

Dietary Hydroxyproline Improves Collagen Contents of the Fillet in Tiger Puffer (*Takifugu rubripes*)

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Abstract: The present study was done to clarify whether dietary hydroxyproline (Hyp) was effective for improvement of collagen contents of the fillet in tiger puffer (*Takifugu rubripes*). We investigated collagen contents in the cranial, middle and caudal parts of the fillet in Experiment 1. The caudal part of the fillet had a high level of collagen compared with other two parts. In Experiment 2, effects of dietary Hyp (1%) on the performance and collagen contents of the fillet were determined at 37 and 81 days of feeding. Growth and feed efficiency was comparable between the control and Hyp groups over the experimental period. Collagen contents were decreased with growth in the control group, but dietary Hyp prevented the reduction in collagen contents in the fillet.

Key words: Collagen, feed efficiency, growth, hydroxyproline, tiger puffer

INTRODUCTION

Collagen is the main component of the connective tissue membranes in the muscles of fish, joining individual myotomes and is responsible for the integrity of the fillets. Hydroxyproline (Hyp), differing from proline by the presence of a hydroxyl group attached to the gamma carbon atom, is a major component of the protein collagen. Both Hyp and proline play key roles for the stability of collagen. Hyp is produced by hydroxylation of the amino acid proline by the enzyme prolyl hydroxylase following protein synthesis as a post-translational modification. Vitamin C is a cofactor for prolyl hydroxylase (Nelson and Cox, 2005). Li *et al.* (2007) investigated the interactions between dietary vitamin C levels (33, 79, 135 and 424 mg kg⁻¹ of wet mass feed) and growth rate on the collagen and cross-link contents of fast muscle in farmed juvenile Atlantic salmon (*Salmo salar* L.). In healthy juvenile salmon reared under controlled growth conditions, the dietary vitamin C inclusion of 79 mg kg⁻¹ was sufficient to produce the required synthesis of soluble muscle collagen. On the other hand, a small decrease in post-translational modifications of the collagen (leading to cross-linking)

was accompanied with an increase in growth rate, but were independent of vitamin C concentration. To authors' knowledge, however, no information was available for dietary proline or Hyp involved in collagen contents in fish.

Fish species containing more collagen are sliced thinner than those containing less collagen. Skipjack or tuna are sliced as thick as 0.7 cm and puffer fish are sliced as thinly as possible (Hatae, 2007). This fact means that collagen contents in puffer fish has important keys for the texture.

Accordingly, the present study was done to clarify whether dietary Hyp modifies collagen contents in tiger puffer.

MATERIALS AND METHODS

Experiment 1

Sample collection: Tiger puffer (*Takifugu rubripes*) with body weight 586±21 g was killed and the fillet was obtained. The samples obtained were frozen by liquid nitrogen and stored at -30°C until assay. About 2.5 g of dissolved sample was sliced from the cranial, middle and caudal parts of the fillet for collagen assay.

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Table 1: Composition of experimental diets (%)

Ingredients	Control	Hydroxyproline
α -Starch	9.3	9.3
Guar gum	1.0	1.0
Carboxymethylcellulose	1.5	1.5
Sodium alginate	1.0	1.0
Fish oil	10.0	10.0
Fish meal	75.0	74.0
Hydroxyproline	0.0	1.0
Vitamin premix	0.3	0.3
Calcium phosphate	1.0	1.0
Choline chloride	0.5	0.5
Mineral mixture	0.2	0.2
Vitamin C	0.2	0.2
Chemical composition		
Moisture (%)	38.9	39.4
Crude protein (%)	32.3	32.7
Crude fat (%)	9.9	9.8
Crude ash (%)	8.9	9.0
Gross energy (kcal/g)	2540.0	2534.0
Calorie/protein ratio	79.0	77.0

Experiment 2

Animals and diets: Total 90 fish were used in the present study. Forty five fish were reared in a round tank (4,000 L) with water (flow through) at 19-22°C. Two diets were used: a control diet and a control diet supplemented with 1% Hyp at dry matter basis. Hyp was a gift from Kyowa Hakko Kogyo Co., Ltd, Tokyo, Japan. The compositions of experimental diets are shown in Table 1. The fish were given diets *Ad libitum* as a single moist pellet at 9:00 and 14:00.

Sample collection and preparation: The first sampling of fillet was done at 37 days of experiment (13 fish in the control group and 12 fish in the Hyp group) and at 81 days (12 fish in both the control and Hyp groups). The sample was obtained from the middle part of the fillet and treated as mentioned in Experiment 1.

Analytical procedures: Samples were finely chopped and hydrolyzed in 10 mL of 6 N HCl for 24 h at 20°C and then kept for 10 h at 120°C with a block heater (ALB-121, Asahi technoglass Co. Ltd., Tokyo, Japan). The hydrolyzed sample was filtered and adjusted to 50 mL with distilled water. The sample (1 mL) was evaporated with a centrifugal evaporator (CVE-3100, Eyela Co. Tokyo, Japan). Evaporated samples were diluted with distilled water. A 0.2 mL sample was mixed with 0.4 mL isopropanol, followed by mixing with 0.2 mL of oxidant solution (75% wt/vol chloramine T and acetate/citrate buffer, pH 6.0, were mixed at a ratio of 3:1). The mixed sample solution was kept for 4 min at room temperature (17-20°C). After adding 2.6 mL Ehrlich's reagent solution, the sample was incubated at 60°C for 25 min. After cooling with water for 2-3 min, the sample solution was diluted with 6.6 mL isopropanol. Hyp concentration in the sample

solution was determined using a spectrophotometer at 560 nm (Bergman and Loxley, 1963). Total collagen content was calculated by multiplication of Hyp content by 13.8 (Sato *et al.*, 1989), because muscle collagen in 22 fish species contain 7.26% Hyp in average.

Satistical analysis: Data were statistically analyzed by one-way analysis of variance (ANOVA) in Experiment 1 and three-way ANOVA in Experiment 2. Multiple regression analysis was applied for Experiment 2. Significant difference denoted $p < 0.05$. Values are presented as means \pm S.E.M. Statistical analysis was made using a commercially available package, Stat View (Version 5, SAS Institute, Cary, U.S.A., 1998).

RESULTS AND DISCUSSION

Table 2 demonstrates the performance of tiger puffer fish given diets with or without dietary Hyp at two feeding periods. All the performances including body weight gain and feed efficiency were comparable between the control and Hyp groups over the experimental period. The results indicated that dietary Hyp at 1% level was not toxic for tiger puffer fish.

Figure 1 shows collagen contents of tiger puffer fish in the different parts of the fillet. Collagen contents were significantly higher in the caudal than in the cranial and middle parts. It was demonstrated in human that strenuous exercise stimulated muscle growth and muscle protein synthesis with indeed collagen synthesis in muscle, tendon and bone (Rennie, 2005). Accordingly, the present results suggested that tiger puffer use their caudal fin to swim and then collagen synthesis is enhanced in the caudal part of the fillet.

Figure 2 demonstrates the change in collagen contents of the fillet with or without dietary Hyp in tiger puffer. The collagen content was significantly ($p < 0.01$) higher in the Hyp group than in the control. The collagen content significantly ($p < 0.0001$) decreased when days went on. No significant sexual difference was detected. The following equation was obtained by multiple regression analysis.

$$\begin{aligned} \text{Hyp content in the fillet (mg/g fillet)} &= 6.588 \\ &+ 0.230 (\text{SE } 0.212) \text{ Hyp} - 0.017 \\ &+ 0.004 (\text{SE } 0.564, \text{df } 53) \text{ Day} \end{aligned}$$

where, Hyp equals 0 for the control and 1 for the Hyp groups. Ito *et al.* (2000) reported that collagen content in the muscle of Matsukawa (*Verasper moseri* Jordan et Gilbert) increased with growth. The results obtained here were reverse, since collagen contents were reduced with

Table 2: Effects of dietary hydroxyproline on the performance of tiger puffer

Parameters	Control	Hydroxyproline
First trial (37 days)		
Total initial body weight (g)	26795.0	26880.0
Number of fish	45.0	45.0
Average initial body weight (g)	595.0	597.0
Total final body weight (g)	29931.0	30409.0
Average final body weight (g)	665.0	676.0
Total body weight gain (g)	3136.0	3529.0
Total feed intake (g)	9894.0	10401.0
Feed efficiency (%)	31.7	33.9
Second trial (44 days)		
Total initial body weight (g)	21065.0	22675.0
Number of fish	32.0	33.0
Average initial body weight (g)	658.0	687.0
Total final body weight (g)	24658.0	25867.0
Average final body weight (g)	771.0	784.0
Total body weight gain (g)	3593.0	3192.0
Total feed intake (g)	9422.0	9422.0
Feed efficiency (%)	38.1	33.9

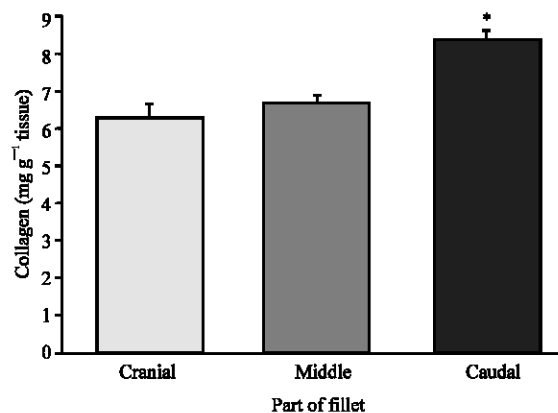


Fig. 1: Collagen contents in the cranial, middle and caudal parts of the fillet in tiger puffer. Results are expressed as means with S.E.M. The number of fish used in was 10. *Significantly different from others at $p < 0.05$

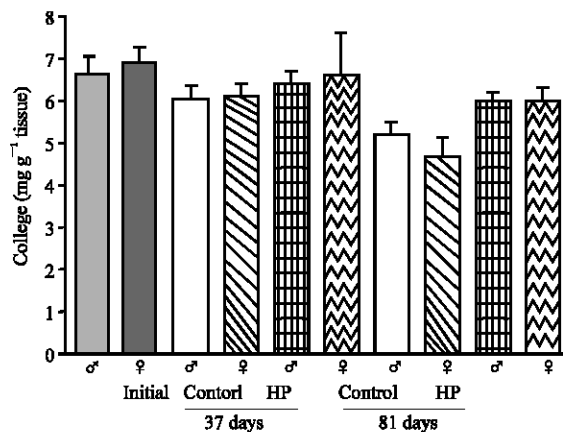


Fig. 2: Changes in collagen contents in the middle part of the fillet in tiger puffer after Hyp treatment. Results are expressed as means with S.E.M. The number of fish used in was 2-10

growth in the control group. On the other hand, skeletal muscle collagen concentration was dependent on growth rate in farmed Atlantic salmon, being lower in high growth rate than in low growth rate (Li *et al.*, 2007). Collagen contents are greatly associated with the growth of fish, but it may be different roles dependent upon species.

The reduction in collagen contents in the control group may imply that the production of Hyp is reduced due to the decreased activity of prolyl hydroxylase. However, Hyp was directly applied here and clearly demonstrated that dietary Hyp prevented the reduction in collagen contents with growth in tiger puffer. It should be determined in the future whether the prolyl hydroxylase activity changes with growth.

CONCLUSION

In conclusion, dietary Hyp was available for keeping the collagen contents of tiger puffer fish without any toxicity.

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