Protein digestion in juvenile turbot (Scophthalmus maximus) and effects of dietary administration of Vibrio proteolyticus

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Accepted 11 November 1999

Abstract

The trial was made to study the protein digestion in juvenile turbot during passage of digesta from stomach to rectum. Moreover, diet supplementation with the potential probiotic bacteria, Vibrio proteolyticus, was evaluated with regard to protein digestion. For a 3-week period, fish (25–30 g) were fed by oral intubation and received either a liquid mixture consisting of 40% nonpurified control diet and 60% water or this mixture supplemented with 10^10 viable V. proteolyticus ml^-1. Daily dry matter intake was 1.5% body weight. As digesta progressed from the stomach to the foregut, hindgut and rectum, the increase in apparent nitrogen digestibility was accompanied by higher ammonia contents, suggesting substantial involvement of the microbiota in protein degradation in the distal segments of the gastrointestinal tract. Water-soluble nitrogen contents were significantly higher in the foregut, presumably corresponding with considerable protein digestion by secreted endogenous enzymes in this digestive segment. Over 65% of the soluble protein in all four parts of the tract had a MW ≤ 10,000. The amount of soluble protein and peptides with MW < 1000 decreased significantly during transit. This was also found for the proportion of the 10,000–20,000 MW proteins, whereas the highest MW category (> 200,000) increased. Ingestion of V. proteolyticus tended to stimulate apparent nitrogen digestibility (P < 0.1). This effect corresponded with increased protein degradation in the proximal intestine as
was shown by the significantly elevated fraction of soluble proteins with MW < 1000.

1. Introduction

In turbot hatcheries, considerable mortality and consequently economic losses may occur due to vibriosis. This disease is mainly caused by *Vibrio anguillarum*. Nowadays, the prophylactic and therapeutic control of vibriosis is based on oral administration of antibiotics. However, such treatment may cause the development of resistant bacteria (Aoki et al., 1985) and yield residues in fish. An alternative treatment could be the administration of probiotic bacteria that exert inhibitory effects against *V. anguillarum* and support the natural host microbial defense mechanisms. Preferentially, such bacteria should be of host origin as they must be able to colonize the gastrointestinal tract (Conway, 1989; Hansen and Olafsen, 1989). *V. proteolyticus* appears to fulfill these conditions as in vitro experiments have shown that this bacterial strain exerted inhibitory effects against *V. anguillarum* (Grisez, 1997). Moreover, preliminary experiments indicated that these bacteria are able to colonize the gastrointestinal tract of turbot juveniles. The potential role of *V. proteolyticus* as a prophylactic agent against vibriosis is currently under further investigation. Another practical aspect of the use of probiotics in fish diets that must be clarified is their possible positive effect on feed efficiency. In this context, the present study dealt with the potential of dietary *V. proteolyticus* supplementation on protein digestion in juvenile turbot. However, the actual knowledge of the extent of protein digestion and absorption in this species, at this age, is greatly inadequate. Thus, an additional aim of this study was to obtain information in this regard. Following the feeding of a diet with or without *V. proteolyticus* supplementation, the progress of protein digestion was examined as digesta passed through the alimentary tract. Therefore, apparent nitrogen digestibility was measured in the different segments of the gastrointestinal tract. Molecular weight (MW) profiles of soluble proteins were determined as well. This latter technique has been successfully used in experiments with young poultry and piglets (Sklan and Hurwitz, 1980; Asche et al., 1989). Additional parameters related to protein degradation were also measured such as water-soluble nitrogen, ammonia and pH.

2. Materials and methods

2.1. Fish husbandry

Turbot (*Scophthalmus maximus*) juveniles (25–30 g) were obtained from a commercial farm (France Turbot, France). They were maintained in six 20-l tanks, each containing six fish. Each tank was connected to a separate system of recirculating artificial seawater that was continuously filtered and aerated. The temperature of the

Keywords: Turbot; *Scophthalmus maximus*; Protein digestion; *Vibrio proteolyticus*; Probiotics
water during the trial was kept between 18°C and 20°C, salinity was 3.6%, oxygen saturation was between 80% and 90% and nitrite content varied between 0.05 and 0.1 mg l⁻¹. The experimental protocols and procedures were approved by the Animal Use and Care Committee at the Catholic University of Leuven, Belgium.

2.2. Dietary treatments

The basal diet was formulated to contain 570 g kg⁻¹ crude protein and consisted mainly of nonpurified ingredients (Table 1). In the 3-week experimental period, fish were fed individually twice daily by oral intubations of a liquid diet consisting of 40% ground basal diet and 60% water. Daily dry matter intake was set at 1.5% body weight. Two dietary treatments were tested: the basal diet (C) vs. the basal diet supplemented with 10¹⁰ V. proteolyticus (VP) ml⁻¹ diet–water mixture. Preliminary trials had revealed that under these circumstances, colonization of the digestive tract with this bacterial strain occurred. Diet–water mixtures were freshly prepared at each time of feeding. Each diet was fed to 18 fish. All fish in the same tank were subjected to the same treatment. The diets contained 5 g kg⁻¹ chromic oxide (technical grade, 98%) to mark the particulate material and to provide a measurement of protein digestibility.

2.3. Sampling

On the sampling day, all fish were fed in the morning and killed 8 h later. In a preliminary experiment, it was found that this time period resulted in the presence of digesta in the overall digestive tract. The stomach, foregut (from the pyloric caeca to the

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Ingredients and chemical composition of the basal diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient</td>
<td>g kg⁻¹</td>
</tr>
<tr>
<td>Herring meal</td>
<td>580</td>
</tr>
<tr>
<td>Dried whey</td>
<td>50</td>
</tr>
<tr>
<td>Blood meal</td>
<td>100</td>
</tr>
<tr>
<td>Condensed milk solubles</td>
<td>25</td>
</tr>
<tr>
<td>Wheat middlings</td>
<td>63</td>
</tr>
<tr>
<td>Fish oil</td>
<td>120</td>
</tr>
<tr>
<td>Molasses</td>
<td>35</td>
</tr>
<tr>
<td>Mineral premix*</td>
<td>0.5</td>
</tr>
<tr>
<td>Vitamin premixd</td>
<td>22</td>
</tr>
<tr>
<td>Chromic oxide</td>
<td>5</td>
</tr>
</tbody>
</table>

* Dietary ingredients were obtained from a local supplier.
* Proximate analyses were performed using AOAC (1990) procedures.
* Provided per kilogram diet: 75 mg Zn as ZnSO₄; 20 mg Mn as MnSO₄; 1.6 mg Cu as CuSO₄; 10 mg I as KIO₄.
* Provided per kilogram diet: 2.25 mg retinyl acetate; 0.015 mg cholecalciferol; 500 mg α-tocopheryl acetate; 28 mg menadione sodium bisulfite; 50 mg thiamin hydrochloride; 55 mg riboflavin; 30 mg pyridoxine.HCl; 115 mg calcium pantothenate; 220 mg nicotinic acid; 0.6 mg D-biotin; 0.06 mg cyanocobalamin; 13 mg pteroylmonoglutamic acid; 132 mg myo-inositol; 3.5 g choline chloride; 1 g ascorbic acid.
middle of the intestine, hindgut (distal half of the intestine) and rectum were dissected according to Koven et al. (1994). The contents from the digestive segments were gently extruded and collected separately. As the amount of digesta from individual fish was insufficient to carry out the analyses, the samples from three fish were pooled.

2.4. Digesta analyses

Following killing, the pH and ammonia content of the digesta in the different intestinal segments were measured immediately. The pH was determined with a Hamilton Slimtrode (Filter Service, Belgium). Ammonia was measured with an ion-selective electrode (Orion, 95-1201, Ankersmit, Belgium) following extraction of the samples with 0.1 mol l⁻¹ HCl (Byrne and Power, 1974).

The digesta were freeze-dried and subsequently ground using a pestle and mortar. Nitrogen was determined by the Kjeldahl method. Chromic oxide was measured spectrophotometrically following dry destruction of the samples at 550°C and oxidation of Cr₃⁺ to Cr⁶⁺ with sodium molybdate (Fenton and Fenton, 1979). In order to determine the amount of water-soluble nitrogen in the digesta, a 2% suspension of the freeze-dried sample was prepared. Following homogenization by stirring, the mixture was centrifuged (25,000 × g for 30 min at 5°C), the supernatant decanted, the residue resuspended in water (in the same volume as was used in the first extraction step), again centrifuged and the supernatant added to the previous one; the combined supernatant was filtered and analyzed for nitrogen.

Soluble proteins in the digesta were separated by size exclusion, high-performance liquid chromatography according to a modified procedure of Asche et al. (1989). Proteins were fractionated on a TSK-SW2000 column (7.5 mm i.d. × 60 cm; Toyo Soda, Japan). Elution of the fractions was performed with a 0.1 mol l⁻¹ sodium phosphate buffer (pH 7.0) containing sodium dodecyl sulphate (1 g l⁻¹) at a flow rate of 0.3 ml min⁻¹. Conditions were isocratic. The pressure varied between 3.5 and 5.5 kg cm⁻² (pump model 600E, Millipore, Belgium). In order to protect the analytical column, an in-line filter and a 7.5 mm i.d. × 7.5 cm pre-column were used; moreover, samples were filtered through a 0.45-μm membrane filter before injection. Eluted proteins were detected spectrophotometrically at 210 nm (model 160, Beckman Instruments, Belgium). The reference solution consisted of thyroglobulin, serum albumin, ovalbumin, β-lactoglobulin A, chymotrypsinogen, ribonuclease, insulin chain B, glucagon and bacitracin (Sigma-Aldrich, Belgium).

Apparent nitrogen digestibility was calculated from the nitrogen and chromic oxide contents in the diets and the digesta using the general formula for digestibility (Vanhoof and De Schrijver, 1996).

2.5. Statistical analyses

Data were analyzed by two-way ANOVA (General Linear Model program; SAS Institute, 1988) with dietary treatment and digestive segment as main effects. When the effects from ANOVA were significant, Tukey’s contrasts were used to differentiate among means. Significant difference was established at P < 0.05. Instances in which
was considered as trends. Values are reported as means ± SD. In a few instances, all analyses could not be completed due to insufficient sample size.

3. Results and discussion

The extent of apparent protein digestion (Table 2) increased in both dietary treatment groups as digesta moved from the stomach down to the rectum. As expected, there was no substantial apparent nitrogen digestibility measured in the stomach. Although gastric proteolytic enzymes may be produced and secreted in juvenile turbot, the stomach is generally not considered as a site for absorption of amino acids and peptides. In light of the large standard errors that were associated with the apparent nitrogen digestibility data in the stomach, conclusions regarding dietary effects should not be drawn from the slightly negative mean value in the control group and the slightly positive mean value in the *V. proteolyticus*-supplemented group. As compared with the apparent nitrogen digestibility in the hindgut, the digestibility in the proximal gut tended to be lower ($P < 0.1$). This lower digestibility may result from influx of endogenous nitrogen originating from the intestinal mucosa, the bile and pancreatic secretions. Substantial protein degradation also occurred in the rectum as differences in apparent nitrogen digestibility were observed between hindgut and rectum ($P < 0.1$). To what extent the inherently present gastrointestinal microflora was involved in protein breakdown could not be derived from the present study since no tests on microbial activity were carried out. On the other hand, more concrete information was obtained concerning the role of the bacteria which were added to the diet. The ingestion of *V. proteolyticus* resulted in increased apparent nitrogen digestibility, mainly when measured at the site of the hindgut and rectum ($P < 0.1$). These effects suggest that the tested bacterial strain was able to stimulate protein degradation in juvenile turbot as soon as pH requirements were fulfilled. In the stomach, pH values were generally lower than 5, whereas pH values in the gut and rectum were higher than 6 and 7, respectively (Table 2). As compared with control group, the *V. proteolyticus* group showed elevated ammonia content in the digesta of the hindgut ($P < 0.05$) and rectum ($P < 0.1$), indicating that the supplemented proteolytic bacteria were active in these parts of the digestive tract. As a consequence, the increased ammonia concentrations following ingestion of *V. proteolyticus* corresponded with the earlier mentioned higher apparent nitrogen digestibilities in the distal digestive segments. As in other species, it appears that also young turbot shows the highest microbial proteolytic activity in the distal parts of the digestive tract since in both treatment groups, ammonia contents were significantly increased in hindgut and rectum as compared with the upper gut.

With respect to soluble nitrogen, no significant effects due to feeding regimen were found (Table 2). In contrast, there were significant differences in relation with the digestive segment. In the stomach, the content of water-soluble nitrogen was, on average, 18% of total nitrogen. This was clearly the lowest value that was measured in the gastrointestinal tract, showing little protein digestion in the stomach as was also concluded from the apparent nitrogen digestibility data. The highest water-soluble nitrogen content (on average 70%) was measured in the foregut. Thereafter, there was a
Table 2

Apparent nitrogen digestibility (%), water-soluble nitrogen (%), ammonia content (\(\mu\)mol g\(^{-1}\)) and pH in gastrointestinal digesta of juvenile turbot fed a diet with or without supplemented *V. proteolyticus*\(^a\).

| Diet     | Stomach | Foregut | Hindgut | Rectum | Stomach | Foregut | Hindgut | Rectum | Pooled SD | ANOVA (\(P\) value)\(^b\) |
|----------|---------|---------|---------|---------|---------|---------|---------|---------|-----------|----------------|----------------|
| Diet C   | 3.1\(^c\) | 37\(^c\) | 53\(^{a,b}\) | 63\(^{a,b}\) | 2.0\(^d\) | 43\(^{a,b}\) | 63\(^{a,b}\) | 77\(^a\) | 9 | 0.09 | 0.001 |
| Diet VP  | 17\(^c\) | 72\(^c\) | 43\(^{b}\) | 40\(^b\) | 20\(^c\) | 68\(^b\) | 47\(^b\) | 33\(^b\) | 8 | 0.25 | 0.01 |
| pH       | 4.7\(^c\) | 6.8\(^b\) | 6.6\(^b\) | 7.6\(^a\) | 4.3\(^c\) | 6.3\(^b\) | 6.7\(^b\) | 7.8\(^a\) | 0.5 | 0.35 | 0.01 |

\(^a\) Values are means, \(n = 5\) or 6. Means within a row without common superscript letter are significantly different (\(P < 0.05\)) as determined by Tukey’s contrasts.

\(^b\) \(P > 0.1\) for diet\(\times\)digestive segment interaction for any measured parameter.

\(^c\) nd, not detectable.
decline until the average of 36% in the rectum. Most probably, the elevated amounts of water-soluble nitrogen in the proximal intestine were related to the release of endogenous enzymes by the host and to considerable digestion of dietary and metabolic protein. Apparently, the production of water-soluble nitrogen compounds in the proximal part of the gut occurred too rapidly to be readily absorbed. Conversely, the decline in water-soluble nitrogen in the distal part of the gut was presumably related to less proteolytic activity and a comparatively higher absorption rate. Moreover, the utilization of water-soluble nitrogen for microbial growth may not be excluded in the distal segments of the alimentary tract.

Protein digestion was also examined by measuring the MW profiles of soluble proteins and peptides as digesta passed through the digestive tract. Table 3 gives the percentage partition of total soluble protein in MW categories for each segment of the gastrointestinal tract. Strikingly, the sum of the peptides and proteins, with MW lower than 10,000, represented between 69% and 85% of the total soluble protein in all four segments. Generally, this percentage was not significantly affected by dietary treatment or digestive segment. Characteristically, within the fractions with MW ≤ 10,000, the 2000–5000 MW range was predominant in each digestive region and represented between 25% and 35% of total soluble protein. The proportion of the smallest proteins (< 1000 MW) decreased significantly as digesta progressed down the tract, reflecting absorption by the host and possibly also utilization by the microflora in the distal parts of the tract. Supplementation of the diet with *V. proteolyticus* resulted in a significantly increased proportion of the lowest MW category in the foregut and in a tendency toward elevated proportions in the hindgut (P < 0.1). Thus, ingestion of *V. proteolyticus* tended to move protein degradation more proximal in the gut. Obviously, these observations were paralleled by enhanced apparent nitrogen digestibility values in the *V. proteolyticus* group.

The proportion of the 10,000–20,000 MW protein category decreased gradually during the transit of digesta in the tract, but there was no effect from the dietary regimen. The proportion of the 20,000–50,000 MW proteins was generally less than 5% in all parts of the tract, in spite of the fact that the digestive enzymes belong to this MW category. It is not known whether this observation was due to poor production of endogenous enzymes in juvenile turbot as this may be the case in other young animals or to fast hydrolysis after being secreted (Bergner et al., 1980). An additional explanation for this apparent discrepancy is the high dilution rate of endogenous nitrogen in the digestive tract because of the very high protein content in the diet (760 g kg⁻¹). Asche et al. (1989) also reported no substantial proportions of the 20,000–50,000 MW range in the intestine of piglets notwithstanding the feeding of a diet containing only 220 g kg⁻¹ protein. As an explanation, the authors suggested that the endogenous enzymes were rapidly hydrolyzed.

As compared with the stomach, soluble proteins having MW higher than 50,000 accumulated further down in the tract. In the control group, the sum of their proportions increased, on average, from 3% in the stomach to 16% in the foregut, 19% in the hindgut and 26% in the rectum. A similar increase in the *V. proteolyticus* group was noted. The reported increasing proportions of heavier soluble proteins in the tract were presumably related to disappearance of lower MW proteins by digestion, absorption and
Table 3
Molecular weight (MW) profiles (% total protein fraction) of the soluble proteins and peptides in gastrointestinal digesta of juvenile turbot fed a diet with or without supplemented *Vibrio proteolyticus*.

<table>
<thead>
<tr>
<th>MW range (kDa)</th>
<th>Diet C</th>
<th>Diet VP</th>
<th>Pooled SD</th>
<th>ANOVA (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stomach</td>
<td>Foregut</td>
<td>Hindgut</td>
<td>Rectum</td>
</tr>
<tr>
<td>&lt; 1</td>
<td>17</td>
<td>14</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>1–2</td>
<td>15</td>
<td>11</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>2–5</td>
<td>31</td>
<td>30</td>
<td>35</td>
<td>33</td>
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<tr>
<td>5–10</td>
<td>17</td>
<td>18</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>10–20</td>
<td>13</td>
<td>8</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>20–50</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>50–100</td>
<td>1.5</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>100–200</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>200–500</td>
<td>0.5</td>
<td>8</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>&gt; 500</td>
<td>0.5</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

²Values are means, *n* = 5 or 6. Means within a row without common superscript letter are significantly different (*P* < 0.05) as determined by Tukey’s contrasts.

³*P* > 0.1 for diet × digestive segment interaction for any protein category.
microbial utilization as well as to synthesis of microbial protein in the distal segments. This followed, in particular, from the proportions of the proteins with MW > 200,000: this protein fraction increased in the control group from 9% in the foregut to 19% in the rectum ($P < 0.1$) and in the V. proteolyticus group from 6% in the foregut to 24% in the rectum ($P < 0.05$). With respect to the dietary V. proteolyticus treatment, it was found that the 200,000–500,000 MW fraction tended to increase in the hindgut and the rectum, referring to the presence of more microbial protein in the V. proteolyticus group as compared with the control group.

These are the first results that document protein digestion in different parts of the alimentary tract in juvenile turbot. In summary, the observed increase in apparent nitrogen digestibility during the transit down to the rectum was paralleled by a steady decrease in small proteins (< 1000 MW) in the soluble fraction of digesta, whereas the proportion of larger proteins (> 200,000) increased. Furthermore, the results demonstrated that in the prevailing experimental conditions, dietary fortification with V. proteolyticus moved protein degradation more to the proximal part of the gut and tended to enhance apparent nitrogen digestibility. In animal nutrition, such effects would generally be considered as beneficial. However, as in this study, the observed positive effect on apparent protein digestion was probably highly related to increased proteolytic activity exerted by bacteria and not by fish enzymes; it remains to be elucidated whether the formed protein degradation products contribute to nitrogen retention in the host. Controlled long-term dose–growth response investigations are suggested to further clarify the nutritional significance of the present findings.

Acknowledgements

The authors thank D. Vermeulen and R. Van Houdt for the technical assistance. The present study was supported by grant G.0063.96 from the Fund for Scientific Research-Flanders (Belgium).

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