over the apical membrane and into the enterocyte (Daniel 2004). Transported peptides are normally hydrolyzed intracellularly by cytoplasmic peptidases so mainly free amino acids are transported out of the basal membrane and into the portal circulation (Ganapathy et al. 2006). However, small peptides that are resistant to hydrolysis may enter the blood and this has led to an intensive investigation in humans because many drugs, pro-drugs and bioactive peptides gain entry to the systemic circulation via PepT1 (for a review see Brandsch et al. 2008). Investigations into the role of PepT1 in nutrient uptake and how it is stimulated in fish

will therefore not only give important information for production of better feed, but may also give information on how the transporter can be utilized as a transporter of bioactive compounds.

A number of studies have been conducted on mechanisms regulating the activity of PepT1 in mammals (for a review see Adibi 2003), but only two known studies have been targeting the dietary effects on PepT1 activity in fish (Gonçalves et al. 2007; Amberg et al. 2008). Gonçalves et al. (2007) studied the regional expression of PepT1 mRNA along the intestine of Asian weatherloach introduced to three different dietary conditions. For a period of one month the fish were either fed a protein rich diet, a carbohydrate rich diet or starved. No significant differences in PepT1 expression was found between diets for any of the investigated segments. However, the high protein diet contained only 9 % more protein than the carbohydrate diet and the results could be further confounded by the fact that the diets varied in energy and levels of other components (lipid, fiber, moisture and ash). In the study of Amberg et al. (2008), where PepT1 gene expression was studied in Atlantic cod larvae fed either zooplankton or enriched rotifers, it was found that when the larvae started exogenous feeding a slight increase in expression of PepT1 occurred, but with no differences between diets. Dietary differences were found in larger larvae (> 0.15mg dry weight), where a higher expression of PepT1 was found in fish fed zooplankton compared to the rotifer fed groups. However, a change from one diet to the other did not alter these differences, allowing the authors to suggest that other factors than the feed affected the expression of PepT1 in Atlantic cod larvae. No chemical analysis was conducted on the two diets in the study, so it is therefore not known if the dissimilarity in dietary composition had the potential to generate differences in PepT1 expression. The dietary effects on regulation of PepT1 expression and activity can be more important in other life stages of fish, so more thorough investigations need to be conducted. In higher vertebrates PepT1 is found to be regulated by its substrates in a number of studies. In humans, PepT1 appears to be up-regulated by the presence of dipeptides both at the protein and mRNA level (Thamotharan et al. 1998; Walker et al. 1998). In both studies Caco-2 cells were used to study how incubation of the cells together with glycyl-L-glutamine affected the uptake of glycylsarcosine (Gly-Sar). In the study by Thamotharan et al. (1998) there was no

increase in uptake after two hours but a significant increase after 24 hours. Inhibition of translational mechanisms abolished this effect suggesting that increased activity of PepT1 was based on increased levels of PepT1 mRNA and subsequent protein synthesis. Walker et al. (1998) demonstrated that cells incubated for three days had a significant increase in transport abilities, which was caused by higher numbers of active PepT1 proteins and increased stability and synthesis of PepT1 mRNA. Dietary protein has also found to up-regulate expression and activity of PepT1 in rats (Erickson et al. 1995; Shiraga et al. 1999), where Shiraga et al. (1999) also showed that the amino acid phenylalanine up regulated the amount of PepT1 mRNA and protein. In rats, developmental differences in regional expression of PepT1 has also been found (Shen et al. 2001), with the highest expression of PepT1 in both small and large intestine up to five days after birth. After this period the expression of PepT1 is reduced to undetectable levels in the colon and to 25 % of the activity observed in the small intestine, a change that is postulated to be caused by a change from a protein rich milk diet (during nursing) to a more omnivorous adult feeding regime. Deprivation of food also seems to have a stimulatory effect on PepT1. Using immunostaining and ultrastructural visualization of rat intestine Ogihara et al. (1999) found a significant increase in membrane bound PepT1 protein in rats that had been starved for four days. Further, Naruhashi et al. (2002) found that rats that had been starved for two days showed a significant increase in PepT1 mRNA in the small intestine.

In summary, the studies on higher vertebrates discussed above demonstrate that dietary composition and nutritional status are important factors regulating PepT1 activity both at mRNA and protein level. Although it is possible that PepT1 in fish are regulated in similar ways, more detailed studies are necessary to understand how the dietary ingredients in fish feed affect the activity of the transporter. The differences in environmental conditions for terrestrial and marine vertebrates could make the functional characteristics of PepT1 in teleost differ from the general vertebrate model. Marine teleost continuously drink water as part of their adaptation to the hyperosmotic sea water and this lead to a continuous loading of ions into the intestinal lumen (Marshall and Grosell 2006). This could result in different luminal osmotic and ionic working conditions for the nutrient transporters in terrestrial vertebrates and marine teleost. Further, the high variability in life history strategies and biological settings among fish

species would make conclusions for fish in general based on results obtained from one species speculative. In a nutritional aspect this is supported by the findings that both proteolytic activity and nutrient absorption capacities vary in different species of fish under the same experimental conditions (Buddington et al. 1987; Hidalgo 1999). In relation to PepT1 some special features have been observed in the two studies where the PepT1 has been sequenced for fish that could alter the functional characteristics of the transporter (Verri et al. 2003; Rønnestad et al. 2007). The unique short amino acid sequence found in the extra cellular loop in Atlantic cod PepT1 (Rønnestad et al. 2007) is not observed in other species and could affect the function of the transporter. In the study of zebrafish PepT1 by Verri et al. (2003) it was found that extracellular alkalization led to an increase in dipeptide uptake, which is in contrast to the mammalian model where increase in peptide transport is found during extracellular acidification (Ganapathy and Leibach 1983). It is therefore suggested that species specific studies should be conducted when studying the functionality of PepT1. With this thesis we therefore set out to obtain information on the dietary regulation of spatial PepT1 transport activity and mRNA expression in the intestine of juvenile Atlantic cod.

1.4 Goal and hypothesis

The overall goal of this study was to investigate how fractions of peptides with different chain length included in the diet affected the spatial transport capacities and mRNA expression of PepT1 in the post-gastric digestive tract of Atlantic cod. These results where planned to be obtained through the following steps:

1. Establish a protocol to measure transport of peptides across the brush border membrane in Atlantic cod.
2. Optimize the protocol for relative quantification of PepT1 gene expression in Atlantic cod.
3. Use these protocols to assess the transport capacities and mRNA expression of PepT1 in pyloric caeca and in four equally long segments from the remainder of the intestine. And to determine how the activity in these intestinal regions was affected when one third of fish meal of the diets was replaced with peptides of

different size or free amino acids. The regional expression was to be studied at two different sampling points to see if any changes in activity occurred over time.

The hypothesis is that inclusion of dietary short chained peptides will lead to an increase in the concentration of available substrates for the PepT1 in the intestinal lumen. In order to absorb the high levels of peptides it is expected an increase in PepT1 activity (transport capacities and/or mRNA expression). It is anticipated that that short peptides in the diet will require less proteolytic work and cause increased activity of PepT1 in the proximal regions of the intestine. If the absorptive capacity for peptides in the proximal part of the intestine become saturated it is hypothesised that there will be additional mobilization of PepT1 in distal regions of the intestine that are expected to be less active in peptide absorption.

2. Materials and methods

The activity of PepT1 in the pyloric caeca and five equally long segments from the pyloric ceca to the anus of juvenile Atlantic cod was investigated. Dietary regulation of the transporter was examined by feeding five groups of fish diets containing approximately 42 % protein either as fish meal or approximately 30% of the fish meal substituted by different fractions of fish protein hydrolysate or by free amino acids. In order to describe the dietary effect on PepT1 two analytical approaches was selected. The first method quantify the transport capacity of PepT1 directly by the use of brush border membrane vesicles (BBMV) while the latter method assess the level of mRNA coding for PepT1 by quantitative reverse transcriptase polymerase chain reaction (q-RT-PCR). For all groups of fish the activity of PepT1 was investigated after 14 and 46 days on experimental diets.

2.1 Fish rearing and experimental conditions

Juvenile Atlantic cod (8-15 grams) were obtained from Real Salmon AS, Eikelandosen, Norway. During the acclimatization and experimental period from mid May to early August 2007 fish were reared in the basement of the High Technology Centre, University of Bergen, Norway. The fish were divided in to five 1000 litre fibreglass tanks and acclimatized to experimental conditions for four weeks (T = 9ºC and 24 h light). All groups were hand-fed until visual satiety twice a day (7 days/week), and the amount food delivered was recorded. During the acclimatization period the fish were fed a 2.5 mm pelleted fishmeal diet (FM diet in Table 1). Three weeks into the acclimatizing period all fish were anaesthetized using 50mg L^{-1} tricain methanesulfonate (MS-222) (Argent Chemical Laboratories Inc., WA, USA) and weight and length were measured before pit-tag was inserted in the abdomen (Trovan MicroTransponder ID162A, Identify UK Ltd). In order to let the fish restore from tagging all fish were kept one more week in their tanks on the FM diet before control sampling and transition to experimental diets. The protocol was approved by the local representative for the Norwegian State Board of Biological Experiments with Living Animals.

2.2 Diets

All diets were provided by Dr. Anders Aksnes at NOFIMA, as 2.5 mm extruded pellets, produced to be isoproteic, isolipidic and isoenergetic, and designed to vary only in protein composition. Overview on ingredients and chemical composition of the different diets is presented in Table 1.

Table 1 - Ingredients and chemical composition of diets

Ingredient (%)	FM	FH	UFR	NFR	FAA
Fish meal 268/06	51.8	35.3	35.1	35.1	34.6
Raw wheat 209/06	48.0	56.0	58.0	54.0	36.5
Fish hydrolysate	0.0	14.4	0.0	0.0	0.0
Ultra filtration retenate	0.0	0.0	13.2	0.0	0.0
Nano filtration retenate	0.0	0.0	0.0	13.0	0.0
Fish oil [1]	12.2	14.1	14.1	14.1	14.0
Vitamin mix [2]	1.0	1.0	1.0	1.0	2.0
Mineral mix [3]	0.4	0.4	0.4	0.4	0.4
Betafine [4]	0.4	0.4	0.4	0.4	0.4
Inositol [5]	0.03	0.03	0.03	0.03	0.0
Lysine-HCl	10.0	10.0	10.0	10.0	0.0
Dicalcium phosphate	17.6	17.6	17.6	17.6	0.0
Amino acid mix [6]	0.0	0.0	0.0	0.0	12.1
Proximate composition					
Dry matter (DM, %)	95.9	95.2	94.7	94.6	95.5
Moisture (%)	4.1	4.8	5.3	5.4	4.5
Crude Protein (% DM)	43.1	43.2	41.9	41.7	41.5
Crude Fat (% DM)	19.6	19.3	18.7	20.0	19.1
Carbohydrate [7]	26.2	26.3	27.5	27.1	30
Ash (% DM)	7.0	6.4	6.6	5.8	4.9
Energy (calculated) (Mj/kg)	22.6	22.5	22.1	22.5	22.6

[1] NorSeaOil, Norsildmel, Norway.
[2] Provided per kg of feed: vitamin D_3, 3000 I.E.; vitamin E, 160 mg; thiamin, 20 mg; riboflavin, 30 mg; pyrodoxine-HCl, 25 mg; vitamin C, 200 mg; calcium pantothenate, 60 mg; biotin. 1 mg; folic acid, 10 mg; niacin, 200 mg; vitamin B_{12}, 0.05 mg; menadion bisulphite, 20 mg.
[3] Provided per kg of feed: magnesium, 56 mg; potassium, 450 mg; zinc, 90 mg; iron, 56 mg; manganese, 11 mg; copper, 5.6 mg.
[4] Betafin BCR, Finnsugar Bioproducts, Finland.
[5] Danisco Animal Nutrition, Finland.
6 Provided as percentage of total: aspartic acid, 9.36; glutamic acid, 13.54; hydroxyprolin, 0.96; serine, 4.15; glycine, 6.13; histidine, 5.19; arginine, 7.38; threonine, 4.20; alanine, 6.05; proline, 4.04; tyrosine, 3.42; valine, 4.84; methionine, 3.08; isoleucine, 4.20; leucine, 7.30; phenylalanine, 3.91; lysine, 9.74; cysteine, 1.44; tryptophan, 1.07.
[7] Carbohydrate calculated as 100 % - %protein - %fat - %ash - %moisture.

Diets contained approximately 42 % crude protein (CP), provided either as fish meal (FM diet) or approximately 30 % of the fish meal substituted by either fish hydrolysate (FH diet), retenate after ultra filtration of fish hydrolysate (UFR diet) or retenate after nano filtration of fish hydrolysate (NFR). The plan was to include permeate after nano filtration to the last diet, but this was not possible due to technical reasons. Therefore, for

the last diet a mix of free amino acids was instead used to substitute 30 % of the fish meal (FAA diet). On dry weight basis all diets were calculated to contain approximately 30 % carbohydrates and 20 % fat. A detailed description on production of ingredients and feed together with the chemical analysis is provided in literature elsewhere (Aksnes et al. 2006a; Aksnes et al. 2006b; Aksnes et al. 2006c).

Table 2 - Content of free amino acids (% of total protein)

Amino acid	FM	FH	UHR	NFR	FAA
Aspartate	0.04	0.24	0.31	0.35	1.76
Glutamate	0.25	0.53	0.66	0.64	3.6
Hydroxyproline	< 0.01	0.02	0.02	0.04	1.34
Serine	0.04	0.21	0.28	0.21	1.3
Aspargine	0.02	0.04	0.06	0.07	0.04
Glutamine	0.15	0.26	0.33	0.2	1.99
Glycine	0.01	0.03	0.06	0.08	0.03
3-amino-propanoic acid	0.01	0.05	0.06	0.04	0.01
Taurine	0.87	1.13	1.36	1.07	0.57
Histidine	0.07	0.13	0.15	0.16	1.57
4-amino-butanoic acid	0.02	0.03	0.06	0.1	0.07
Citrulline	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
Threonine	0.05	0.2	0.26	0.26	1.19
Alanine	0.24	0.47	0.65	0.53	1.22
Carnosine	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Arginine	0.11	0.34	0.39	0.55	2.33
Proline	0.04	0.15	0.21	0.19	1.29
Anserine	0.05	0.33	0.43	0.46	0.05
Tyrosine	0.03	0.19	0.16	0.21	0.61
Valine	0.07	0.3	0.4	0.41	1.51
Methionine	0.02	0.19	0.25	0.25	1.05
Cysteine	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Isoleucine	0.04	0.25	0.32	0.35	1.33
Leucine	0.08	0.58	0.76	0.79	2.38
Phenylalanine	0.04	0.28	0.35	0.32	1.12
Tryptophan	0.03	0.08	0.09	0.09	0.34
Ornithine	0.03	0.04	0.13	0.07	0.02
Lysine	0.13	0.43	0.63	0.73	2.49
Total free amino acids	2.44	6.5	8.38	8.17	29.21

Free amino acid composition of the different diets is presented in Table 2. With higher levels of 17 out of the 28 investigated amino acids (with the exception of ornithine, cysteine, anserine, arginine, carnosine, citrulline, 4-amino-butanoic acid, taurine, 3-amino-propanoic acid, glycine and aspargine), the FAA diet contained the highest amounts of free amino acids (making up close to 30 % of the protein). With the exception of cysteine, citrulline and ornithine, the FM diet contained the lowest amount of both individual and total free amino acids. The FH, UHR and NFR diets contained equal

amounts of all amino acids, with a slightly lower total free amino acid concentration in the FH diet.

Details on the fractional composition of fish hydrolysate and filtered fish hydrolysate included in the FH, UFR and NFR diet is presented in Table 3. All fish hydrolysate material contained small or undetectable fractions of the largest peptides (10 000-20 000 Da). The total fish hydrolysate (included in FH diet) contained the highest amount of all peptide fractions except the medium sized peptides (1000-5000 Da), and the lowest amount of anserine and taurine of the three. The retenate fish hydrolysate after ultra filtration (included in UFR diet) contained the lowest amount of the shortest peptides and free amino acids, but approximately 90 % of the protein as an even distribution for short to large chained peptides. FH retenate after nano filtration (included in NFR diet) contained the highest amount of the second smallest peptide fractions (100-1000 Da) and the lowest amount of large peptides (5 000-10 000 Da).

Table 3 - Chemical composition of fish hydrolysate and filtered FH

Ingredient (g/kg DM)	FH	UFR	NFR
Yield by fractionation (%)	100.0	57.0	29.0
Crude protein	913.0	972.0	959.0
Lipid	<1.0	<1.0	<1.0
Ash	78.0	15.0	64.0 [1]
Free amino acids (% of protein)	10.4	1.7	14.8
Peptides 10.000-20.000 Da [2]	<1.0	<1.0	<1.0
Peptides 5.000-10.000 Da [2]	35.9	35.0	19.7
Peptides 1.000-5.000 Da [2]	9.7	25.8	12.4
Peptides 100-1.000 Da [2]	36.1	28.0	58.8
Peptides <100 Da [2]	16.0	6.7	9.0
Anserine (g/kg prot)	27.5	48.0	41.2
Taurine (g/kg prot)	11.0	17.0	17.0

[1] Include some chloride from HCl added for preservation.
[2] Size fractioned as described in Aksnes et al. 2006b.

2.3 Fish sampling

Two fish from each tank were sampled on the day before feeding with experimental diets (T0) and ten fish from each tank 14 days (T1), 34 days (T2) and 46 days (T3) after T0. Before dissection the fish where anaesthetized using 50mg L^{-1} MS-222 and killed with a blow to the head. All dissecting work was done on ice. The weight and length of each fish was measured before the whole intestine was removed by cutting right before the pyloric caeca and right before the anus. The intestine was divided into five segments consisting

of the pyloric caeca and the remainder of the intestine divided into four equally long parts (Figure 1). From each intestine a small section right after the pyloric caeca was sampled for use in other studies (in situ hybridization). To make sure that the relative length of all the segments were the same for each intestine a template form was used (Figure 2). When the intestine (without the pyloric caeca) was

Figure 1 - *Segments sampled from each intestine. Segment 1 (S1) being the pyloric caeca and the following segments (S2-S5) equally long parts from the remainder of the intestine. The short piece between the pyloric caeca and segment 2 was sampled for other studies.*

stretched over the template the part covering the dark section of the template was cut out (sample for other studies) and the rest of the inte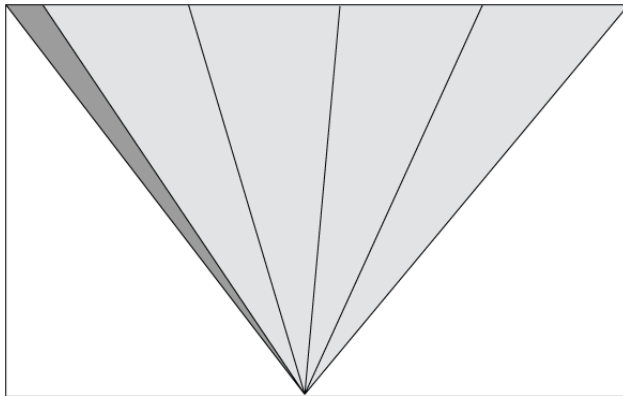stine divided into four equally long parts according to the light sections on the template. The intestine was emptied of any leftover feed by gently stroking the content out and each segment was rinsed thoroughly in a phosphate-buffered saline (PBS) solution (145 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, pH 7.2) before gently dried with lab paper. The segments were then wrapped in pre-labeled aluminum foil, frozen in liquid nitrogen and stored at - 80 ºC until further use.

Figure 2 - *Template to cut intestine. Dark narrow part to the left was used to sample a short segment of the intestine that where to be used in other studies. The remainder of the intestine was cut into four equally long parts according to the light sections of the template.*

2.4 Isolation of BBMV and electrogenic transport measurements

BBMV produced from intestine have been successfully used in a number of transport studies both in humans (Ganapathy et al. 1986; Malo and Berteloot 1991), rabbit (Ganapathy et al. 1984), rat (Cassano et al. 1984) and fishes like European eel (*Anguilla*

anguilla) (Storelli et al. 1986; Verri et al. 1992; Maffia et al. 1997; Verri et al. 2000), African tilapia (Reshkin and Ahearn 1991; Thamotharan et al. 1996), Pacific copper rockfish (*Sebastes caurinus*) (Ahearn et al. 1992) and Antarctic ice fish (*Chionodraco hamatus*) (Maffia et al. 2003). No known studies have been performed where BBMV have been isolated from Atlantic cod or any other gadoid fishes. Methodical training for this procedure was conducted at the University of Salento, Italy, and during the stay work was done on the preparation of an article describing the method which is now published in Aquaculture Nutrition (Verri et al. 2008). Details on the method and the materials used are described in this article and is attached as Appendix A. Briefly, starting from intestinal mucosal scrapings it is possible to obtain the brush-border membranes of the enterocytes in a right (luminal) side out vesicular form (called brush-border membrane vesicles, or BBMV) through initial homogenization and following steps of a) precipitation in the presence of Mg^{2+} and hyposmotic medium and b) centrifugation (described in detail by Storelli et al. 1986). To assess the quality of the isolated BBMV, a comparison is made between the specific activity of brush-border membrane-bound enzymes (for instance alkaline phosphatase, leucine aminopeptidase and maltase) in the final BBMV fraction and in the homogenate from the mucosa (prior to isolation of vesicles) (Storelli et al. 1986). A 12-to-18-fold increase in the activity in one or more of such membrane-bound enzymes (often called enrichment factor or yield) is generally considered adequate to indicate that an enriched fraction of BBMV has been obtained after the biochemical isolation process (Prof. Tiziano Verri, personal communication). In parallel, a negligible enrichment in other membrane-bound enzymes (such as the Na^+/K^+-ATPase, that is a marker for the basolateral membrane), as well as in organelle-specific enzymes and cytosolic enzymes, rules out the possibility that the BBMV fraction is contaminated by other cellular components (Storelli et al. 1986).

BBMV are osmotically active (i.e. the vesicular membrane separates the extravesicular from the intravesicluar space) and when used in conjunction with radioactive or fluorescent tracers can be utilized to monitor a variety of transport phenomena across membrane. The potential-sensitive fluorescent dye 3,3'-diethylthiadicarbocyanine iodide (DiS-C$_2$(5)) is ideal to spectrophotometrically detect changes in membrane potential in conjunction with BBMV (Verri et al. 2008). When an

inside-negative membrane potential is artificially generated in BBMV by using an outwardly-directed 100-to-1 K^+ gradient and the K^+ ionophore valinomycin, the fluorescent dye (DiS-C_2(5)) binds to the vesicles causing a rapid decline in the measured fluorescence. As the artificially-induced membrane potential diminishes, the dye is steadily released to the extravesicular medium as a function of time, thus causing an increase in fluorescence. Electrogenic transport via membrane transporters (that induce charge movement across the vesicle membrane) significantly affect the membrane potential dissipation rate, and consequently the fluorescence signal. As di- and tripeptides cross the membrane together with hydrogen ions via PepT1 (Daniel 2004), PepT1-mediated transport activity can be monitored by comparing the fluorescence changes in the extravesicular medium in the presence and absence of extravesicular peptides. Based on this difference it is possible to calculate the transport rate of peptides into the vesicles. The method can also be utilized to measure electrogenic transport of other compounds like amino acids and sugars (Verri et al. 2008), and may also have a potential to study the transport of peptide-mimicking drugs since many of these are co-transported via PepT1 (Rubio-Aliaga and Daniel 2002; Brandsch et al. 2008).

2.5 Q-RT-PCR

Q-RT-PCR is a powerful tool that can be used to detect even the smallest amount of RNA (reviewed by Kubista et al. 2006). RNA isolated from tissue or cells can with the help of the enzyme reverse transcriptase be synthesized into complementary DNA (cDNA), a discovery that awarded David Baltimore, Renato Dulbecco and Howard Martin Temin with the 1975 Nobel Prize in medicine (The Nobel Foundation 2008). Through the polymerase chain reaction (PCR), first explained by Mullis et al. (1986), the cDNA can be amplified exponentially to yield high amounts of product. By using gene specific primers, PCR can be used to amplify a targeted gene sequence in the cDNA (Kubista et al. 2006). It is possible to measure this amplification process real-time (real-time PCR) by using fluorescent probes that emit fluorescence when binding to double stranded DNA (amplified cDNA) (Bustin 2002). The number of amplifications it takes for the fluorescent to reach a threshold value is called the crossing point or the cycle threshold (Ct), and the time needed to reach this threshold will depend on the starting concentration

of the targeted sequence. Although it is possible to perform absolute quantification of a gene (reviewed by Bustin 2000), relative quantification based on normalization to a standard is preferred when doing comparative analysis (Pfaffl 2001). A reference gene is often used as standard and is a gene that is present in the investigated tissue which expression is not affected by different experimental conditions (Kubista et al. 2006). In a PCR this means that the Ct value should be the same for all compared samples when an equal amount of template is used. However, stable expression of a reference gene is rarely found in all tissues under different experimental conditions, so for a given experimental setup, multiple potential reference genes should be evaluated in order to find the most suited candidate (Thellin et al. 1999). GeNorm is a Visual Basic applet for Microsoft Excel developed by Vandesompele et al. (2004), and is often used when determining the most stable reference gene among a number of candidates. This applet runs a pair wise comparison of the variation in relative quantities for every investigated gene, ranking the stability of each gene with an M-value. M-value lower than 1.5 is an indication for good stability, and the gene showing the lowest value should be selected as an internal control gene (Vandesompele et al. 2004). Once an appropriate reference gene is obtained, the expression of the gene of interest (GOI) can then be related to the expression of this reference gene, and the relative quantities calculated by the following formula:

$$RQ = 2^{Ct_{REF} - Ct_{GOI}}$$

Equation 1 (Kubista and Sindelka 2007)

Where RQ is the relative quantity and Ct_{REF} and Ct_{GOI} is the Ct value for the reference gene and the gene of interest respectively. However, this formula assumes that both reference gene and gene of interest have been amplified with 100 % efficiency, a criterion that in most cases are not met (Kubista et al. 2006). The efficiency of the amplification process depend on the purity of the template used and should be evaluated as a number of substances and reaction conditions have been found to inhibit or enhance the PCR (for a review see Wilson 1997). Components affecting amplification can often be diluted out, so a method frequently used to determine amplification efficiency is to calculate the linearity of a cDNA dilution series (Rasmussen 2001). By running a linear regression on

plotted Ct values against the cDNA concentration the slope of this curve can be used to calculated the amplification efficiency according to the formula:

$$E = 10^{[-1/slope]}$$

Equation 2 (Rasmussen 2001)

Where E is amplification efficiency and given as a value between 1 and 2, where 2 equals 100 % efficiency. Once amplification efficiency of both reference gene and gene of interest has been determined relative gene expression can be calculated according to the formula:

$$NE = \frac{(E_{GOI})^{Ct_{GOI}}}{(E_{REF})^{Ct_{REF}}}$$

Equation 3 (Muller et al. 2002)

Where NE is normalized gene expression, E_{GOI} amplification efficiency of gene of interest, E_{REF} efficiency of reference gene and Ct_{GOI} and Ct_{REF} crossing point values for gene of interest and reference gene respectively. This method was used to determine the PepT1 mRNA expression in segments of Atlantic cod intestine.

2.6 RNA isolation

For each diet (FM, FH, UFR, NFR and FAA) three fish (total of 3x5 segments for each diet) was randomly selected from the sampled f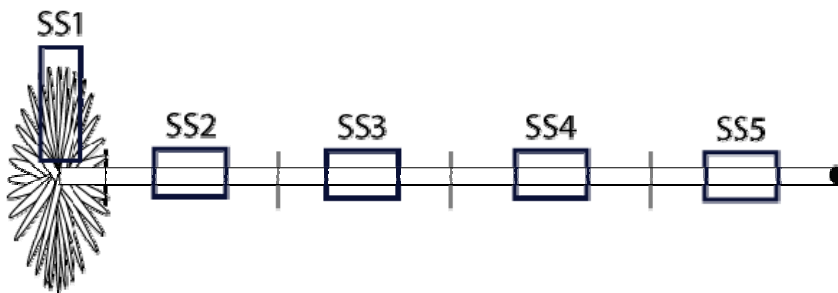ish at T1 and T3 (14 and 46 days after start of feeding with experimental diets). A sub-sample of 60 +/- 10 mg tissue was taken from each segment for RNA isolation. The location from which the sub-samples were taken is illustrated in Figure 3. Dissecting of segments was done on dry ice to prevent thawing of

Figure 3 - *Illustrating where each sub-sample for RNA isolation were taken from the intestinal segments. Sub-sample 1 from the pyloric caeca was taken from the tip to the base of the caeca and sub-sample 2-5 taken from the center part of each segment.*

tissue before it was added to the extraction medium. Total RNA was isolated using TRI-reagent (Sigma, MO, USA) according to the manufacturer's protocol. Tissue was homogenized for 20 seconds in 1 ml TRI-reagent using Lysing Matrix D tubes (MP Biomedicals, OH, USA) in a FastPrep FP120 (Savant Instruments Inc, NY, USA). After precipitation with isopropanol and washing with ethanol the RNA pellet was dissolved in 44 µl diethylpyrocarbonate (DEPC) treated water (Ambion, TX, USA).

2.7 DNAse treatment

In order to remove any genomic DNA the RNA was treated with RQ1 RNase-Free DNase (Promega Corporation, WI, USA) according to manufacturer's recommendations with DNase Stop reaction substituted by phenol:chloroform extraction. In an additional precipitation step the (DNase treated) RNA was added 9 ml Sodium Acetate and 250µl 100 % ethanol and stored for 2 hours at -20ºC. Precipitated RNA was then washed in ethanol and re-suspended in 30-50µl DEPC-treated water depending on size of RNA pellet.

2.8 Quantification and quality control of RNA

Purity and concentration of RNA was measured using a ND-1000 spectrophotometer (NanoDrop Technologies, NC, USA). Integrity of the RNA was checked by running 1µg RNA on an ethidium-bromide stained 1 % agarose gel with 1x TAE buffer. Gels were then subjected to ultraviolet light and photographed in a GDS 7500 White/Ultraviolet Transilluminator (UVP, CA, USA).

2.9 cDNA synthesis

For each sample 4µg (0.2µg/µl) of RNA was synthesized to cDNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, CA, USA) with Oligo(dT)$_{20}$ primers according to manufacturer's protocol, but with RNase H treatment omitted. For every diet RNA from one segment was selected as negative reverse transcription (-RT) and prepared without the addition of the reverse transcriptase enzyme. The enzymatic reaction was incubated using a Peltier Thermal Cycler 200 (MJ Research Inc., MA, USA).

2.10 Calculation of amplification efficiency and working concentrations, and evaluation of reference genes

Atlantic cod elongation factor 1 alpha (EF1A), ubiquitin (Ubi) and Ribosomal protein S9 (S9) was selected as reference gene candidates. For sequence of forward and reverse primers for all genes see Table 4. CDNA from all segments from two fish (UFR diet) where pooled and two dilution series was made (2x and 10x) to be used in calculation of amplification efficiency and working concentration for gene of interest and all reference genes. With PepT1 specific primers the 2x dilution series was run in triplicates on a PCR plate (as described in section 2.11 Real-time PCR), and for each reference gene the 10x dilution series was run in triplicates with corresponding primers. To calculate the amplification efficiency Ct values and concentrations were plotted in Microsoft Excel and the slope of the dilution curve calculated by linear regression. Amplification efficiency of codPepT1 and the three reference genes were then determined according to *Equation 2*, proposed by Rasmussen (2001). Amplification efficiency in percentage was calculated using "QPCR Standard Curve Slope to Efficiency Calculator" (Stratagene ® 2008)

Table 4 – Nucleotide sequence of primers for PepT1 and reference gene candidates

Gene	*GenBank reference*	*Amplicon length*	*Sequence forward and reverse primers*
codPepT1	AY921634	103	F: 5'-GGC TTT TAT TGC TGC TGC TC-3' R: 5'-ACC GGC CAA GTT AAA GAC CT-3'
EF1A	CO541820	93	F: 5'-CCCCTC CAG GAC GTC TAC AAG-3' R: 5'-GGC AGA GCC ACC GAT CTT C-3'
S9	CO542669	84	F: 5'-TCT TTG AAG GTA ATC CAA CTT CAT CTT-3' R: 5'-CGA GGA TGT AAT CCA ACT TCA TCT T-3'
Ubiquitin	CO542553	69	F:5'-GGC CGC AAA GAT GCA GAT-3' R:5'-CTG GGC TCG ACC TCA AGG T-3'

All reference gene primers were provided at the courtesy of Pål Olsvik. Primers for Cod PepT1 are published in Amberg et al (2008).

Because cDNA was synthesized using oligo-dT primers (thereby transcribing only mRNA) the final concentration of cDNA was not known. Determination of cDNA working concentrations were therefore based on pre-transcription total RNA

concentrations (0.2 µg/µl). The working concentration was selected as a common value that was within the linear phase of the dilution curves for all investigated genes. This was found to be a dilution to 0.03 µg/µl from an initial 0.2 µg/µl total RNA (approximately seven fold dilution of cDNA).

The M-value was calculated for every reference gene to find which of the three that had the most stable expression among samples (Vandesompele et al. 2004). For each of the five intestinal segments cDNA was pooled from three different diets (FM, FH and FAA). For each reference gene the five pooled samples was then run in triplicates on a PCR plate (as described in section 2.11 Real-time PCR) to obtain the Ct values. Relative quantities calculated from mean Ct values were then evaluated using geNorm (Vandesompele et al. 2004). The reference gene that gave the lowest M-value was selected as internal control gene to be used on the experimental plates, and to be related to PepT1. To confirm the stability of the selected reference gene Ct values from all experimental plates were evaluated in geNorm. For each diet the stability among segments was investigated, and by comparing reference gene Ct values from different plates the stability among segments from different diets and from different times was also evaluated.

2.11 Real-time PCR

cDNA was amplified in a PTC-200 (MJ Research, MA, USA) with a Chromo 4 Continuous Fluorescence Detector (Bio-Rad, CA, USA) managed by the software RJ Opticon Monitor 3.2.32 (Bio-Rad). The PCR plates used was Semi-skirt 96CLR, MicroSeal PCR Plates covered with Optical clear Microseal "B" Film (Bio-Rad). Programmed amplification process consisted of 6 minutes polymerase activation at 95ºC followed by 40 cycles at 95ºC for 30 s, 56ºC for 30s, 72ºC for 30 s. After last cycle there was a final extension at 72ºC for 10 minutes before the amplification process was ended. Amplification of product was detected using SYBR Green PCR Master Mix (Applied Biosystems, CA, USA). cDNA from each segment was amplified in triplicates both for PepT1 and for the reference gene (total of six wells for each sample). In order to detect any variation between plates duplicates of a plate-to-plate control was added to each plate, which consisted of pooled cDNA from all segments in two fish (UFR diet). Preliminary

tests on cDNA from a number of segments showed no amplification of the –RT sample or the non-template control (NTC) (water, primers and SYBR Green) for either PepT1 or the reference gene, so on all experimental plates one well was dedicated to the –RT (PepT1 primers) and one well to NTC (reference gene primers). With 5 µl cDNA, 2.5 µl (150 ng) forward primer, 2.5 µl (150ng) reverse primer and 10 µl SYBR Green the final volume in each well was 20 µl.

2.12 Calculation of Mean Normalized Expression

After all plates were run a manual threshold value was selected (0.08) in the Q-PCR software (RJ Opticon Monitor 3.2.32, Bio-Rad) that was significantly above the background, and within the exponential phase of the amplification plot for all samples. The Ct values was then exported in to a Microsoft Excel sheet and the triplicates from each sample sorted so that the lowest, median and highest Ct-value from PepT1 was related to the lowest, median and highest Ct-value of the reference gene respectively (Simon 2003). GeNorm results from the plate-to-plate control gave M-values lower than 1.0 for both PepT1 and reference gene, but to minimize the effect of variation between plates the Ct values from every plate was also normalized towards the plate-to-plate controls according to a formula proposed by Kubista and Sindelka (2007):

$$Ct_{norm} = Ct_r^{gene} - Ct_r^{p2p} + \frac{\sum Ct_{All-p2p}}{m} \qquad \textbf{\textit{Equation 4}}$$

Where Ct_{norm} is the Ct value normalized to plate-to-plate variance, Ct_r^{gene} the measured Ct value, Ct_r^{p2p} measured plate-to-plate Ct value on the respective plate, m the number of plates and $\Sigma Ct_{All-p2p}$ the sum of all plate-to-plate Ct values.

The normalized Ct values was then used to calculate mean normalized expression (MNE) using the Excel Visual Basic applet qGene (Muller et al. 2002).

2.13 Statistical analysis

MNE values were checked for normality using a Shapiro-Wilk's test and homogeneity of variance was investigated using a Levene's test. MNE values showed homogeneity of variance but normality tests failed (Shapiro-Wilk`s test: $p<0.05$). MNE values were then $\log_2$ transformed as suggested by Kubista and Sindelka (2007), and normality and homogeneity of variance re-tested. The tests showed normality and homogeneity of variance for $\log_2$ transformed values which were used in further statistical analysis. The effect of diet and time on MNE in each segment was investigated using a two-way analysis of variance (ANOVA). Differences in expression between segments within each diet were investigated for both times by repeated measures one-way ANOVA. In cases where significant differences were found a post-hoc Tukey HSD test was used to determine which groups differed. For the post-hoc of the repeated measures ANOVA the critical value was estimated using df_{error} and $MS_{within\ segments}$ substituted by MS_{error} in Tukey HSD formula (Hays 1994). To look for correlation between PepT1 gene expression and growth or size a Pearson correlation test was done between expression in each segment and the respective fish specific growth rate and condition factor. (For calculation of specific growth rate and condition factor see subscript Table 5). All statistical analysis except the post hoc Tukey HSD was performed using the statistical software R version 2.6.1 (The R Foundation for Statistical Computing, www.r-project.org). Tukey HSD test following repeated measures ANOVA showing significant differences between groups was calculated manually using Microsoft Excel. P-values from 0.1 to 0.05 were considered as trends and p-values smaller then 0.05 were considered statistically significant.

3. Results

3.1 Fish growth and physiological performance

With temperatures stable around 9 ºC, a steady water flow and oxygen levels never below 90 % no technical difficulties occurred during the experimental period and only two fish died. Only minor differences in growth parameters were observed between groups (Table 5, from Dr. Pedro Gómez Requeni, unpublished data). No significant difference in condition factor (CF) was found between groups either for T1 or T3. Fish fed the FAA diet had a significant higher specific growth rate (SGR) then the FM, FH and UFR groups from T0 until sampling at T1 (one-way ANOVA; $P<0.01$, F-value=6.29, 4 df). No significant difference in SGR was found between groups from the T2 to T3 sampling or overall during the whole experimental period (T0 – T3). At the T1 sampling the fish fed the UFR diet showed a lower hepatosomatic index (HSI) then the other groups (one-way ANOVA; $P<0.01$, F-value=4.98, 4 df). At T3 no statistically differences were found between groups HSI.

Table 5 - Growth and physiological performance for the different diets at T1 and T3

	FM	*FH*	*UFR*	*NFR*	*FAA*
CF [a]					
T1	0.83 ± 0.06	0.84 ± 0.08	0.85 ± 0.07	0.85 ± 0.08	0.86 ± 0.08
T3	0.91 ± 0.08	0.89 ± 0.05	0.88 ± 0.1	0.86 ± 0.08	0.88 ± 0.07
SGR (%) [b]					
T0 – T1	1.62 ± 0.51^{A}	1.66 ± 0.52^{A}	1.58 ± 0.36^{A}	1.75 ± 0.54^{AB}	1.97 ± 0.54^{B}
T2 – T3	1.68 ± 0.48	1.70 ± 0.35	1.80 ± 0.52	1.65 ± 0.35	1.68 ± 0.46
T0 – T3	1.73 ± 0.28	1.75 ± 0.37	1.76 ± 0.33	1.70 ± 0.28	1.78 ± 0.41
HSI [c]					
T1	9.2 ± 1^{A}	8.8 ± 1.6^{A}	7.2 ± 2.2^{B}	9 ± 1.7^{A}	9.3 ± 1.6^{A}
T3	10 ± 1.4	9.6 ± 1.8	9.3 ± 1.6	10.1 ± 1.4	9.4 ± 2.8

Results from Dr. Pedro Gómez Requeni, unpublished data. Values are mean ± standard deviation, n=18 for HSI, n=90 for CF and SGR at T0-T1, n≈20 for CF and SGR at T2-T3 and T0-T3. Values within row showing different capital superscript letter are statistically different. Table is only showing data relevant for T1 and T3 sampling.
[a] Condition factor (CF): $(100 \times \text{weigth (g)})/\text{length (cm)}^3$
[b] Specific growth rate in % (SGR): $100 \times ((\ln \text{final weight (g)} - \ln \text{initial weight (g)}) / \text{days}))$
[c] Hepatosomatic index (HSI): $(\text{liver weight (g)} / \text{body weigth (g)}) \times 100$

3.2 Isolation of BBMV and electrogenic transport measurements

A successful preparation of BBMV were made from intestines of European eel, and electrogenic transport measurements were conducted (Verri et al. 2008, Appendix A).

However, within the time-frame available for this thesis we failed to prepare and utilize BBMV from Atlantic cod intestine in transport studies. Both tests on frozen intestines of adult cod (1.5 – 2 kg) and preparations from fresh intestines from juvenile cod (200-300 g) were unsuccessful. The spectrofluorometric measurements showed that there was no difference in fluorescence quenching when there was a difference in intra- and extravesicular K^+-concentration (outwardly directed 100mM to 1mM K^+ gradient) compared to when concentrations were the same inside and outside the vesicles (no K^+ gradient, intracellular K^+ concentration equal to extracellular K^+ concentration equal to 100 mM), indicating lack of membrane potential (data not shown). Significant yield in brush-border membrane-bound enzymes was obtained (enrichment factors; alkaline phosphatase 15.1, leucine aminopeptidase 12.9) which indicated successful isolation. Transport measurements were tested with different concentrations of vesicles, valinomycin and fluorescent dye (DiS-C_2(5)) and at two different temperatures (20 and 7ºC). Changing these parameters did not allow us to study transport, so we were unable to establish a working protocol for Atlantic cod before the start of the feeding experiment.

3.3 RNA isolation and Q-RT-PCR

Quality control of RNA samples gave a 280/260 nm ratio between 1.95 - 2.10 and a 260/230nm ratio between 2.1 - 2.3 and pictures of the gels gave clear 18S and 28S bands with a ratio 2:1 in strength for all samples (data not shown). Amplification efficiency of the different genes was found to be 1.96 (96.41 %) for PepT1, 1.99 (98.84 %) for EF1A, 1.87 (86.6 %) for S9 and 1.92 (92.03 %) for Ubi. GeNorm evaluation of the different reference genes (S9, Ubiquitin and EF1A) showed that EF1A had the lowest M-value (0.885), which was selected as reference gene when calculating the relative expression of PepT1. GeNorm evaluation of EF1A Ct values obtained from all experimental plates yielded M-values lower than 1.0 for all investigated combinations (segment : diet : time), confirming the stability of EF1A among samples and sampling points. No background was found in the NTC or -RT wells.
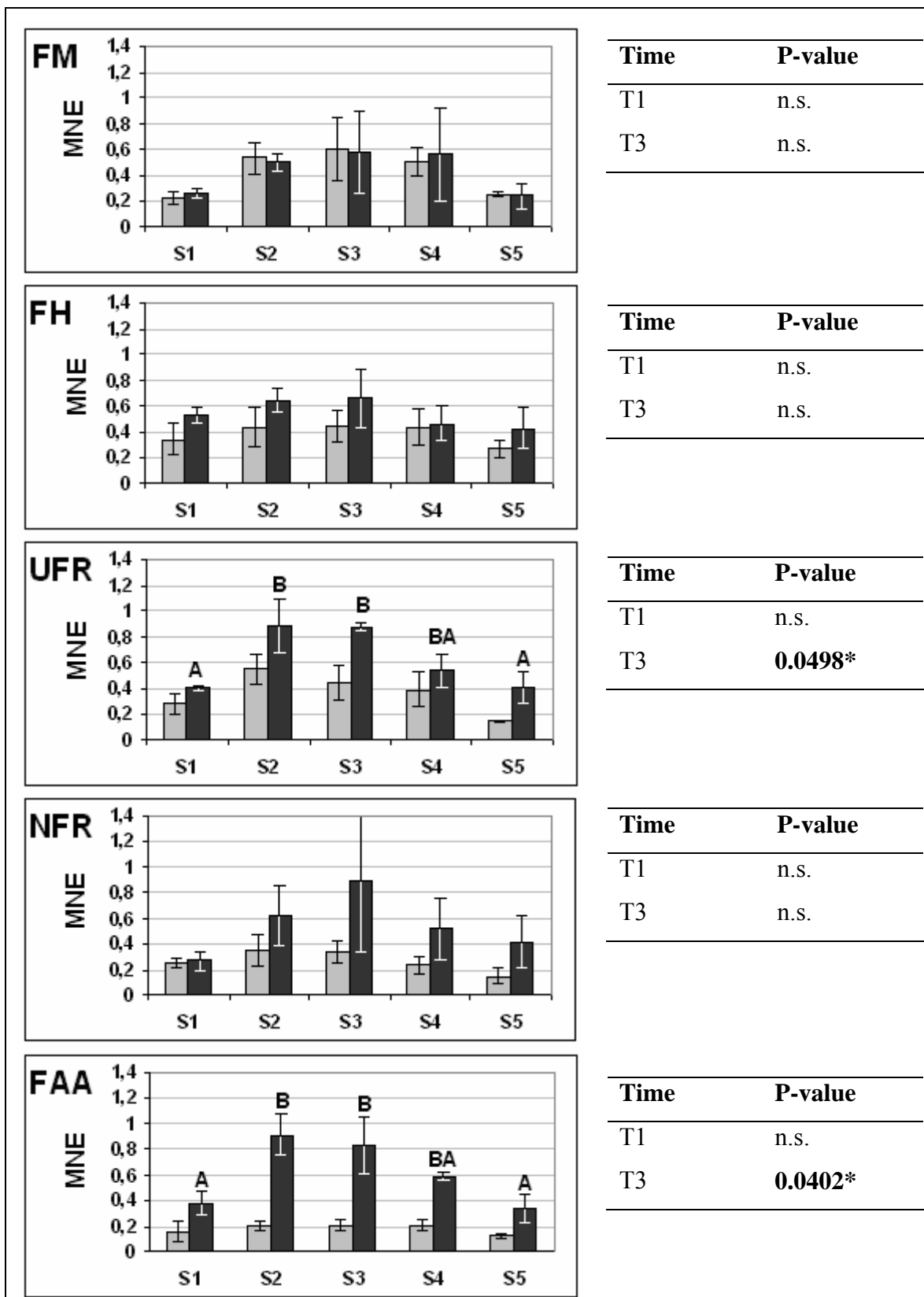
The image contains five bar charts (FM, FH, UFR, NFR, FAA) with accompanying P-value tables.

FM

Time	P-value
T1	n.s.
T3	n.s.

FH

Time	P-value
T1	n.s.
T3	n.s.

UFR

Time	P-value
T1	n.s.
T3	**0.0498***

NFR

Time	P-value
T1	n.s.
T3	n.s.

FAA

Time	P-value
T1	n.s.
T3	**0.0402***

Figure 4 – *See next page for legend.*

Figure 4 – *Regional mean normalized gene expression of PepT1 in fish fed different experimental diets; either all protein in diet as fish meal (FM) or approximately 30 % of the fish meal substituted by either fish hydrolysate (FH), retenate after ultra filtration of fish hydrolysate (UFR), retenate after nano filtration of fish hydrolysate (NFR) or a mix of free amino acids (FAA). All graphs showing mean normalized expression (MNE) +/- standard error for the two sampling points; 14 days (T1) (light bars) and 46 days (T3) (dark bars) on the experimental diets. Regions in each diet are sub-samples from pyloric caeca (S1) and four equally long segments from the remainder of the intestine (S2-S5). Tables opposite to graph show results from repeated measures ANOVA for the two different sampling points. Different letters denote significant differences (P<0.05) in PepT1 gene expression between segments.*

3.4 PepT1 gene expression

For all diets PepT1 was found to be expressed in all five segments (*Figure 4* and *Figure 5*). No correlation was found between expression in individual segments and the respective fish SGR or CF (data not shown).

3.4.1 Regional expression of PepT1

The intestinal PepT1 expression profile for each diet is shown in Figure 4. In the FM diet there was a trend at T1 (P=0.086, F-value=3.20, 4 df) and it appears as there is a slightly higher expression of PepT1 in S2, S3 and S4 than in pyloric caeca (S1) and S5 (light bars FM graph *Figure 4*). A similar expression profile was observed at T3 but statistical tests did not suggest any differences between segments (P=0.15, F-value=2.1, 4 df). In the FH diet no significant difference in expression of PepT1 between segments were found for any of the sampling points (FH graph *Figure 4*). At T3 a significant difference was found between segments in the UFR diet (P=0.0498, F-value=3.84, 4 df) (dark bars UFR graph *Figure 4*). The post-hoc test showed that the expression of PepT1 was higher in S2 and S3 than that in pyloric caeca (S1) and S5. A trend could also be observed for T1 (P=0.07, F-value=3.53, 4 df), and at both T1 and T3 a gradually decline in expression from the proximal to the distal intestine after the pyloric caeca could be observed, with expression in the two last segments (S4 and S5) similar to that observed in the pyloric caeca. In the NFR fed fish equal expression was found in all segments at both T1 and T3 (NFR graph *Figure 4*). Significant difference in expression between segments was found for the FAA diet at T3 (P=0.04, F-value=4.2, 4 df) (dark bars FAA graph *Figure 4*). The expression of PepT1 in segment 2 and 3 was higher then in the pyloric ceca (S1) and S5, giving a profile very similar to that observed for the UFR diet at T3. No significant difference in expression of PepT1 was observed between segments at T1 which showed an even and low expression in all segments (light bars FAA graph *Figure 4*).
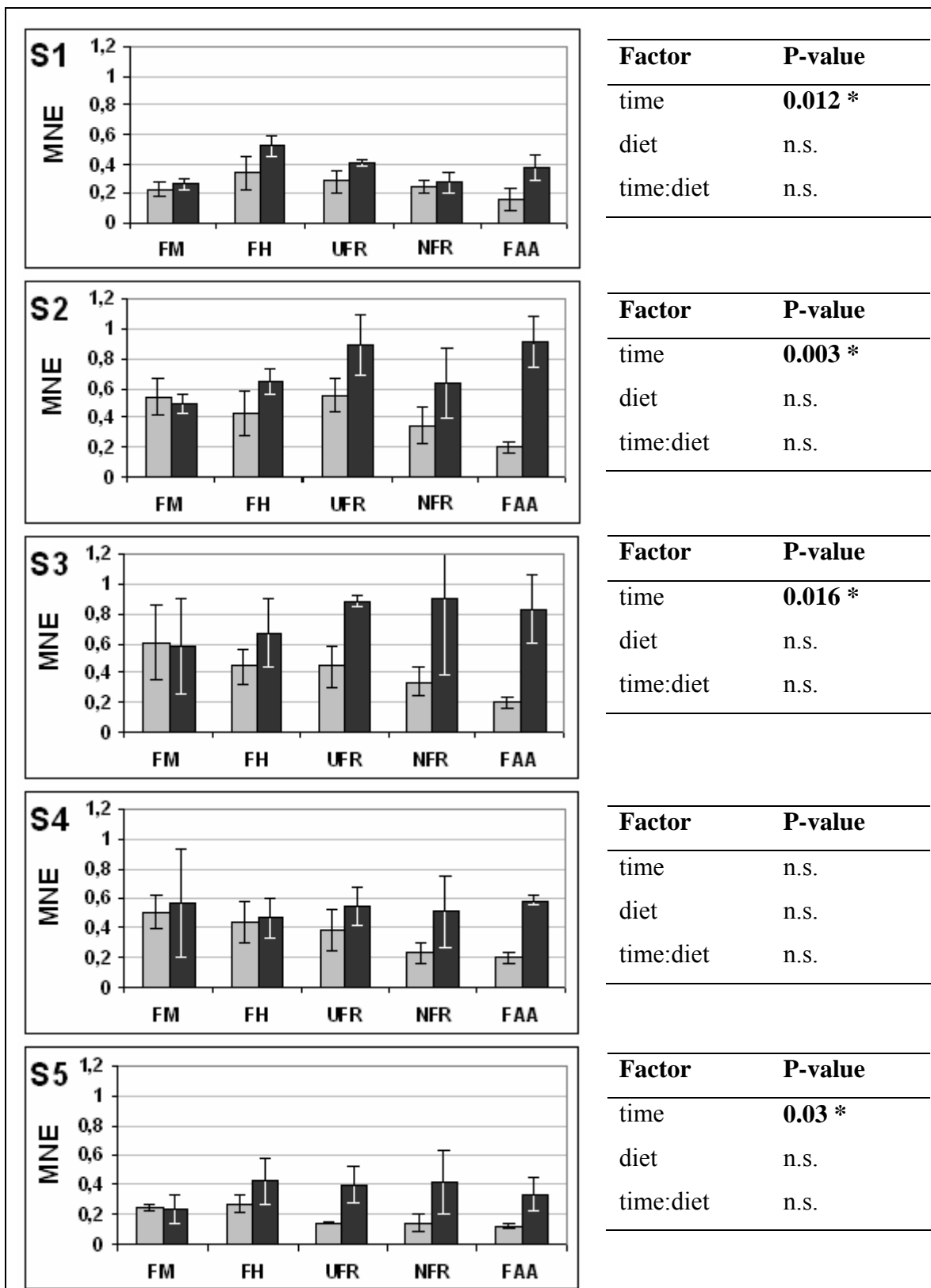
Factor	P-value
time	**0.012 ***
diet	n.s.
time:diet	n.s.

Factor	P-value
time	**0.003 ***
diet	n.s.
time:diet	n.s.

Factor	P-value
time	**0.016 ***
diet	n.s.
time:diet	n.s.

Factor	P-value
time	n.s.
diet	n.s.
time:diet	n.s.

Factor	P-value
time	**0.03 ***
diet	n.s.
time:diet	n.s.

Figure 5 – *See next page for legend.*

Figure 5 – *Mean normalized gene expression of PepT1 in pyloric caeca (S1) and four equally long segments from the remainder of the intestine (S2-S5) for fish fed different experimental diets. Either all protein in diet as fish meal (FM) or approximately 30 % of the fish meal substituted by either fish hydrolysate (FH), retenate after ultra filtration of fish hydrolysate (UFR), retenate after nano filtration of fish hydrolysate (NFR) or a mix of free amino acids (FAA). All graphs are showing expression at the two sampling points; 14 days (T1) (light bars) and 46 days (T3) (dark bars) on experimental diets. Tables opposite to graph show results from Two-way ANOVA on the effect of time , diet and the interaction between time and diet.*

3.4.2 Effect of diet and time on PepT1 expression

A comparison between diets averaged MNE in each segment is shown in Figure 5. No significant difference in PepT1 expression between diets was found in any of the segments. The expression of PepT1 was found to be higher at T3 then at T1 in all segments except S4 (*Figure* 5), but not related to any specific diets as no interaction between diet and time was found.

4. Discussion

4.1 Fish growth and physiological performance

Since the focus of this thesis was to assess the dietary effect on regulation of PepT1, only the main observations from the growth experiment will be discussed. Further details on the dietary effect on growth performance and metabolism are due to be reported in ongoing studies (Pedro Gómez Requeni, unpublished). The small differences in growth parameters (CF, SGR or HSI) that were found between the groups suggest that Atlantic cod can utilize both fish hydrolysate and free amino acids when these levels are included in the diet. This is in accordance with the findings of Aksnes et al. (2006b), who substituted fishmeal in a high plant protein diet for Atlantic cod with either approximately 27 % total fish hydrolysate, ~16 % ultra filtrated retenate, ~ 8 % nano filtrated retenate or ~ 4 % nano filtrated permeate. No significant difference in CF or SGR was found between groups after 89 days on these experimental diets. The SGR was however lower (~1.3 %) then in the current study, and is probably related to the high levels of various plant protein (520g kg^{-1}) that where included to the study by Aksnes et al. (2006b). This is supported by the finding of Hansen et al. (2007a) who showed that plant protein inclusion levels of 50% or more in diets for Atlantic cod caused a significant lower SGR then groups of fish fed no or 25% plant protein included.

In post-smolt Atlantic salmon, there were no differences in SGR between groups of fish when up to 24 % of the fish meal was substituted by fish hydrolysate (Hevroy et al. 2005). The beneficial effect seems to be even better when substituting fish meal with hydrolysate in a diet containing some plant material for this fish. This is shown by the finding of Refstie et al. (2004) where the overall growth rate of the salmon increased when up to 36 % of the fish meal was substituted by fish hydrolysate in a diet containing 10 % soy bean meal. An interesting topic to study further could therefore be to investigate the growth of Atlantic cod fed increasing levels of fish hydrolysate in diets containing moderate to low levels of plant protein.

Similar inclusion levels of free amino acids as used in the FAA diet in the current study have been found to be beneficial both for protein synthesis and growth when substituting intact protein in diets for Atlantic salmon (Espe and Lied 1994). In the

current study the overall SGR for the FAA diet was similar to that in the other groups, which suggest that also juvenile Atlantic cod can utilize free amino acids at these inclusion levels. Espe and Lied (1994) discussed whether the increased protein synthesis is associated with increased levels of insulin, which is an important hormone both in amino acid uptake and muscle growth in fish (Matty 1986; Rungruangsak-Torrissen et al. 1999; Rungruangsak-Torrissen and Sundby 2000). The higher SGR observed from T0 to T1 for the FAA diet in the currents experiment could therefore be associated with a better incorporation of dietary protein and the involvement of insulin could be important. However, the exact reason for this high SGR during the first period might be more explainable once more metabolic data is available (Dr. Pedro Gómez Requeni, unpublished).

In the study by Aksnes et al. (2006b) it was found a slightly lower HSI in cod fed the diets containing retenate after ultra- and nano filtration. A lower HSI were also found at T1 in the current study for the UFR fed fish. However, the diet containing retenate after nano filtration (NFR) did not show a lower HSI then the other diets (FM, FH and FAA), and at T3 all groups showed similar HSI. Thus, it seems that the diets used in the currents study do not cause any differences in relative liver weight over time.

Even though the groups in the current study showed similar growth parameters the dietary treatments could have caused some differences in feed and protein utilization between groups. This is demonstrated by the reduced feed efficiency of Atlantic cod fed diets containing retenate after filtration of fish hydrolysate (Aksnes et al. 2006b) and the lower protein efficiency with the use of hydrolysate in diets for post-smolt salmon (Hevroy et al. 2005). Based on the feed delivered to each tank in the current study the amount of food consumed by each kg of fish seemed to be stable and the same among groups during the whole experimental period (Dr. Pedro Gómez Requeni, unpublished). However, as no replicate data is available it is not possible to accurately calculate feed and protein utilization. The costs associated with the growth performance found in the groups this study is therefore not known and should be investigated in future studies.

Studies on Atlantic cod have showed that the gastric evacuation of chyme from the stomach to the intestine is fairly even from 6 - 12 up to 48-60 hours after feeding (depending on dietary treatment) (Lyndon et al. 1993; Hansen et al. 2006). In the study

by Lyndon et al. (1993) plasma levels of free amino acids measured in the hepatic portal vein was high from 12 hours postprandial until the last measurement was conducted after 24 hours. In the current experiment fish from all diets were sampled approximately 24 hours after last feeding and during dissection it was observed that all intestines had a fairly even distribution of chyme in the lumen (data not shown). It is therefore safe to assume that this is a period of active absorption where a potential dietary effect on PepT1 could be detected.

4.2 Isolation of BBMV

The reasons for the unsuccessful preparations of BBMV from Atlantic cod intestine are not known. Successful preparations of BBMV from other fishes have been made earlier in both eel (Storelli et al. 1986; Verri et al. 1992; Maffia et al. 1997; Verri et al. 2000), tilapia (Reshkin and Ahearn 1991; Thamotharan et al. 1996), rockfish (Ahearn et al. 1992) and even Antarctic ice fish (Maffia et al. 2003). Based on these findings and that successful preparation was made from eel intestine using the exact same chemicals and protocol (Verri et al. 2008, Appendix A), some special considerations apparently needs to be made when preparing BBMV from Atlantic cod intestine. Our results clearly indicated enrichment in brush-border membrane-bound enzymes, which suggest that the method applied to Atlantic cod allow isolation of BBMV. What appeared to be a missing membrane potential could be caused by three factors. Either the brush-border membrane fractions do not form vesicles, or if vesicles are present, valinomycin either fails to generate a negative membrane potential or the membrane potential is quickly neutralized by leakage or transport of ions into the vesicles. Within the time available for this thesis we were unfortunately unable to conduct further investigations into these problems. Future problem solving should include new preparations of BBMV from Atlantic cod intestine and studies of the vesicular suspension with microscopic techniques to determine if vesicles are present (Bozzola and Russell 1998). If vesicles are not formed or brush-border membrane fractions are loosely connected (thereby causing leakage), further optimisation and tests on the preparation protocol needs to be conducted. If vesicles are present in the suspension the reason for the undetectable membrane potential should be investigated further. Other ionophores than valinomycin (such as nigericin that

acts as an H^+, K^+ and Pb^{2+} ionophore) might be tested to see if they are able to generate a membrane potential (Prof. Tiziano Verri, personal communication). The presence and role of other transporters in the vesicles should also be investigated, as transport of ions over the BBM could neutralize the membrane potential. Thus, in order to determine how and why Atlantic cod differ from other investigated species further experiments with suggested problem solving steps should be conducted.

Since we were not able to use this method in measuring the transport capacity of PepT1 in the intestinal regions, it is not known how the dietary treatments will affect the activity of membrane-bound PepT1 protein. No studies have been found where substrates stimulate the activity of PepT1 without affecting de novo synthesis of mRNA. However, in a study with human Caco-2 cells by Walker et al. (1998) there was a non-proportional relationship between increased levels of PepT1 mRNA and increased dipeptide transport, and it was concluded that increased mRNA stability and transcription also contributed to some of the increased transport. It is also possible that the dietary differences could have some indirect effect on PepT1 activity, since some studies have shown that hormones and signalling pathways can regulate PepT1 activity without affecting mRNA expression. Early studies on PepT1 regulation have shown that activation of protein kinase C (PKC) have a negative effect on PepT1 mediated transport of the dipeptide Gly-Sar in Caco-2 cells (Brandsch et al. 1994), an inhibitory effect that was found to be on membrane bound PepT1 protein and not at the gene expression level. Subsequent studies of the signal pathway found that this activation of PKC and subsequent inhibition of PepT1 was related to increased activity of cyclic AMP (cAMP) (Muller et al. 1996). The latter study therefore concluded that the cAMP-mediated activation of PKC (and protein kinase A) is an important route in down regulation of PepT1 activity. In the sequence of Atlantic cod there appeared to be three cAMP/cGMP-dependent protein kinase motifs but no protein kinase C phosphorylation site (Rønnestad et al. 2007). Although it is likely that these regulatory mechanisms are different in Atlantic cod, further studies on the effect of secondary messengers on PepT1 activity needs to be conducted to verify this assumption. Nielsen et al. (2003) found that short-term treatment with insulin had a positive effect on Gly-Sar uptake when administered to the basolateral side of Caco-2 cells. This positive effect by insulin has also been found in a earlier study by Thamotharan et al. (1999),

where pre-incubation of Caco-2 cells in medium containing 5nM insulin stimulated Gly-Gln uptake two fold compared to control. The conclusion from both studies is that insulin treatment of Caco-2 cells either led to an activation of membrane bound PepT1 protein or a mobilization from a preformed cytoplasmic pool, without affecting the amount or stability of PepT1 mRNA. Insulin is an important hormone involved in anabolic processes during fish metabolism (Matty 1986; Navarro et al. 1999; Buddington and Krogdahl 2004), and increased levels of this hormone correlate with higher concentrations of plasma free amino acids in Atlantic salmon (Rungruangsak-Torrissen and Sundby 2000). As the level of plasma free amino acids seems to be affected by level of hydrolyzed protein or free amino acids included in the diet (Espe 1993; Berge et al. 1994; Espe and Lied 1994; Espe et al. 1999) it is possible that a consequent change in insulin levels could affect the activity of PepT1 without affecting levels of mRNA. Another regulatory mechanism of PepT1 was suggested in a study by Berlioz et al. (2000) where an agonistic stimulation of alpha-2-adrenergic receptors increased transport of the antibiotic cephalexin in a differentiated human Caco-2 cell line. Because of the rapid response to the agonist and the kinetic properties of transport it was suggested that the positive effect was caused by increased mobilization of PepT1 protein to the apical membrane. Although no information is available on the presence or function of the alpha-2-adrenergic receptor in Altantic cod intestine, high affinity for agonists and antagonists have indicated its presence in goldfish intestine (Bakker et al. 1993). However, to pursue the idea that these regulatory mechanisms either via neural, insulin or cAMP and protein kinase pathways applies in Atlantic cod further studies needs to be conducted. In any case, it is still important to keep in mind that even though the relationship between levels of PepT1 protein and mRNA in most situations are closely connected (Daniel 2004), the findings above show that it is possible that the dietary treatments used in the current experiment could have some effects on PepT1 activity outside of what is possible to detect with Q-RT-PCR.

4.3 Q-RT-PCR

The use of same sample size during isolation and same RNA quantity for cDNA synthesis is important for normalization when comparing samples (Huggett et al. 2005). In the current experiment the sub-samples taken from the intestinal segments were of the same size and amount of RNA used for cDNA synthesis were the identical for all samples. The OD values obtained from the RNA quality control of all samples is right in the area of what is considered highly purified RNA, and the clear 28S and 18S bands obtained from electrophoreses gel is good indication of low degradation and high integrity of RNA (Bustin and Nolan 2004; Fleige and Pfaffl 2006). As good quality RNA is essential for successful real-time RT-PCR (Pfaffl 2002), these results show that a good foundation was made for reverse-transcription and the consecutive PCR for all samples.

A stable reference gene is essential to obtain accurate gene quantification data, and should be thoroughly evaluated as the activity of most genes will be regulated under certain conditions (Thellin et al. 1999). The low M-value obtained from geNorm investigations of the experimental plates confirms that the expression of codEF1A remained stable both among intestinal segments, between dietary treatments and times. EF1A has also been found to be a reliable reference gene in a study by Lilleeng et al. (2007) were intestinal gene expression in Atlantic cod was studied under two different dietary treatments. Further, in a study to find the most stable reference gene in Atlantic salmon, Olsvik et al. (2005) compared multiple reference candidates and found EF1A to be the best choice. All in all this suggest that EF1A is a reliable reference gene and should be included when evaluating candidate reference genes in fish.

Amplification of genomic material other than the targeted sequence could be a potential source of error and is often caused by the presence of unwanted DNA molecules or primer dimer formation (Kubista et al. 2006). Since no background was found in the NTC and -RT wells in this study it therefore indicates that the Ct values are obtained from amplified mRNA only.

4.4 PepT1 gene expression

The results from this study show that Pept1 is expressed through the whole intestine of juvenile Atlantic cod, confirming results from previous studies both in adult (Rønnestad

et al. 2007) and larval Atlantic cod (Amberg et al. 2008). Thus, indicating that PepT1 is important in protein uptake, and that the whole intestine probably is involved in peptide transport in this fish. In the study by Rønnestad et al. (2007) a weaker signal of PepT1 gene expression was found in the most distal intestine indicating a reduced activity of the transporter in this region for adult Atlantic cod. In the current study the expression of PepT1 in the last segment was found to be similar to at least two of the preceding segments, and with the exception of the UFR and FAA diet at T3 showed no statistical difference in expression compared to the other segments for any of the other diets (Figure 5). Studies with Atlantic salmon have concluded that the dietary protein requirements decline with increasing fish size (Einen and Roem 1997), so it is possible that for the juvenile fish used in the current experiment PepT1 activity in needs to be high the whole intestine in order to meet the high demand of amino acids for growth. During dissection in the currents study no clear distal chamber of the intestine could be observed (own unpublished observations), a feature that is found in intestine of adult cod (Refstie et al. 2006b; Rønnestad et al. 2007). These anatomical differences could cause some developmental differences in the function of PepT1 in this region. Developmental change in PepT1 expression has been found in the intestine of rats in a study by Shen et al. (2001). During the nursing period when rats where on a protein rich milk diet there was an even and high expression of PepT1 in the whole intestine including colon. Just prior to weaning the levels of PepT1 mRNA and protein showed a rapid decrease in all segment and to low or undetectable levels in colon, a change that was suggested to be caused by a change from a protein rich milk diet to feeding diets consisting of mainly carbohydrates. It is however not determined whether the high PepT1 expression observed during suckling in these rats was a response to optimize growth (and survival) at an early age or a direct response of PepT1 to the high protein levels in the diet. The dietary protein levels used in the current study (approximately 42 % DM) is close to what is considered to give optimal protein retention for Atlantic cod this size (Lied and Rosenlund 1983; Rosenlund et al. 2004). In a study by Førde-Skjærvik et al. (2006) the digestibility of different nutrients was investigated along the intestine of Atlantic cod and it was found an active digestion of protein in the whole intestine including the most distal region in fish fed a protein rich fish meal diet (57% CP). High levels of dietary protein have been found to

increase the intestinal PepT1 mRNA expression in both rats (Erickson et al. 1995) and birds (Chen et al. 2005). So a reason for the stable expression of PepT1 in the whole intestine (including the most distal region) in the current study could therefore be that the luminal peptide content is close to levels that are giving maximum retention, thereby calling for a mobilization of transporters in the whole intestine to meet the high levels of available protein. However, how the level of protein included in the diet affects the expression and activity of PepT1 in Atlantic cod is still a topic that needs to be investigated.

The lack of correlation between PepT1 expression in each segment and SGR or CF in the current study is in accordance with the results from Asian weatherloach by Gonçalves et al. (2007), where groups of fish that had a significant difference in both growth and final bodyweight showed the same intestinal PepT1 expression. Thus, even though protein is the main source of growth in fish (Houlihan 1988) and transport of short peptides via PepT1 in Atlantic cod seems to be an important route for protein absorption (Rønnestad et al. 2007; Amberg et al. 2008; Own results), the relationship is probably more complex.

4.4.1 Regional expression of PepT1

In a study on Atlantic halibut (*Hippoglossus hippoglossus*) filling of the pyloric caeca was caused by retrograde contractions in the nearby proximal intestine after the pyloric caeca, moving chyme in an posterior to anterior direction (Rønnestad et al. 2000). The narrow area between the pyloric sphincter and the entrance to the pyloric caeca in Atlantic cod intestine indicate that also this fish has to utilize similar mechanisms in order to fill the pyloric caeca (own unpublished observations). When the chyme fills the pyloric caeca from the subsequent intestinal region, thereby exposing the two areas to the same luminal content, one might expect to find a similar regulatory effect on the expression of PepT1 in S1 and S2. However, since a small proportion just after the pyloric caeca was removed in the current study (see section 2.3 Fish sampling) a significant part of the intestine involved in this retrograde movement of chyme was not investigated. The expression in S2 might therefore be more related to activity in the early mid intestine than proximal intestine.

The only known study that have been looking at dietary effects on the regional expression of PepT1 in fish is the study on Asian weatherloach by Gonçalves et al. (2007). As expected no expression of PepT1 was found in the most distal part of the intestine in this fish as this region is utilized as an accessory air-breathing organ (Gonçalves et al. 2007), while it in the preceding intestine was a gradual decline in PepT1 expression in a proximal to distal direction. This expression profile was however not different between groups feed either a protein rich diet, carbohydrate rich diet or starved during the experimental period (one month). The regional profile in higher vertebrates appears to be a fairly even expression of PepT1 in duodenum, jejunum and ileum with low or undetectable levels in colon (Erickson et al. 1995; Freeman et al. 1995; Miyamoto et al. 1996; Chen et al. 1999; Shen et al. 2001; Rome et al. 2002; Chen et al. 2005), although expression of PepT1 in the colonic area have been found in bears (Gilbert et al. 2007), suckling rats (Shen et al. 2001) and under certain intestinal pathological conditions in humans (reviewed by Adibi 2003). A change in profile as a response to diet have however not been found, although there appears to be a general increase in PepT1 expression in the whole small intestine as a response to a high (quality) protein diet (Erickson et al. 1995; Chen et al. 2005; Gilbert et al. 2008). As demonstrated in vitro, the presence of dipeptides in incubation medium for human Caco-2 cells has been found to up regulate PepT1 mRNA levels (Thamotharan et al. 1998; Walker et al. 1998). The results from the current study show that the dietary inclusion of peptides also could have an effect on the regional expression of PepT1 in the intestine of juvenile Atlantic cod. The FH diet had the highest level of the shortest peptides included (Table 1) and there was a similar expression in all segments including the pyloric caeca in the fish fed this diet. The UFR and FAA diets had only small or no levels of short chained peptides included and both showed a higher expression of PepT1 in S2 and S3 then pyloric caeca and S5 at T3, a tendency that was also observed for the UFR diet at T1. Based on the levels of PepT1 mRNA this indicates that for fish fed the FH diet the relative involvement of PepT1 in the proximal regions of the intestine (e.g. pyloric caeca) is higher than in diets containing less short peptides (UFR and FAA). Similar to the FH diet the statistical tests showed that there were no differences in PepT1 expression between segments for the NFR fed fish, which also suggests that high amounts of slightly larger

peptides (100-1000 Da) would cause involvement of PepT1 in the pyloric caeca similar to that observed in other segments. The higher PepT1 expression observed in S2 and S3 in the UFR and FAA diets could therefore be caused by a lack of or low amounts of short chained peptides included in the diet. Consequently that this region have a higher function in protein absorption as some proteolytic work needs to be conducted on the dietary protein before the substrate is available for PepT1 (Ganapathy et al. 2006). This could also explain the trend that was observed in the FM diet at T1, indicating that there was a lower expression of PepT1 in pyloric caeca (S1) and S5 than in S2, S3 and S4. As the diet had all its protein from fish meal and not included any peptides this suggests that some degradation of protein is necessary before peptides can be transported via PepT1 also for this group. However, a similar trend was not observed at T3 where the statistical test showed that there were no significant differences between segments. It is therefore not possible to conclude that there is any lower relative involvement of PepT1 in pyloric caeca compared to other segments in fish fed the FM diet. Gilbert et al. (2008) compared groups of broiler chicks fed equal amounts of either a high quality protein diet (soybean meal) or a low quality protein diet (corn gluten meal), and found that chicks fed the high quality diet to have the highest intestinal PepT1 mRNA expression. It is well known that, to date, high-quality fish meal is the optimal marine protein ingredient in pelleted feed for carnivorous fish. Thus, since the FM diet contained approximately 14% more fish meal then the other diets it could therefore be that the higher levels of good quality protein would allow a better utilization of digested protein thereby allowing a more rapid absorption of peptides. Further, the fish fed the FM diet had been on this diet since the start of the experimental period, meaning that they would have had a significant longer time (one month) then fish fed the other diets to adapt an optimal utilization of the feed. However, to investigate this further the regulatory effects on PepT1 by protein quality and the transporters postprandial adaptation to dietary conditions should be investigated in more detail.

The profile observed at T3 for the FAA diet was not observed at T1, when there was a low and equal expression in all segments. The low PepT1 mRNA levels did not seem to affect growth performance in any way as the FAA diet was found to have the highest SGR from T0 to T1 (Table 5). As discussed earlier and in the study by Espe and

Lied (1994), high levels of dietary free amino acids lead to higher plasma free amino acids levels and probably have an regulatory effect on the levels of circulating insulin. Because insulin have found to stimulate the activity of membrane bound PepT1 without affecting mRNA levels (Thamotharan et al. 1999; Nielsen et al. 2003) it is possible that the fish have maintained dipeptide transport due to activation of membrane-bound PepT1 or recruitment from a preformed cytoplasmic pool. Ferraris et al. (1988a) found no difference in intestinal PepT1 mediated carnosine transport after two weeks for groups of mice fed diets containing 54 % protein either as intact casein, hydrolyzed casein or free amino acids. This suggests that in mice the transporter is still present and active after longer periods on diets with high levels of free amino acids. However, to what extent the level of PepT1 mRNA were affected in these mice are not known. Given the time the fish in the current experiment were fed the FAA diet before the T1 sampling (two weeks) and with enterocyte turnover time of just days (Kryvi and Totland 1997) it would be reasonable to expect elevated *de novo* synthesis of PepT1 mRNA during this period. Shiraga et al. (1999) found that the amino acid phenylalanine (Phe) up regulated the amount of PepT1 mRNA and stimulated Gly-Sar transport in BBMV. If this amino acid also functions as an inducer of PepT1 activity in Atlantic cod a positive effect should also be expected in the current study as the FAA diet contained more than 3 times higher levels of Phe compared to all the other diets (Table 2). Further, Daniel (2004) reported that luminal free amino acids inhibit dipeptidases in the BBM, so with less hydrolysis of dipeptides one might expect more substrate to become available for PepT1. Thus, as evidence suggest a positive effect of free amino acids on PepT1 activity the reason for the low and stable expression at T1 for the FAA diet is probably related to other regulatory mechanisms outside of what is possible to identify with the data available in this thesis. More detailed studies should therefore be conducted to investigate the long term postprandial effects of amino acids (and possibly indirectly insulin) on PepT1 expression in Atlantic cod.

4.4.2 Effect of diet and time on PepT1 expression

Shiraga et al. (1999) included 20 % of the dipeptide Gly-Phe in an otherwise protein free diet to adult rats. After four days on experimental diets the group fed diets containing the

dipeptide showed approximately 2.5 fold higher expression of PepT1 protein and mRNA in the intestine than rats fed protein free diets. However, even with these relatively high inclusion levels of pure dipeptide the expression was only slightly higher than PepT1 expression in rats fed 20 and 50 % intact protein (casein) included to the diet. In another study on mice the transport of carnosine was measured using everted intestinal sleeves prepared from animals fed a protein rich diet with 54 % of the diet either as casein, partly hydrolyzed casein or free amino acids (Ferraris et al. 1988a; Ferraris et al. 1988b). After two weeks of feeding these diets there were no differences in carnosine uptake in any of the segments investigated (duodenum, jejunum and ileum). The results from these two studies seem to be in line with the findings in the currents study. As the statistical analysis and Figure 5 shows, there was no significant difference in PepT1 mRNA expression between diets in any of the segments. Thus, inclusion of fractions of peptides or free amino acids at the levels used in this study do not seem cause any changes in PepT1 expression above or below that found in groups fed other diets for any of the intestinal regions investigated. Gonçalves et al. (2007) found that there was no difference in PepT1 expression in the intestine Asian weatherloach fed either a protein rich diet, carbohydrate rich diet or starved for one month. In the study on Atlantic cod larvae no difference in PepT1 expression was found between groups fed either zooplankton or enriched rotifers (Amberg et al. 2008). Thus, the results from the current study seem to add support to the findings in these studies, suggesting that dietary properties might be less important in the regulation of PepT1 expression in fish. However, as the results suggest that a higher inclusion of short peptides seem to cause a higher relative involvement of PepT1 in the pyloric caeca (see 4.4.1 Regional expression of PepT1), upcoming studies should assess the dietary effect on PepT1 expression when higher levels of short chained peptides are included to the diet (for instance permeate after nano filtration of fish hydrolysate (Aksnes et al. 2006b)).

From the start of the weaning period (7-14 postpartum) until 24-30 days postpartum there is an increase in the levels of PepT1 mRNA and protein in the small intestine of rats (Miyamoto et al. 1996; Shen et al. 2001). In both studies it is suggested that rise in PepT1 activity could be an adaptation for better utilization of low protein diet and/or to optimize growth and survival at an early age. In the current experiment the fish

fed the fish meal diet (FM) had a longer time (1 month) to adapt to the dietary conditions compared to the other groups, so if the higher expression found at T3 compared to T1 were related to dietary adaptation one might expect to find a more pronounce difference between sampling points in fish fed diet were fish meal was substituted (FH, UFR, NFR and FAA). However, no interaction between time and diet was found (Figure 5) so the higher expression at T3 found for most segments do not seem to be caused by any ongoing adaptation to the dietary conditions. Thus, it would seem that for juvenile Atlantic cod some developmental or growth related changes in PepT1 expression occur during this life stage.

5. Conclusion and future perspectives

PepT1 appears to be involved in peptide absorption in the whole intestine of juvenile Atlantic cod as the transporter is expressed along the whole post-gastric intestine. Compared to adult Atlantic cod where it has been indicated a lower involvement of PepT1 in the most distal region (Rønnestad et al. 2007), the results from the current study suggest that for juvenile cod the involvement of the most distal segment seems to be similar to that of other regions of the intestine.

With an overall higher activity of PepT1 in most segments at T3 then at T1 the results also suggest that some developmental changes in PepT1 expression occur during growth of juvenile Atlantic cod.

Although a higher inclusion level of short peptides did not lead to expression of PepT1 above that observed for diets not containing or with larger peptides, the hypothesis is partly confirmed by the fact that for diets where fish meal is substituted (FH, UFR, NFR and FAA) the expression of PepT1 mRNA indicate that the relative involvement of the pyloric caeca in peptide transport is lower compared to the two following segments for diets without or with low levels of short peptides included (UFR and FAA diet), and that for diets containing the highest amount of short chained peptides the involvement of PepT1 in the pyloric caeca in peptide transport is similar to that observed in the rest of the intestine (FH and NFR). Since no differences in PepT1 expression was found in the last segment, none of the dietary treatments used seemed to cause any increased activity in the most proximal intestine due to saturation of PepT1 activity in early intestine. The last segment also showed a similar expression to that in the preceding segment for all diets suggesting that the dietary protein composition used in this study mainly cause regulation of PepT1 at the mRNA level in the proximal and mid intestine.

With the use of fish hydrolysate as a substitution for fish meal in pelleted feed for Atlantic cod it might therefore be beneficial to include some short peptides in order to stimulate an earlier absorption of protein. How and if this could improve protein retention is however one of many questions that still needs to be answered. Thus, to better

understand the function of PepT1 in Atlantic cod, the following topics should be investigated in future studies:

- Many studies on higher vertebrates have shown that the expression and activity of PepT1 increase with increasing levels of dietary protein (Erickson et al. 1995; Shiraga et al. 1999; Chen et al. 2005; Gilbert et al. 2008). To what extent dietary protein levels affect the transporter in marine teleost is not known. This is important information for Atlantic cod and other important aquaculture species to better understand the relationship between PepT1 and growth.

- PepT1 has received much attention in human not only because of its nutritional importance but because of its role in transport of β-lactam antibiotics, angiotensin-converting enzyme inhibitors and other drugs (Brandsch et al. 2008). To what extent PepT1 is involved in the transport of the antibiotics and other drugs currently used in aquaculture feed for Atlantic cod and other important aquaculture species is not known. Further investigations into these mechanisms could allow a better understanding on how to stimulate a better uptake of these compounds.

- The kinetics of peptide transport via PepT1 in Atlantic cod should investigated, either by optimizing the BBMV protocol (Verri et al. 2008) or using other methods like everted sleeves (Karasov and Diamond 1983), *Xenopus laevis* oocytes (Daniel 2000) or Ussing chamber (Ray et al. 2002).

- Hormones like insulin, epidermal growth factor, leptin and thyroid hormone, have been found to have a regulatory effect on PepT1 in mammals (Adibi 2003). These aspects need to be investigated in relation to PepT1 in fish to better understand the regulatory mechanisms of the transporter.

- The increasing use of alternative dietary protein sources also introduces potential anti nutrients and other bioactive ingredients into the intestinal lumen. How these components affect PepT1 expression and transport properties should be investigated.

- Finally it would be interesting to see how the PepT1 profile in the intestine of wild Atlantic cod relates to the profiles found in this experiment.

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APPENDIX A

A rapid and inexpensive method to assay transport of short chain peptides across intestinal brush-border membrane vesicles from the European eel (*Anguilla anguilla*)

T. VERRI[1], A. DANIELI[1], S. BAKKE[2], A. ROMANO[1], A. BARCA[1], I. RØNNESTAD[2], M. MAFFIA[1] & C. STORELLI[1]

[1] *Department of Biological and Environmental Sciences and Technologies, University of Salento (formerly University of Lecce), Lecce, Italy;* [2] *Department of Biology, University of Bergen, Bergen, Norway*

Abstract

Membrane potential depolarization due to electrogenic peptide transport activity was examined in eel (*Anguilla anguilla*) intestinal brush-border membrane vesicles (BBMV) by monitoring the fluorescence quenching of the voltage-sensitive dye 3,3′-diethylthiadicarbocyanine iodide. Our experimental approach consisted of generating an internal negative membrane potential mimicking *in vivo* conditions and measuring membrane potential depolarization due to different extravesicular dipeptides. Peptide-dependent membrane potential depolarization was observed in both the presence and absence of extravesicular Na$^+$ and was inhibited by diethylpyrocarbonate, which is consistent with the involvement of electrogenic, Na$^+$-independent, H$^+$-dependent peptide transport activity. Kinetic analysis indicated that peptide-dependent membrane potential depolarization is a saturable process ($K_{m,app} \sim 1.5$ mmol L^{-1}) and that within the 0.1–10 mmol L^{-1} peptide range a single carrier system is involved in the transport process. Our results suggest that a peptide transport activity, kinetically resembling the PepT1(Slc15A1)-type-mediated H$^+$/peptide cotransport action, can be monitored in eel intestinal BBMV using an easy and inexpensive fluorescence assay.

KEY WORDS: cyanine dye, di/tripeptide transport, DiS-C$_2$(5), membrane potential, PepT1, Slc15A1

Introduction

Hydrolysis of dietary proteins leads to high levels of short chain peptides (di- and tripeptides) in the intestinal lumen during the digestive process. Released di- and tripeptides are either further hydrolysed to their constituent amino acids or directly taken up in intact form into intestinal epithelial cells (for a comprehensive review, see e.g. Daniel 2004). At the apical membrane of enterocytes, transport of di- and tripeptides is mediated by a single carrier system, namely PepT1(Slc15a1) (for a recent review of the transporters of the Solute Carrier 15 family, see e.g. Daniel & Kottra 2004). PepT1 functions as a Na$^+$-independent, H$^+$-dependent transporter of a large variety of di- and tripeptides. It is electrogenic and responds to both application of inwardly directed transmembrane H$^+$ gradients (pH$_{out}$ < pH$_{in}$) and the (internal negative) transmembrane electrical potential (for details, see Daniel 2004). PepT1 is also responsible for the transport of orally active drugs, such as β-lactam antibiotics, aminopeptidase and angiotensin-converting enzyme inhibitors, δ-aminolevulinic acid and many selected pro-drugs (for a review, see e.g. Rubio-Aliaga & Daniel 2002).

With regard to fish, peptide transport has initially been described in brush-border membranes of European eel (*Anguilla anguilla*) intestine (Verri *et al.* 1992, 2000; Maffia *et al.* 1997), tilapia (*Oreochromis mossambicus*) intestine (Reshkin & Ahearn 1991; Thamotharan *et al.* 1996), rockfish (*Sebastes caurinus*) intestine and pyloric caeca (Thamotharan *et al.* 1996), Atlantic salmon (*Salmo salar*) intestine (Bakke-McKellep *et al.* 2000; Nordrum *et al.* 2000), rainbow trout (*Oncorhynchus mykiss*) intestine (Bogé *et al.* 1981; Nordrum *et al.* 2000) and icefish (*Chionodraco hamatus*) intestine (Maffia *et al.* 2003). Interestingly, some

of these early studies pointed out that in fish, as in mammals (for a recent review, see e.g. Daniel 2004), a single peptide can be more efficiently absorbed than a mixture of the identical amino acids (Bogé *et al.* 1981; Reshkin & Ahearn 1991). In fish brush-border membrane vesicle (BBMV) preparations, carrier-mediated uptake of radiolabelled peptides is stimulated by transmembrane electrical potential and to a less extent by an inwardly directed transmembrane H^+ gradient (Reshkin & Ahearn 1991; Thamotharan *et al.* 1996; Maffia *et al.* 1997; Verri *et al.* 2000). Furthermore, intravesicular acidification is observed with the addition of di- and tripeptides to the extravesicular medium (Verri *et al.* 1992, 2000; Maffia *et al.* 1997, 2003). Diethylpyrocarbonate (DEP), which efficiently inhibits peptide transport in mammalian BBMV (Miyamoto *et al.* 1986; Kramer *et al.* 1988; Kato *et al.* 1989), also inhibits peptide transport in fish BBMV (Verri *et al.* 1992, 2000; Thamotharan *et al.* 1996; Maffia *et al.* 1997, 2003). Such biochemical features have recently been corroborated by the molecular and functional characterization of a piscine PepT1-type peptide transporter, i.e. the zebrafish PepT1 (Verri *et al.* 2003). At present, based on such experimental results, the assessment of intestinal peptide transport activity and function in fish is a highly predictable procedure.

During the past decade, the bulk of biochemical, physiological and molecular information collected about the functional activity of the fish intestinal peptide transporter has paralleled the concept that teleosts can efficiently utilize dietary di- and tripeptides for development, growth and metabolism, and consequently, that balanced peptide-based diets or peptide rather than amino acid supplementation would be highly advantageous in solving the problem of nutritional inadequacy of formulated feeds for cultured fish (a still open debate; see e.g. Zambonino Infante *et al.* 1997; Dabrowski *et al.* 2003, 2005; Aragão *et al.* 2004; Zhang *et al.* 2006; Rønnestad *et al.* 2007). In this context, the availability of easy and inexpensive assays to rapidly evaluate intestinal transport of peptides in fish might be instrumental in the investigation of which peptides (i.e. families/groups) are best and/or selectively absorbed and thus assist in the choice of the best combination of peptides to supplement. In fact, current methodology in peptide transport research is largely based on the use of highly expensive and not always accessible radiolabelled (^{3}H or ^{14}C) di- and tripeptides, that are not commercially available and can be obtained only after very costly custom synthesis (5000–15 000 € per peptide). This has limited the chances of study to a few model peptides. In the present study, we report for the first time the effect of extravesicular dipeptides on an artificially imposed internal negative membrane potential in BBMV from eel intestine by monitoring the fluorescence quenching of the voltage-sensitive dye 3,3′-diethylthiadicarbocyanine iodide (DiS-C$_2$(5)). By using this non-radioactive method, we were able to assess both saturable peptide-dependent membrane potential depolarization and kinetics, suggesting that carrier-mediated peptide transport activity can be easily monitored using this voltage-sensitive dye. This is particularly desirable in studies of peptide transport, as the fluorescence method can be used to test for many kinds of peptides and peptide-like drugs, thus opening the possibility for large-scale screenings of their absorption in fish.
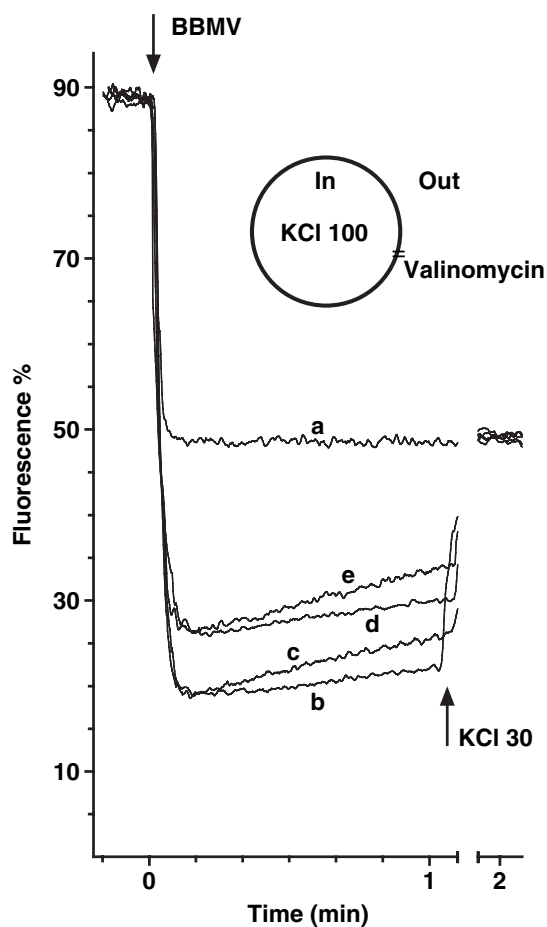
Materials and methods

Materials

European yellow eels (*Anguilla anguilla*), 200–250 g, were obtained from a commercial source, Ittica Ugento (Lecce, Italy), and kept in seawater aquariums until use. All chemicals were reagent grade and purchased from Merck (Darmstadt, Germany). Valimomycin was obtained from Sigma (St Louis, MO, USA). The cyanine dye DiS-C$_2$(5) was obtained from Eastman Kodak (Rochester, NY, USA).

BBMV preparation

Brush-border membrane vesicles were prepared from the intestine of yellow eels as described elsewhere (Storelli *et al.* 1986). The preparation was based on a selective precipitation, in the presence of ethylene glycol-bis(β-aminoethylether)-N,N'-tetraacetic acid and $MgCl_2$ 12 mmol L^{-1}, of all cellular components, with the exception of the brush-border membranes. After the last centrifugation step, BBMV were resuspended in suitable buffer (KCl 100 mmol L^{-1}, mannitol 100 mmol L^{-1}, HEPES 20 mmol L^{-1} adjusted to pH 7.4 with Tris), centrifuged at 50 000 *g* for 30 min, and resuspended again in a small volume (100–200 μL) of the same buffer by passing them 30 times through a fine-gauge needle. To ensure complete equilibration of the extravesicular buffer components into the intravesicular osmotically active space, BBMV were kept on ice for at least 1 h before starting the experiment. Protein concentration was measured using the Bio-Rad Protein Assay Kit I (Bio-Rad Laboratories, Segrate, Italy) using lyophilized bovine plasma γ-globulin as a standard. Before starting the experiment, protein concentration was adjusted to 6 mg mL^{-1}.

Fluorescence quenching measurements

The fluorescence of DiS-C$_2$(5) was measured as previously described (Cassano *et al.* 1988) using a Perkin-Elmer LS-50B spectrofluorometer equipped with an electronic stirring system and a thermostabilized (25 °C) cuvette holder and controlled by a personal computer running the Perkin-Elmer Fluorescence Data Manager software (Perkin-Elmer Life and Analytical Sciences, Waltham, MA, USA). Excitation and emission wavelengths were 645 and 665 nm, respectively, and both slit widths were set to 10 nm. Into a glass cuvette 10 μL of a 0.6 mmol L^{-1} dye solution (in ethanol), 10 μL of a 1 mmol L^{-1} valinomycin solution (in ethanol) and 1960 μL of an extravesicular cuvette buffer were added. For sake of clarity, details of the composition of the extravesicular cuvette buffers are shown in Figs 1 and 4. The fluorescence intensity of the mixture was set to 90 arbitrary fluorescence units and the reaction started by injecting 20 μL of a BBMV suspension (120 μg of proteins) into the cuvette. Under our experimental conditions, intra- and extravesicular buffers had the same ionic strength, pH,

anion concentration and osmolarity. Fluorescence signals were recorded every 0.1 s and the rate of fluorescence quenching dissipation was calculated from the slope through the data points collected during the dissipation of the artificially imposed internal negative membrane potential (see Fig. 1). Fluorescence quenching dissipation signals were linear up to 1 min after the addition of the vesicles as judged by regression analysis with correlation coefficients (by the least squares fit) of 0.98–0.99 for each experimental condition.

Calculation of kinetic parameters

The rates of depolarization due to peptide transport were fitted to the following Michaelis–Menten-type equation:

$$\Delta F = (\Delta F_{max} \times [S])/(K_{m,app} + [S]) \tag{1}$$

where ΔF_{max} is the maximal fluorescence response produced by the peptide, $[S]$ is the extravesicular peptide concentration

Figure 1 Dissipation of an internal negative membrane potential in the presence of extravesicular peptides. BBMV were prepared in a buffer containing mannitol 100 mmol L^{-1}, HEPES 20 mmol L^{-1} adjusted to pH 7.4 with Tris, KCl 100 mmol L^{-1}. Twenty microlitres of the vesicle suspension was injected (see upper arrow) into a cuvette buffer containing (final concentration) mannitol 100 mmol L^{-1}, HEPES 20 mmol L^{-1} adjusted to pH 7.4 with Tris, valinomycin 5 μmol L^{-1}, DiS-C$_2$(5) 3 μmol L^{-1}, ethanol 1% and: (1) KCl 100 mmol L^{-1} (control; trace a); (2) KCl 1 mmol L^{-1}, choline chloride 99 mmol L^{-1} (trace b); (3) KCl 1 mmol L^{-1}, choline chloride 99 mmol L^{-1}, Gly-L-Pro 10 mmol L^{-1} (trace c); (4) KCl 1 mmol L^{-1}, NaCl 99 mmol L^{-1} (trace d); (5) KCl 1 mmol L^{-1}, NaCl 99 mmol L^{-1}, Gly-L-Pro 10 mmol L^{-1} (trace e). When present, peptides iso-osmotically replaced mannitol. In order to rapidly abolish the artificially imposed internal negative membrane potential, 20 μl of a 3 mol L^{-1} KCl solution were added into the cuvette (30 mmol L^{-1} final concentration) at the time indicated (lower arrow), which resulted in a rapid return of the fluorescence signal towards values equal to the control (KCl 100 mmol L^{-1}; trace a). In the figure, five traces are superimposed. Please note *x*-axis gap (1.10–1.95 min interval) with trace breaks. A BBMV is representatively depicted as a circle, with 'in' meaning inside and 'out' outside. KCl 100 indicates the concentration of KCl inside the vesicle (KCl 100 mmol L^{-1}). Refer to a–e for KCl, choline chloride, NaCl and Gly-L-Pro concentrations outside the vesicle. With low K$^+$ concentration outside (KCl 1 mmol L^{-1}) and in the presence of valinomycin, an outwardly directed K$^+$ gradient is generated, which results in an internal negative membrane potential. Membrane potential dissipation rate is influenced by extravesicular cations and/or Gly-L-Pro (traces b–e). KCl 30 indicates the concentration of KCl into the cuvette (KCl 30 mmol L^{-1}) that is used to abolish the internal negative membrane potential.

and $K_{m,app}$ is the peptide concentration yielding one half ΔF_{max}. Kinetic parameters $\pm$ standard error (SE) over the estimate parameter were determined by non-linear regression analysis using the software GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA).

Statistical analysis

Each experiment was repeated at least three times using BBMV prepared from four to six animals. Within a single experiment, each data point represents three to five replicate measurements. Data points reported in the figures are given as mean $\pm$ SE. SE bars are shown wherever they exceed the size of the symbols.

Statistical analysis was carried out using Student's *t*-test for unpaired samples or two-way analysis of variance (ANO-VA), as appropriate. When indicated, *post hoc* tests (Bonferroni) were also performed. GraphPad Prism 4 software was used for statistical analysis. A *P* value of less than 0.05 was considered to be statistically significant.

Results

Rheogenic transport of peptides in eel intestinal BBMV

In order to generate an internal negative membrane potential mimicking *in vivo* conditions, BBMV preloaded with 100 mmol L^{-1} KCl were injected into a buffer containing the K^+ ionophore valinomycin, KCl 1 mmol L^{-1} and choline chloride 99 mmol L^{-1}. In this way, an internal negative membrane potential was artificially created which led to a corresponding fluorescence quenching, which then slowly dissipated with time (Fig. 1, trace b). Fluorescence quenching dissipated more rapidly when the dipeptide glycyl-L-proline (Gly-L-Pro) was also present in the extravesicular medium (Fig. 1, trace c), which suggested that Gly-L-Pro may activate a specific rheogenic pathway in eel intestinal BBMV. BBMV also were injected into a buffer containing NaCl instead of choline chloride, a more physiological experimental condition (Fig. 1, trace d). With Na$^+$ replacing choline, the rate of membrane potential dissipation was higher than that recorded in the presence of choline, indicating, as expected, that Na$^+$ is more permeable than choline across eel intestinal BBMV. Also in this case, a consistent increase in the rate of membrane potential dissipation with respect to trace d (Fig. 1) could be observed in the presence of Gly-L-Pro (Fig. 1, trace e). Treatment of eel intestinal BBMV with 2 mmol L^{-1} DEP totally abolished Gly-L-Pro-dependent

membrane depolarization in both the choline and Na$^+$ media (Fig. 2).

Dissipation of the fluorescence quenching dependent on extravesicular dipeptides (namely, Gly-Gly, Gly-L-Ala and Gly-L-Pro) was compared to that dependent on its component amino acids glycine (Gly), L-alanine (L-Ala) and L-proline (L-Pro), on the amino acid L-phenylalanine (L-Phe) and on the monosaccharide D-glucose, all (control) substrates for which rheogenic transport in eel intestinal BBMV has previously been demonstrated using the same experimental set up as described in this study (Cassano *et al.* 1988, 1990; Maffia *et al.* 1990). In general, extravesicular dipeptides induced membrane depolarization in eel intestinal BBMV to a lesser extent than amino acids and D-glucose (Fig. 3).
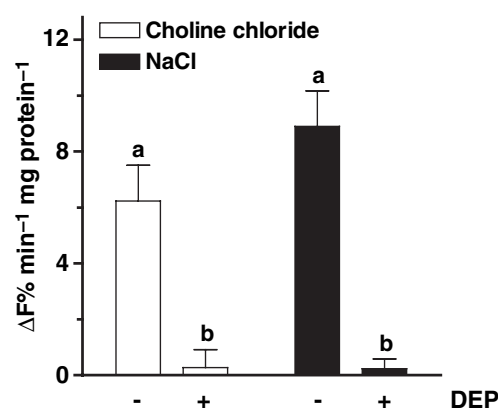

Figure 2 Inhibition of peptide-dependent fluorescence quenching dissipation by diethylpyrocarbonate (DEP). BBMV were incubated for 1 h at 20 °C in a buffer containing mannitol 280 mmol L^{-1}, K$_2$HPO$_4$/KH$_2$PO$_4$ 20 mmol L^{-1}, pH 6.4 either in the presence (from a 100 mmol L^{-1} ethanol stock solution) or in the absence (ethanol only) of DEP 2 mmol L^{-1}. Final ethanol concentration in the media did not exceed 1%. To eliminate the excess of DEP, which affects DiS-C$_2$(5) fluorescence signal, DEP-treated and not-treated BBMV were diluted in 35 ml of a buffer containing mannitol 100 mmol L^{-1}, HEPES 20 mmol L^{-1} adjusted to pH 7.4 with Tris, KCl 100 mmol L^{-1} and centrifuged at 50 000 *g* for 30 min. This washing procedure was repeated twice. The resulting BBMV were preloaded with a buffer containing mannitol 100 mmol L^{-1}, HEPES 20 mmol L^{-1} adjusted to pH 7.4 with Tris, KCl 100 mmol L^{-1} and used for the transport measurements. Dissipation of the internal negative membrane potential due to extravesicular peptide was measured in the presence of extravesicular choline or Na$^+$ (as shown in Fig. 1). Net rates of peptide-dependent fluorescence quenching dissipation are reported in the figure. Data were analysed to assess the effect of DEP (factor I: + or −), the effect of extravesicular medium (factor II: white or black colours) and their possible interaction based on a balanced two-way analysis of variance (two-way ANOVA). Mean values differed for DEP treatment only ($F = 56.75$, df = 1, $P < 0.05$). Mean values with shared letters were not significantly different.
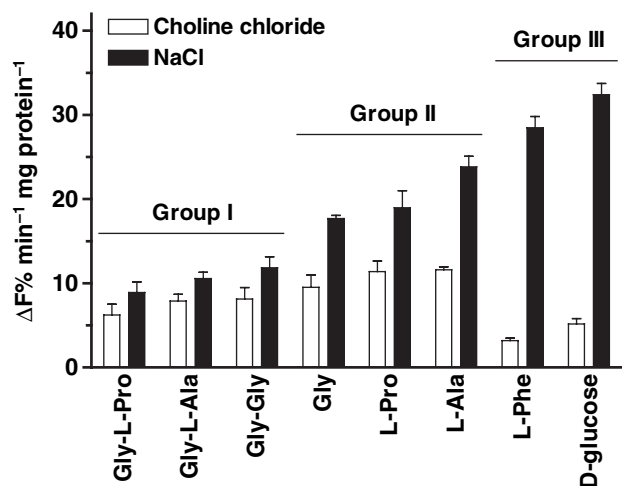
Figure 3 Dependence of transport rate (fluorescence quenching dissipation) on extravesicular peptides, amino acids and sugars. Dissipation of the internal negative membrane potential due to extravesicular substrates Gly-L-Pro, Gly-Gly, Gly-L-Ala, Gly, L-Pro, L-Ala, D-Phe and D-glucose was measured in either choline or Na^+ medium as described in Fig. 1. Net rates of substrate-dependent fluorescence quenching dissipation are reported in the figure as obtained by subtracting the dissipation rate measured in the absence of substrate (control; see Fig. 1, trace b and d) to the rate obtained in the presence of substrate (see Fig. 1, trace c and e). Data were analysed in order to assess the effect of the type of substrate (factor I: group I, group II and group III), the effect of extravesicular medium (factor II: white or black colours) and their possible interaction based on a balanced two-way analysis of variance (two-way ANOVA). Group I was composed of Gly-L-Pro, Gly-Gly and Gly-L-Ala (dipeptides), group II of Gly, L-Pro and L-Ala (component amino acids) and group III of L-Phe and D-glucose. Mean values differed significantly for type of substrate ($F = 26.20$, df $= 2$; $P < 0.05$), for extravesicular medium ($F = 160.90$, df $= 1$, $P < 0.05$) and for interaction between the two factors ($F = 41.95$, df $= 2$, $P < 0.05$). Interaction was due to the different response of L-Phe and D-glucose in the presence of the different extravesicular medium (i.e. group III is composed of substrates that are translocated across the membrane by Na^+-dependent transport processes only, and therefore in the absence of sodium a very low membrane potential depolarization is generated). Finally, application of the Bonferroni *post hoc* test showed that there are differences between mean values of group II ($t = 5.73$, df $= 4$, $P < 0.05$) and group III ($t = 13.19$, df $= 2$, $P < 0.05$) for different extravesicular media, but not for group I.

Dipeptides also induced depolarization in both choline and Na^+ medium (see also Fig. 2), which was to be expected, as dipeptides are cotransported into the cell with H^+ and not with Na^+. Furthermore, it was observed that Gly, L-Pro and L-Ala induced depolarization in both choline and Na^+ medium (Fig. 3), with depolarization in the presence of Na^+ being higher than that obtained in its absence. This behaviour is also expected as such amino acids are substrates of

two different classes of transport systems, one H^+-dependent the other Na^+-dependent, that are located on the apical membrane of intestinal epithelial cells (for recent reviews see Brandsch 2006; Bröer *et al.* 2006; Thwaites & Anderson 2007), including eel intestinal epithelium (Storelli *et al.* 1986; Cassano *et al.* 1988, 1990; Maffia *et al.* 1990; Ingrosso *et al.* 2000). Furthermore, as expected, both L-Phe and D-glucose induced depolarization in the presence of Na^+ only (Fig. 3) as their transport occurs via Na^+-dependent transport systems (for a recent review see Bröer *et al.* 2006).

In summary, these results suggest that in eel intestinal BBMV the presence of extravesicular peptides can promote significant membrane depolarization, as detected by the use of a voltage-sensitive fluorescent dye.

Kinetics

Kinetics was initially determined by monitoring Gly-L-Pro-dependent membrane depolarization with increasing concentrations of Gly-L-Pro in the presence of extravesicular choline (Fig. 4a) or Na^+ (Fig. 4b). In both cases, peptide-dependent depolarization was a hyperbolic function of peptide concentration. The corresponding Woolf–Augustinsson–Hofstee plots (Segel 1975) are also shown (see insets to Fig. 4a,b), and these suggest that the peptide-dependent membrane potential depolarization is due to a single carrier-mediated process. Kinetic analysis performed using Gly-Gly as a substrate confirmed the occurrence of a single carrier-mediated process for dipeptide transport in eel intestinal BBMV. Kinetic parameters, calculated by non-linear regression analysis (see Materials and methods, equation 1), are summarized for both substrates in Table 1.

In order to exclude the possibility that depolarization was not entirely due to the peptide but also to its component amino acids (which could be present in the extravesicular medium as hydrolysis products, thus contributing to the depolarization effect), we performed a kinetic analysis of Gly-Gly in the presence of saturating concentrations of its component amino acid glycine (Gly). On the same membrane preparation, Gly-Gly-dependent depolarization rate with increasing [Gly-Gly] (Fig. 5, open circles) and Gly-dependent depolarization rate with increasing [Gly] (Fig. 5, open squares) were initially measured (experiment conducted in sodium medium). Kinetic parameters were calculated for both substrates by fitting the Michaelis–Menten equation to rate values using non-linear regression analysis (see Material and methods, equation 1) ($R^2 = 0.993$ for glycyl-glycine and $R^2 = 0.980$ for glycine). Thereafter, fluorescence quenching was measured in the simultaneous presence of Gly
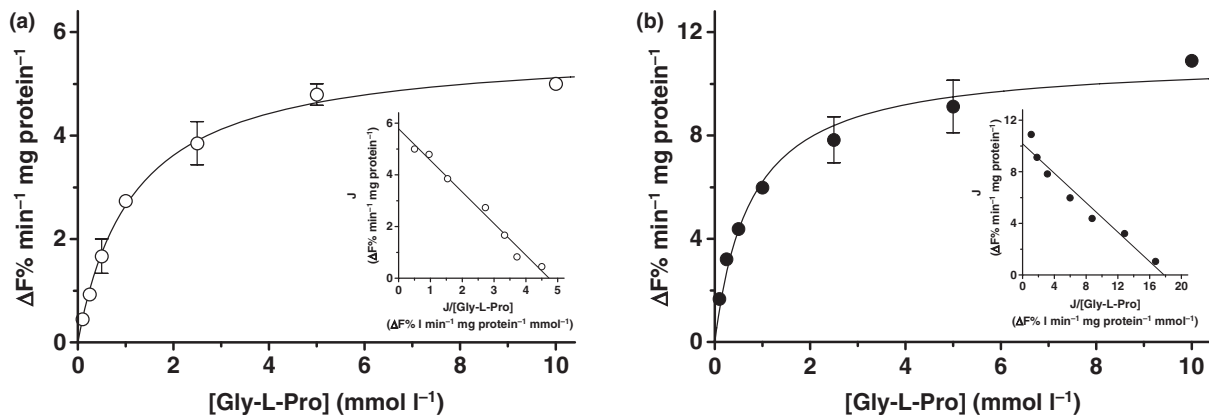
Figure 4 Saturation of initial rate of fluorescence quenching dissipation versus extravesicular peptide concentration. BBMV were prepared in a buffer containing mannitol 100 mmol L^{-1}, HEPES 20 mmol L^{-1} adjusted to pH 7.4 with Tris, KCl 100 mmol L^{-1}. Twenty microlitres of the vesicle suspension was injected into a buffer containing (final concentration) mannitol 100 mmol L^{-1}, HEPES 20 mmol L^{-1} adjusted to pH 7.4 with Tris, valinomycin 5 μmol L^{-1}, DiS-C$_2$(5) 3 μmol L^{-1}, ethanol 1%, either choline chloride 99 mmol L^{-1}, KCl 1 mmol L^{-1} (a) or NaCl 99 mmol L^{-1}, KCl 1 mmol L^{-1} (b), and increasing concentrations (from 0.1 to 10 mmol L^{-1}) of the peptide Gly-L-Pro compensated for by decreasing mannitol concentrations. Net peptide-dependent transport rate was determined by subtracting data of fluorescence quenching dissipation obtained in the absence of peptide (as a reference, see Fig. 1, trace b and trace d) from data obtained in the presence of increasing peptide concentrations (see Fig. 1, trace c and trace e). The lines represent the best curve fitting the data and were obtained by an iterative non-linear regression program (see Materials and methods, equation 1) [$R^2 = 0.997$ for the experiment reported in (a) and $R^2 = 0.978$ for the experiment in (b)]. Calculated kinetic parameters for this experiment were as follows: $K_{m,app} = 1.16 \pm 0.08$ mmol L^{-1}, $\Delta F_{max} = 5.71 \pm 0.12$ $\Delta F\%$ min^{-1} mg^{-1} protein for the experiment reported in (a) and $K_{m,app} = 0.78 \pm 0.13$ mmol L^{-1}, $\Delta F_{max} = 10.94 \pm 0.51$ $\Delta F\%$ min^{-1} mg^{-1} protein for the experiment in (b). In the *insets*, experimental data of the initial rates of peptide-dependent fluorescence quenching dissipation reported in the main bodies of the figures are transformed according to Woolf–Augustinsson–Hofstee.

10 mmol L^{-1} and increasing Gly-Gly concentrations (Fig. 5, closed circles). The dashed line in Fig. 5 was obtained using the kinetic parameters reported in the legend to the figure according to the following equation:

$$\Delta F = \{\Delta F_{max}^{Gly} \times [Gly]/(K_{m,app}^{Gly} + [Gly])\} + \{\Delta F_{max}^{Gly-Gly} \times [Gly-Gly]/(K_{m,app}^{Gly-Gly} + [Gly-Gly])\} \quad (2)$$

and represents the depolarization expected if Gly and Gly-Gly have completely different pathways. Conversely, the dotted line in Fig. 5 was calculated using the same parameters as above according to the following equation:

$$\Delta F = (\Delta F_{max}^{Gly} \times [Gly]/(K_{m,app}^{Gly} + \Delta F_{max}^{Gly-Gly} \times [Gly-Gly]/K_{m,app}^{Gly-Gly})/(1 + [Gly]/K_{m,app}^{Gly} + [Gly-Gly]/K_{m,app}^{Gly-Gly}) \quad (3)$$

and represents the depolarization expected in the case that Gly and Gly-Gly would have mutually competed for a common transport system (competitive inhibition, see also Cassano *et al.* 1990). The rate values measured in the presence of both substrates (closed circles) approximated the dashed line (two distinct pathways and no inhibition)

($R^2 = 0.911$), thus suggesting that the two measured transport phenomena occur via distinct carriers and do not interfere with each other.

Discussion

A large variety of peptides are generated in the gut lumen during normal digestion of dietary proteins. Large quantities of di- and tripeptides are absorbed intact through the gut mucosa and this is the primary mechanism for absorption of dietary nitrogen (for a recent review see Daniel 2004). Interestingly, many of these dietary peptides also exhibit significant biological activity, and are systemically active in microgram quantities (for recent reviews see e.g. Zaloga & Siddiqui 2004; Rutherfurd-Markwick & Moughan 2005). PepT1, located at the apical membrane of the enterocyte, is the transport system that endorses, channels and regulates the transepithelial transport of di- and tripeptides into the intestinal epithelium, thus representing the physiological route for the intestinal absorption of such substrates (see e.g. Daniel 2004; Daniel & Kottra 2004). Hundreds of dipeptides and thousands of tripeptides may be substrates of PepT1, although with highly differing transport efficiencies (for a

Table 1 Kinetic parameters for peptide transport in eel intestinal brush-border membrane vesicles

Peptide	Extravesicular medium	Kinetic parameters		
		$K_{m,app}$ (mmol L^{-1})	ΔF_{max} ($\Delta F\%$ min^{-1} mg^{-1} protein)	No. of experiments (n)
Gly-L-Pro	Choline chloride	1.43 ± 0.53	6.46 ± 1.02	3
Gly-L-Pro	NaCl	1.68 ± 1.01	10.86 ± 1.86	3
Gly-Gly	Choline chloride	1.59 ± 0.40	7.60 ± 0.33	4
Gly-Gly	NaCl	2.49 ± 0.84	10.21 ± 2.15	5

Rates were obtained by measuring the initial fluorescence quenching dissipation as reported in Fig. 1: $K_{m,app}$ values are expressed in mmol L^{-1}, ΔF_{max} values are in $\Delta F\%$ min^{-1} mg^{-1} protein. Values are reported as means ± standard errors. Kinetic parameters were calculated with an iterative non-linear regression program by fitting rates of fluorescence quenching dissipation to a Michaelis–Menten equation (see Materials and methods). Extravesicular medium did not significantly affect Gly-L-Pro and Gly-Gly kinetic parameters, as assessed by the application of unpaired Student's t-tests ($P > 0.05$) to calculated $K_{m,app}$ and ΔF_{max} mean values.
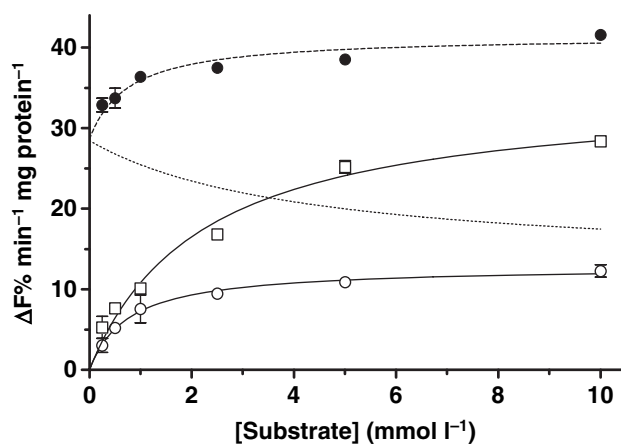

Figure 5 Dependence of cotransport rate (fluorescence quenching dissipation) on the extravesicular concentration of glycyl-glycine (open circles) and glycine (open squares) in BBMV from eel intestine. The experiment was performed in the presence of Na$^+$ gradient (out > in). Experimental conditions were as reported in Fig. 4. By fitting the rate values to a Michaelis–Menten equation, kinetic parameters were calculated and resulted: (1) $K_{m,app}^{Gly} = 2.20 \pm 0.45$ mmol L^{-1}, $J_{max}^{Gly} = 34.71 \pm 2.56$ $\Delta F\%$ min^{-1} mg^{-1} protein for glycine, (2) $K_{m,app}^{Gly-Gly} = 0.76 \pm 0.07$ mmol L^{-1}, $J_{max}^{Gly-Gly} = 23.83 \pm 0.31$ $\Delta F\%$ min^{-1} mg^{-1} protein for glycyl-glycine. Glycyl-glycine transport was also measured in the presence of glycine 10 mmol L^{-1} (closed circles). The dotted and dashed lines were calculated as reported in the text (see Results), assuming that glycine and glycyl-glycine are, respectively, transported by two distinct carriers (equation 2) or by the same carrier (equation 3).

recent classification of PepT1 substrates on the basis of their affinity and transport rate see e.g. Vig *et al.* 2006). Therefore, in order to more easily estimate their individual transport efficiencies and thus select from the variety of di- and tripeptides that can optimally or suboptimally cross the intestinal epithelium via PepT1 in fish, we developed an easy low-cost method to assay transport of single peptides across the intestinal epithelium. Our method, based on the use of a voltage-sensitive fluorescent dye in combination with eel intestinal BBMV, allows for the screening of transport activity of all commercially available di- and tripeptides, and is an alternative to the use of the highly expensive and not always accessible radiotracer-based methods that are routinely used in peptide transport research.

It has previously been shown that in eel intestinal BBMV DiS-C$_2$(5) fluorescence quenching depends linearly on the value of the electrical potential difference across the membrane. In other words, DiS-C$_2$(5) fluorescence increases with membrane depolarization and decreases with membrane hyperpolarization (Cassano *et al.* 1988). It has also been shown that the flux of positive charges generated by Na$^+$/neutral substrate (D-glucose or neutral amino acids) cotransport activities can be measured by monitoring the decay of an artificially imposed internal negative membrane potential (Cassano *et al.* 1988, 1990; Maffia *et al.* 1990). In the present paper, we have extended these findings by showing that in eel intestinal BBMV the decay of the artificially imposed internal negative membrane potential can also be due to a flux of positive charges generated by pure H$^+$/neutral substrate cotransport activities, such as that generated by a PepT1-type transporter in the presence of extravesicular zwitterionic (at pH 7.4) Gly-Gly, Gly-L-Pro and Gly-L-Ala. In the presence of extravesicular zwitterionic peptides, electrogenic transport occurs in eel intestinal BBMV, accelerating the depolarization of the artificially induced membrane potential due to transfer of net positive charges from the exterior to the interior side of the membrane. This happens either in the presence or absence of Na$^+$, suggesting that electrogenicity of peptide transport may be due to movement of cation(s) other than Na$^+$ (e.g. H$^+$) across the brush-border membrane. This conclusion is also corroborated by the fact that Cl$^-$ (100 mmol L^{-1} in both intra- and extravesicular medium) is unable to short-circuit membrane potential with the present experimental set up (Cassano *et al.* 1988). These results are similar to findings obtained in rabbit intestinal BBMV using the voltage-sensitive cyanine dye DiS-C$_3$(5) but with a different experimental set up (Ganapathy *et al.* 1984, 1985). Moreover, as the peptide-dependent depolarization component was totally

inhibited by DEP, the data support the concept that the activity of a H$^+$/peptide cotransport phenomenon was indeed measured under the experimental conditions employed. Further support for the quality of our methodological approach comes from the following pieces of evidence: (1) in the 0.1–10 mmol L^{-1} range, peptides cause depolarization following Michaelis–Menten-type saturation kinetics, with apparent affinity constants in the millimolar range; (2) a single transport system is apparently involved in peptide transport in both the presence and absence of Na$^+$; (3) peptide transport is observable even in the presence of a second substrate which transport activity also generates membrane depolarization (i.e. glycine in our experiments). These results are in full agreement with kinetic data previously obtained in the same (Verri *et al.* 1992, 2000; Maffia *et al.* 1997) and other (Reshkin & Ahearn 1991; Thamotharan *et al.* 1996; Maffia *et al.* 2003) fish models using different experimental approaches. Of interest is the observation that the kinetic parameters of Gly-L-Pro transport measured using our novel fluorometric method ($K_{m,app}$ = 1.43 mmol L^{-1}, in extra-vesicular choline chloride medium and pH 7.4) were absolutely comparable to those we obtained previously using same eel intestinal BBMV experimental preparation, same experimental conditions and the radioactive tracer [^{3}H]Gly-L-Pro ($K_{m,app}$ = 1.27 mmol L^{-1}; Maffia *et al.* 1997). To the best of our knowledge, [^{3}H]Gly-L-Pro and D-[^{3}H]Phe-L-Ala are the only radioactive peptides tested in eel intestinal BBMV so far. Taken together, our results strongly support the idea that the 'low affinity' H$^+$/peptide cotransport activity operating in eel intestinal brush-border membranes can be monitored by means of our simple fluorescence analysis.

In conclusion, we have shown that PepT1-type peptide transport activity can be monitored in eel intestinal BBMV by means of an easy fluorescence assay. This assay is also inexpensive with respect to the current methodology, mainly as it eliminates the problem of the very costly custom synthesis of radiolabelled di- and tripeptides. As pointed out above, the need for radioactive material has significantly limited transport studies to a very few model peptides, such as Gly-L-Pro, Gly-Sar and D-Phe-L-Ala. Such peptides have been chosen as they are resistant to spontaneous hydrolysis in aqueous solution as well as to hydrolysis mediated by brush-border membrane and/or cytosolic enzymes. Our fluorometric assay offers the advantage of analysing a large variety of substrates regardless of their sensitivity to hydrolysis. In fact, all substrates, including readily hydrolysable peptides (such as the dipeptides Gly-Gly and Gly-L-Ala studied in this paper), are tested in the millimolar range and are monitored by means of a very brief (1–2 min) assay procedure. This significantly limits the amount of component amino acids that are released in the assay medium due to the enzymatic degradation that occurs when peptides are in contact with the BBMV preparation.

In the perspective of optimizing diet formulations in aquaculture, an understanding of the basic processes that allow intestinal nutrient absorption is advantageous. Our method may represent a realistic option for screening for the uptake of a variety of peptides and peptide-like molecules in the intestine of commercially relevant cultured fish species. This method is particularly attractive in light of recent data that have clearly demonstrated that not all peptides are effective substrates of PepT1 (Vig *et al.* 2006), in contradiction to the assumption that PepT1 transports all di- and tripeptides equally well. Different peptides generated in the gut lumen during normal digestion of dietary proteins may differ regarding their affinity to the transporter, their rate of transport, whether they are transported at all, and their competitive inhibition of the transport of the other peptides also present in the intestinal lumen. Therefore, not only peptide hydrolysis products obtained after normal digestion from various natural protein sources, but also artificial peptide-based diets, might be either more or less efficacious on the basis of their peptide composition. The method may also be applicable in investigations into the use of small peptides with constituent, nutritionally limiting amino acids rather than the purified amino acids as supplements in feeds.

Acknowledgements

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