Digestive Function of Red Sea Bream Compensates for Dietary Soybean Trypsin Inhibitors

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Abstract: The inhibition of soybean trypsin inhibitor (SBTI) in brown fish meal diets for digestive and absorptive functions was investigated using juvenile red sea bream, Pagrus major, (mean body weight 62.1 g). Apparent protein and fat digestibilities indicated no significant differences among dietary supplements of SBTI at the levels of 0, 0.6, and 6.0 trypsin inhibitory units (TIU)/mg diet during a 22-day rearing period. Gastral digesta indicated an increasing tendency with an increase in dietary TIU at 6 h after feeding (AF), despite no differences among the dietary treatments at 3 h AF. Intestinal digesta of the 6.0 TIU diet group showed a lower tendency than the 0 and 0.6 TIU diet groups at 3 h AF; all groups at 6 h AF gave a similar low level. Basic protease and SBTI-insensitive basic protease activities of intestinal digesta in the 6.0 TIU diet group were significantly higher than the other diet groups at 3 h AF; all diet groups at 6 h AF reached a similar high activities. These suggest that red sea bream compensate dietary SBTI to promote the secretion of basic protease and SBTI-insensitive basic protease from the hepatopancreas in a relatively early time after feeding. Equivalent protein digestibilities among the dietary treatment might be due to the compensation as well as the prolongation of digesta transit time in the SBTI diet group.

Key words: Pagrus major; Soybean trypsin inhibitor; Basic protease; Soybean trypsin inhibitor insensitive protease

Soybean, Glycine max, contained antinutritional factors for humans and land animals. These are soybean trypsin inhibitors (SBTI), phytate, lectines, antigenic and estrogenic flavons, oligosaccharides, and else1). The SBTI among antinutritional factors has been considered to cause digestion and growth depressions in animals1). Defatted and toasted soybean meal (SBM), as an alternative protein source for fish meal in diets, was estimated to be one of valid plant proteins in carp, Cyprinus carpio2), rainbow trout, Oncorhynchus mykiss3), Atlantic salmon, Salmo salar4), red sea bream, Pagrus major5,6), tiger puffer, Takifugu rubripes7), and yellowtail, Seriola quinqueradiata8,9). These reports also indicated differences in the optimum dietary SBM level among fishes, due to the low digestibility and essential amino acid imbalance of SBM.

Our previous report showed that the sensitivity of tryptic and basic proteases for SBTI differed in cultured-fishes10). The highest sensitivity of tryptic protease was in tiger puffer, followed by rainbow trout, striped jack, Pseudocaranx delicatissimus, red sea bream, Japanese striped knifejaw, Oplegnathus fasciatus, bluefin tuna, Thunnus thynnus, purplish amberjack, Seriola dumerili, and yellowtail. While, the highest sensitivity of basic protease was in red sea bream, followed by rainbow trout, purplish amberjack, tiger puffer, Japanese striped knifejaw, bluefin tuna, striped jack, and yellowtail. These orders did not actually reflect on dietary SBM availability of fishes; no remarkable differences were found among rainbow trout20), red

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sea bream\textsuperscript{5}), tiger puffer\textsuperscript{7}), and yellowtail\textsuperscript{8}). The object of the present study, therefore, was conducted to identify how the digestive mechanism and function of red sea bream, possessing highly sensitivity of the digestive proteases for SBTI, confront the dietary SBTI.

**Materials and Methods**

**Dietary Formula and SBTI**

Table 1 displays dietary formula used in the present study. Diet 1, which was composed of 60% brown fish meal, 10% wheat gluten, 9% sardine oil, 12% $\alpha$-potato starch, 5% vitamin mixture\textsuperscript{11}), and 4% mineral mixture\textsuperscript{11}), was a basal diet and added 0.5% Cr$_2$O$_3$ as an indicator. Diets 2 and 3 were respectively prepared to supplement SBTI to diet 1 at the levels of 0.6 and 6 trypsin inhibitor units (TIU)/mg diet.

Commercially available SBTI, which was prepared from soybean by Wako Pure Chemicals (Osaka, Japan), came with no information about purity and contents of Kunitz and Bowman-Birk trypsin inhibitors. The SBTI was 5,300 TIU/mg, and one TIU was defined as decreasing optical density of 0.01/tube under an assay condition\textsuperscript{12}). Fixed amounts of SBTI were dissolved in 10 ml of 0.001 N NaOH and supplemented to diet 1, together with deionized water. Diets were given as a dry pellet after lyophilizing a moist pellet with 3 mm in diameter and stored at -20°C until use.

**Table 1. Formula and proximate composition of diets**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Diet no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
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<tbody>
<tr>
<td>Formula (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brown fish meal</td>
<td></td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Wheat gluten</td>
<td></td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Sardine oil</td>
<td></td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>$\alpha$-Potato starch</td>
<td></td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Vitamin mixture\textsuperscript{*1}</td>
<td></td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Mineral mixture\textsuperscript{*1}</td>
<td></td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Cr$_2$O$_3$ (%)</td>
<td></td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>SBTI*$^2$ (TIU*$^3$/mg diet)</td>
<td></td>
<td>0.06</td>
<td>0.6</td>
<td>6</td>
</tr>
</tbody>
</table>

*1 Halver\textsuperscript{11}.

*2 Soybean trypsin inhibitor (Wako Pure Chemicals).

*3 Trypsin inhibitor units defined by the method of Kakade et al.\textsuperscript{12}.

**Fish and Feeding Protocol**

A brood of red sea bream juveniles, a mean body weight of 62.1 ± 4.5 g, was obtained from the Kinki University Fish Nursery Center, Uragami. After domesticating the fish to rearing conditions on diet 1 for 10 days, three groups of fish of 15 each were respectively introduced into 400-l indoor Guelph tank equipped with a fecal collection column. A flow-through water system was adopted to tanks at 2-l filtrated seawater/min. Each group of fish was fed on one of three diets at 10:00 and 16:00 till satiation for 22 days. Feces were collected by the method cited previously\textsuperscript{13)} at 9:00 everyday from day 3 after the start of feeding trial. Feces samples for every 3 days were pooled, resulting in 6 feces samples totally for each dietary group, and stored at -20°C after lyophilizing. For assaying the inhibition of SBTI to digestive and absorptive functions of the fish, moreover, the hepatopancreas and gastric and intestinal digesta were sampled from each dietary group at 0, 3, and 6 h after feeding (AF) under an equivalent feeding rate (0.7% of body weight), on day 2 after the end of the feeding trial.

The mean water temperature and DO during the feeding trial were 22.6 ± 1.0°C and 3.52 ± 0.62 ml/l, respectively. A photoperiod was conducted on a 12L:12D with fluorescent tubes.

**Assays**

Kunitz and Bowman-Birk trypsin inhibitor contents of the SBTI were assayed using a sodiumdodecyl sulfate-polyacrylamide gel electrophoresis\textsuperscript{14)}. Apparent digestibilities of protein and fat were measured using feces by the indirect method cited by Furukawa and Tsukahara\textsuperscript{15}.

We also inspected the inhibition of SBTI to digestive and absorptive functions in terms of digestive enzymes, which were acidic protease (AP), basic protease (BP), and SBTI-insensitive protease (TI-BP) of the hepatopancreas and gastric and intestinal digesta after feeding. The hepatopancreas and digesta were homogenized with iced-deionized water with a glass homogenizer. Resultant homogenate was centrifuged at 10,000 × g for 20 min. These were con-
ducted under 4°C, and the obtained supernatant as enzyme solution was frozen by liquid nitrogen and stored at -80°C. The AP activity was measured by the casein-Folin method at pH 2.5 and 30°C for 10 min<sup>10</sup>. The BP and TI-BP activities were also measured by the casein-Folin method at pH 8.2 and 30°C for 10 min<sup>10</sup>. Enzyme solution obtained from the hepatopancreas was previously activated by bovine enteropeptidase, as cited in the previous study<sup>10</sup>. One unit of AP, BP, and TI-BP activities was defined as 1 µmole of tyrosine liberated from the substrate for 1 min under the assay conditions.

**Statistical Analysis**

The data were analysed by one-way ANOVA to determine the inhibitory effects of dietary SBTI (<i>p</i> < 0.05). If this was significant, differences between means were identified by Duncan’s Multiple Range Test (<i>p</i> < 0.05)<sup>17</sup>.

**Results**

**Trypsin Inhibitor Content of SBTI and TIU of Diets**

The SBTI used in the present study was composed of 43.5% Kunitz trypsin inhibitor and 50.6% Bowman-Birk trypsin inhibitor. Remained TIU of diets 2 and 3 were 0.46 and 4.42 TIU/mg diet at the end of the feeding trial, respectively.

**Digestibility and Digestive Organ Weight**

Apparent protein and fat digestibilities and digestive organ-somatic indices at the initial and final of the feeding trial are displayed in Table 2. No significant differences among the dietary treatment were found in protein and fat digestibilities. The highest hepatopancreatic-somatic index was obtained in diet 1 group, followed by diets 2 and 3 groups at the end of the feeding trial. Stomach- and intestine-somatic indices indicated no significant differences among the dietary groups.

**Hepatopancreatic BP and TI-BP Activities after Feeding**

Figure 1 shows hepatopancreatic BP and TI-BP activities, which were compared with units per 100 g body weight or per g tissue, at 0, 3, and 6 h AF. The BP activities of the dietary groups indicated slightly decreasing trends with time passing from 0 to 6 h AF, but no significant differences among the dietary treatments at each time AF. The hepatopancreatic TI-BP activities per 100 g of body weight in diet 1 group were higher than diets 2 and 3 groups at 0 h and 3 h AF, but not that at 6 h AF and the activities per g tissue at each time AF.

**Digesta Weight and Its AP, BP, and TI-BP Activities after Feeding**

Figure 2 shows relative gastral and intestinal digesta weights to body weight and their AP, BP, and TI-BP activities at 3 and 6 h AF. Digesta weights were measured on wet weight basis, and activities were expressed as units/g digesta or/100 g body weight.

<p>| Table 2. Apparent digestibilities and digestive organ weight of the fish fed the diets for 22 days |
|--------------------------------------------|-----------------|-----------------|--------------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Initial</th>
<th>Final</th>
<th>Diet group</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Apparent digestibilities (%)&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>97.9±0.6&lt;sup&gt;2&lt;/sup&gt;</td>
<td>95.8±0.9</td>
<td>95.3±0.4</td>
</tr>
<tr>
<td>Lipid</td>
<td>97.2±0.6</td>
<td>97.5±1.3</td>
<td>97.3±0.4</td>
</tr>
<tr>
<td>Organ-somatic indice (%)&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatopancreas&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.77±0.14</td>
<td>3.57±0.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.10±0.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.79±0.16</td>
<td>0.83±0.01</td>
<td>0.78±0.18</td>
</tr>
<tr>
<td>Intestine</td>
<td>1.23±0.20</td>
<td>1.57±0.33</td>
<td>1.32±0.18</td>
</tr>
</tbody>
</table>

<sup>1</sup> n=6.<br><sup>2</sup> Mean±SD.<br><sup>3</sup> n=6.<br><sup>4</sup> Different superscript letters denote significant differences with a low (<i>p</i> < 0.05).
Fig. 1. Changes in basic proteinase (BP) and trypsin inhibitor insensitive BP (TI-BP) activities of the hepatopancreas in fish at 0, 3, and 6 h after feeding the diets. *□, □■, and ■ abbreviate mean protease activities (n=3) of diets 1, 2, and 3 groups, respectively. Different superscript letters on the bar denote significant differences among the dietary treatments (p<0.05).

Fig. 2. Changes in gastric and intestinal digesta weight and their acid protease (AP), basic protease (BP), and trypsin inhibitor insensitive BP (TI-BP) activities in fish at 3 and 6 h after feeding the diets. *□, □■, and ■ abbreviate mean protease activities (n=3) of diets 1, 2, and 3 groups, respectively. Different superscript letters on the bar denote significant differences among the dietary treatments (p<0.05).
No significant differences among the dietary treatments were found in relative gasstral digesta weights and AP activities at 3 h AF. Although an increasing trend with an increasing in dietary TIU was revealed in relative gasstral digesta weights, AP activity in diet 1 group were higher than diets 2 and 3 groups at 6 h AF.

Relative intestinal digesta weights of diet 1 and 2 groups were higher than diet 3 group at 3 h AF. The BP and TI-BP activities of diet 1 and 2 groups were reversely lower than diet 3 group at 3 h AF. No significant differences among the dietary treatments were depicted in relative intestinal digesta weights as well as BP and TI-BP activities at 6 h AF.

Discussion

Defatted and toasted SBM ordinarily has about 6 TIU/mg. Therefore, the SBTI activities of diets 2 and 3 corresponded to 10% and total replacement of dietary ingredients with SBM, respectively. The SBTI activities of diets 2 and 3 fell to 0.46 and 4.42 TIU/mg at the end of the feeding trial, respectively. The TIU reduction might be resulted in dietary preparation and storage. Moreover, the rearing period of the present study was obligated to conduct for 22 days, a relatively short duration, due to the small volume of Guelf tank used. And apparent digestibilities of protein and fat had a possibility to be overestimated, due to leaching of some nutrients from feces in Guelf tank.

No meaningful differences among the dietary treatments were found in apparent protein and fat digestibilities and stomach- and intestine-somatic indices. In hepatopancreas-somatic index, intestinal digesta weight at 3 h AF, hepatopancreatic BP and TI-BP activities expressed as units/100 g body weight at 0 h and 3 h AF, and AP activity in gasstral digesta at 6 h AF, all appeared a decreasing trend with an increase in dietary TIU. The same trend was also obtained in daily weight gain, feed efficiency, and protein efficiency ratio of the dietary groups (0.62%, 47.0%, 0.78 in diet 1 group; 0.52%, 40.3%, 0.66 in diet 2 group; and 0.32%, 28.1%, 0.47 in diet 3 group, respectively). Otherwise, gasstral digesta weight at 6 h AF and BP and TI-BP activities in intestinal digesta at 3 h AF increased with an increase in dietary TIU. These results intensely indicate that red sea bream compensate SBTI in diets with promoting BP and TI-BP secretion into the intestinal lumen in a relative early time after feeding. Thus, lower growth performance of the highest dietary TIU group was informed to be due to the large energy expenditure for heat increment, and low feed intake due to the prolongation of digesta transient time, beside similar nutrient digestibilities obtained in the dietary groups. Ukawa et al.5,18) obtained growth retardation in red sea bream and tiger puffer fed diets included above 30% of SBM, as compared with a control brown fish meal diet. It is suggested that the limitation of SBM level in diets is mainly attributed to SBTI for red sea bream, which have high trypic and BP sensitivities for SBTI10). Refstie et al.4) also reported Atlantic salmon received extracted SBM with low TIU, oligosaccharides, lectin, and antigen grew faster than the fish received toasted SBM. Furthermore, Olli et al.19) had already indicated that Atlantic salmon could maintain nutrient digestibility and growth performance for a certain SBTI in diet, due to increased trypsin secretion. It has not yet been examined how tiger puffer, which have no stomach20) and high protease sensitivities for SBTI10), compensate for dietary SBM and SBM in detail.

The SBTI in diets might reduce the hepatopancreas of red sea bream. It has already shown that SBTI induced the enlargement of the pancreas in rats and chickens, but not pigs and monkeys21,22). Histological inspection should be conducted on the hepatopancreas of red sea bream fed SBTI diets.

In the present study, there appeared no significant differences among ratios of TI-BP to BP activities in intestinal digesta of the dietary groups at 3 h and 6 h AF. This indicated that red sea bream basically confront dietary SBTI through the quantitative compensation of digestive proteases, but not through the qualitative compensation. Holm et al.23,24) indicated the inhibitor-resistant trypsic activity after raw soy-
bean installation were caused by Kunitz and Bowman-Birk trypsin inhibitors in humans and Wister rat. In addition, Takagi et al. showed the improved utility of soy protein concentrate (SPC) with growth in red sea bream. A similar phenomena of SPC had already been detected in yellowtail. The changes in utility coefficients of the fishes for SPC may be due to (1) an increase in the SBTI-resistant pancreatic proteases, (2) an increase in digestive potency for SPC protein as well as carbohydrate, and (3) a decrease in requirements for essential amino acids. It is also reasonable that the hepatopancreas of grown red sea bream fully synthesize and secrete digestive proteases for confronting SBTI under low daily feed intake per body weight, as compared with the juveniles and youngs under high daily feed intake per body weight.

Acknowledgments

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大豆トリプシンインヒビターに対するマダイ消化機能の適応

滝井健二・瀬岡 学・長岡智子・臼井智彦
中村元二・熊井英水・吉澤康子

大豆トリプシンインヒビター（SBTI）を0、0.6および6トリプシンインヒビター単位（TIU）/mg飼料添加した沿岸魚粉配合飼料で、一尾仔のマダイ稚魚（平均体重62.1 g）を22日間飼育して消化機能を比較した。期間中のタンパク質および脂質の消化率に有意な区間差は認められなかった。しかし、6 TIU 飼料区の摂飼3時間後における腸内容物量は他の区に比べて少なく、摂飼6時間後における胃内容物量は逆に多く残存していた。一方、摂飼3時間後における腸内容物の塩基性プロテアーゼ（BP）および SBTI 非感受性 BP（TI-BP）活性は、いずれも6 TIU 飼料区が他の区に比べて高かったが、摂飼6時間後には各区とも上昇して区間差はなかった。以上の結果から、飼料 SBTI に対してマダイは摂飼後の比較的早い時間に、腸内腔への BP および TI-BP 分泌を促進するとともに、消化時間を遅延して消化吸収機能を高く維持する方向に適応することが示唆された。