In vitroとin vivo法によるブリのコーチングルテンミールと魚粉消化性の比較
Comparisons of Corn Gluten Meal and Fish Meal Digestion in Yellowtail (*Seriola quinqueradiata*) by *In vivo* and *In vitro* Approaches

Patricia Yumi Morimoto KOFUJI¹, Yumi MICHIIRO¹, Hidetsuyo HOSOKAWA¹ and Toshiro MASUMOTO¹

**Abstract:** The lower digestibility of corn gluten meal (CGM) has been reported in yellowtail (*Seriola quinqueradiata*). This paper studied the factor(s) involved in such low digestibility both *in vivo* and *in vitro*. The post-feeding profiles of pepsin and trypsin secretion, and trypsinogen activation were investigated *in vivo* by feeding yellowtail (average body weight, 720 g) either a fish meal-based diet (FMD) or a diet containing CGM (CGMD). The activities of pepsin and trypsin in CGMD-fed fish were not significantly different from those in FMD-fed fish. On the other hand, the *in vitro* study revealed that the catalytic ability of stomach and pyloric caeca extracts to digest CGM were lower than in FMD digestion. These results suggested that the lower digestion of CGM compared to fish meal was attributed to the lower catalytic ability of digestive proteases to CGM and not to a lower secretion volume or activation rate of proteases such as pepsin and trypsin.

**Keywords:** Corn gluten meal; Digestive enzymes; Digestion; Yellowtail

Yellowtail (*Seriola quinqueradiata*) is one of the most popular aquaculture species in Japan. They require high dietary protein, the major source of which is fish meal. A reduction in fish meal production has accelerated the necessity for alternative sources of protein (Tacon and Jackson 1985; Watanabe and Kiron 1997; Watanabe et al. 1997, 2000). Consequently, the substitution of fish meal with more abundant plant proteins has become necessary for sustainable yellowtail culture (Takagi et al. 1996; Masumoto et al. 1996). Among plant protein sources, corn gluten meal (CGM) is considered to have high protein level, vitamins B and E, low fiber and no anti-nutritional factors, such as digestive enzymes inhibitors (Regost et al. 1999). Thus, CGM is one of the most popular plant protein sources used to replace fish meal, and it has been widely applied in feeds for many aquaculture species (Robaina et al. 1997; Kikuchi 1999ab; Regost et al. 1999; Moyano-Lopez et al. 1999; Garcia-Carreno et al. 2002; Yamamoto et al. 2002). However, there are variations in the bioavailability of CGM among fish species. For example, the maximal inclusion levels of CGM are reportedly 40% for common carp, rainbow trout and gilthead seabream (Yamamoto et al. 1998; Moyano et al. 1992ab; Robaina et al. 1997) and 20% for turbot and Japanese flounder (Regost et al. 1999; Kikuchi 1999). On the other hand, at CGM inclusion levels above 10%, yellowtail growth is reduced (Shimeno et al. 1993). This low bioavailability of CGM for yellowtail is due to their low digestibility of CGM. The reported apparent protein digestibility (APD) coefficient for yellowtail is less than 50% for fish fed with a CGM diet (Masumoto et al. 1996), while APD values of over 80% have been reported in carp (Smith 1971) and rainbow trout (Cho et al. 1982;
Several digestive enzymes are involved in the digestion of dietary protein. In yellowtail, these enzymes are acid proteases, mainly pepsin, from stomach tissues, and alkaline proteases from the pancreatic tissues surrounding the pyloric caeca. They are stored as inactive zymogen forms, but activated after secretion, which is essential for digestion. Enzymes digest feed efficiently at their optimum pH range, so the lower digestibility of CGM might be due to the inappropriate pH of the intestine. Furthermore, the catalytic activity of enzymes might be lower in CGM digestion compared to fish meal. The aim of the present study is to ascertain if the low digestibility of CGM in yellowtail is attributable to low enzyme secretion rates, low enzyme activation or the low catalytic ability of proteases in CGM digestion.

Materials and Methods

Experimental diets
Two experimental moist pellet diets were formulated (Table 1), a fish meal diet (FMD), composed of brown fish meal as the sole source of protein, and a CGM diet (CGMD), which was a 1:1 ratio of CGM and FMD. The level of CGM inclusion in the CGMD was at a level where young yellowtail showed lowered growth performance (Shimeno et al. 1993).

Experimental conditions and fish
Adult one year yellowtail (Seriola quinqueradiata) (mean body weight: 720 ± 90 g) were obtained from a commercial fish farm in Kochi Prefecture, Japan and maintained in 2 t concrete tanks at Usa Marine Biological Institute at Kochi University (Kochi, Japan). The water temperature during the experiment was approximately 19°C. Prior to the experiment, fish were divided into 2 groups (FMD and CGMD), accustomed to the experimental diets for one week, and starved one day prior to sampling to allow evacuation of the previous feed. In the morning of the experimental day, either the FMD or the CGM was fed to fish ad libitum. Four fish from each treatment were removed from the tanks before feeding (0 time) and 0.5, 1, 2, 4 and 6 hours after feeding. At each sampling time, fish were anesthetized with phenoxyethanol and sacrificed by a blow to the head. The digestive tract was removed and dissected, and the pyloric caeca were isolated together with the surrounding fat tissue, which contains the pancreatic tissue. The stomach, pyloric caeca and intestine were isolated and frozen at −30°C for further analysis. The pH values in the stomach and intestine contents were measured with pH test paper (Duotest, Machery-Nagel, Germany) after each sampling time.

Pepsin activity assay
The stomachs were opened when half-thawed, and the contents were isolated from the tissues by scissors. The surface of stomach tissue, which was covered with mucus and food, was gently scraped away with a scalpel and used for the pepsin activity assay. The stomach tissue and contents were homogenized in distilled water (1:4, w:v) using a homogenizer (Physcotron, Tokyo, Japan) and centrifuged at 10,000 g at 4°C for 20 min. The supernatants were used for the pepsin activity determination according to the method of Anson (1938) with some modifications and using bovine hemoglobin (Sigma H-2500, St. Louis, USA) as the substrate. The reaction mixture consisted of 0.9 ml of 0.1 M sodium citrate buffer at pH 2, 0.9 ml of substrate and 0.2 ml of sample homogenate. After incubation for 30 min at

<table>
<thead>
<tr>
<th>Ingredients*</th>
<th>FMD</th>
<th>CGMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown fish meal</td>
<td>67.3</td>
<td></td>
</tr>
<tr>
<td>Corn starch</td>
<td>13.4</td>
<td></td>
</tr>
<tr>
<td>Feed oil</td>
<td>8.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Vitamin*</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>Mineral*</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>CMC-Na</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>Guar gum</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Corn gluten meal</td>
<td>-</td>
<td>50.0</td>
</tr>
<tr>
<td>Total</td>
<td>100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

*Purchased from Nihon formula feed Co. (Yokohama, Japan).
25°C, the reaction was stopped with 2 ml of 10% (w/v) trichloracetic acid (TCA) solution. The blanks were prepared by the addition of 10% TCA prior to the incubation. The precipitates resulting from the TCA addition were removed by centrifugation for 15 min at 3,500 rpm, and 0.2 ml of the supernatant was reacted with diluted Folin-Ciocalteu reagent. Absorbance was read at 750 nm, and activity was expressed as U/g of dry tissue or content to avoid the effects of moisture and dietary protein. One unit was defined as the number of changes in absorbance in one minute.

**Trypsin activity assay**

The frozen pyloric caeca/pancreatic tissue and intestinal content samples were thawed, homogenized in distilled water (1:4, w:v) using a homogenizer then centrifuged at 10,000 g at 4°C for 20 min. The supernatants were used for trypsin analysis after the precipitates were freeze-dried and weighed. Trypsin activity was determined using synthetic N-benzoyl-L-arginine-p-nitroanilide (L-BAPA:3057, Peptide, Osaka, Japan) as a substrate. The reaction mixture consisted of 1.6 ml of tris buffer (0.1 mM, pH 8.2), 1 ml of L-BAPA (1.3 mM) and 100 µl of tissue homogenate. Trypsin activity was determined by measuring the rate of paranitroaniline (pNA) production during 5 min of reaction at an absorbance of 405 nm. The optimal temperature and pH were determined previously as 25°C and pH 8, respectively. The reactions rates were calculated in units (U), defined as the number of 1 µmol of pNA released in 1 min. Activity was expressed as U/g of dry tissue or digestive content.

**Trypsinogen activation**

Yellowtail trypsinogen was activated in vitro by the incubation of pyloric caeca extracts with tris buffer containing CaCl₂ (50 mM) for 60 min. In this study, trypsin activity measurements taken with trypsinogen activation were called total trypsin activity and those measured without activation were called trypsin activity.

**Catalytic ability of pyloric caeca extracts**

The same source of fish meal and CGM were used in the in vitro study. Yellowtail stomach tissue and pyloric caeca/pancreas homogenates were prepared in a similar manner as described above, and centrifuged to separate the supernatant. Twenty to twenty-five mg of fish meal or CGM was added to 50-ml erlenmeyer flasks. Then, 10 ml of 0.2 M trisodium citrate dehydrate buffer (pH 3.0) for the stomach tissue or 0.1 M NaH₂PO₄·2H₂O with 0.1 M tris buffer (pH 8.0) for the pyloric caeca in vitro digestion, and 11 µl of 34 mg/ml chloramphenicol were added to each flask prior to the addition of an enzyme solution. Following the addition of 1 ml enzyme solution, the contents were mixed quickly and placed in a shaking water bath at 30°C. All flasks were covered with aluminum foil with holes and incubated for 1.5 h and 3 h. After each incubation time, 1 ml of solution from each flask was taken and immediately transferred to micro-tubes, which were heated in a boiling bath (about 100°C) for 3 min to inactivate the proteases. After cooling to room temperature, 800 µl of supernatant was transferred to the test tubes. The contents were diluted with 2,400 µl of 0.01 M phosphate buffer (pH 8.2), and the resultant solution was used for colorimetric reaction with trinitrobenzene sulphonic acid (TNBS) (Ihekoronye 1986).

**Statistical analysis**

The student’s t-test was applied to evaluate any specific differences between the two treatments at a significance level of 0.05.

**Results and Discussion**

There was no difference in pepsin activity before and after activation (data not shown). Pepsinogen is probably activated during the enzyme activity assay, which was performed at an acid pH. Pepsin activity in the stomach tissue of the yellowtail fed with both the FMD and CGMD decreased 30 min after feed intake (Fig. 1). Pepsin was probably secreted within 30 min of feeding in both groups. No statistical differences in tissue pepsin activity were found...
between the two dietary groups. In Atlantic salmon, pepsin was secreted from the stomach tissue within 1 hour of food ingestion, and it was also concluded that pepsinogen is rapidly synthesized and secreted with the onset of feeding (Einarsson et al. 1996). The exact control mechanisms of pepsin secretion in yellowtail have not been determined yet, pepsin secretion in response to the feeding was not affected by either CGMD or FMD (Fig. 2).

Total trypsin (trypsinogen plus trypsin) activity in the pyloric caeca of both dietary groups increased after 30 min of feeding then decreased until 6 hours after feeding. There was no significant difference in the activity between the two dietary groups at each sampling time (Fig. 3). These results indicate that trypsin and trypsinogen secretion are similar in the fish fed with CGMD and FMD.

Total trypsin activity in the intestinal contents of both dietary groups increased (Fig. 4) in parallel to the decreased total trypsin activity in the pyloric caeca tissue. There was no difference in trypsin activity in the intestinal contents of both dietary groups at each sampling time. In the intestine, the percentage of trypsin

![Fig. 1. Pepsin activity in the stomach tissue of yellowtail fed FMD or CGMD.](image1)

![Fig. 2. Pepsin activity in the stomach contents of yellowtail fed FMD or CGMD.](image2)

![Fig. 3. Trypsin activity in the pyloric caeca of yellowtail fed FMD or CGMD.](image3)

![Fig. 4. Trypsin activity in the intestinal contents of yellowtail fed FMD or CGMD.](image4)
activity in relation to total trypsin activity was almost constant at about 80%, and there was no significant difference between the FMD and CGMD-fed fish (Fig. 5). This result suggests that trypsinogen is rapidly and almost all converted to trypsin by the food present in the intestine. There was no significant difference in the percentage of active trypsin between the two dietary groups at each sampling time. These results indicated that trypsinogen activation was similar irrespective of the dietary components. Moreover, no significant differences were found in the pH of the stomach and intestine contents between the FMD and CGMD-fed fish (Figs. 6 and 7). These results indicated that the pH in the stomach or intestinal lumen was probably not responsible for the differences in CGM and FM digestibility in yellowtail.

*In vitro* assays were performed to investigate the differences in yellowtail protease catalytic ability in fish meal and CGM digestion. The results obtained from the stomach and pyloric caeca extracts showed that CGM digestion was significantly lower than FM digestion (Figs. 8 and 9). These results are in accordance with *in vivo* digestibility studies performed previously with this fish species (Masumoto et al. 1996). Therefore, the catalytic abilities of proteases in the yellowtail stomach and pyloric caeca are significantly less effective in CGM compared to FM digestion. In the future, it will be interesting to determine whether fish such as carp and rainbow trout digest CGM *in vitro* better, since they have higher *in vivo* catalytic abilities to CGM (Smith 1971; Cho et al. 1982; Watanabe and Pongmaneerat 1991).

In conclusion, both pepsin and trypsin from the CGMD-fed yellowtail were similarly secreted and activated in the FMD-fed yellowtail. The lower catalytic ability of both the stomach and pyloric caeca digestive enzymes found in the *in vitro* study might be responsible for the lower digestion of CGM by yellowtail.
Fig. 8. Comparison of proteolysis product of FM and CGM incubated with yellowtail stomach crude enzyme, pH 3 (n = 4).

Fig. 9. Comparison of proteolysis product of FM and CGM incubated with yellowtail pyloric caeca crude enzyme, pH 7 (n = 4).

Acknowledgments

This study was carried out with financial support from the Japanese Ministry of Education, Science and Culture. We are grateful to the staff of Kochi University, Usa Marine Biological Institute, for their assistance during the experiment.

References


In vitro と in vivo 法によるブリのコーングルテンミールと魚粉消化性の比較

Patricia Yumi Morimoto KOFUJI・道広夕海・細川秀毅・益本俊郎

コーングルテンミール（CGM）は魚粉代替タンパク質源として養魚飼料に多用されているが、ブリではその消化性が低い。本研究では、消化性が劣る理由を明らかにするために、CGM に対するタンパク質消化性を in vivo と in vitro 法で魚粉と比較した。In vivo 法では、消化管内容物の pH、ベプシンおよびトリプシン活性および活性化トリプシンの割合に魚粉と CGM との間で差が無かった。一方 in vitro 法では、胃と幽門組織抽出物による CGM タンパク質分解量が魚粉に比べ低かった。したがって、ブリの CGM に対する消化性の低さは、タンパク質消化酵素の分泌には関係なく、むしろ分泌された酵素の CGM に対する分解力が劣るためと考えられた。