Replacing Moringa leaf (*Moringa oleifera*) Partially by Protein Replacement in Soybean Meal of Fancy Carp (*Cyprinus carpio*)

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Abstract

Moringa oleifera Lam (Moringaceae) is a highly valued plant, distributed in many countries of the tropics and subtropics. The leaves are the protein source with an adequate profile of amino acids. The present study was undertaken in order to determine the effect of a dietary of moringa leaves on digestibility and growth performance of fancy carp. Fish were fed with diets containing isonitrogenouse and isoenergetic formulated by 20 and 50 g kg^{-1} of moringa leaves to replace protein in soybean. Fish were distributed in 500-liter tanks with flow-through water. Every fish was weighed and after the terminal experiment, all groups' livers and distal intestines were sampled. All fish grew normally ($p>0.05$) but fish fed with protein-replacing moringa leaves at 50 g kg^{-1} were noted to exhibit slightly poor growth performance and feed utilization. The study indicated that the tested moringa leaf diet contains ingredients that could be used for fancy carp diets with possibly up to 20 g kg^{-1} soybean protein replacement without negative effect on growth and digestibility.

Key words: fancy carp, moringa, growth, digestibility

Introduction

Since farming aquatic animals in Thailand was broadly adopted and improved, it has caused a problem of high-priced feed as well as insufficient nutrition. A significant proportion of fish meal possesses a broad range of amino acids, and hence high-priced. There has been an attempt to replace fish meal with soybean meal which possesses good quality of essential amino acids (EAA). As a result, soybean meal, both imported and locally made, is utilized with hope to help decreasing the cost, but as it turns out, still somewhat expensive. Herdsmen, no matter of undersized or oversized farms, are looking for a new cheaper raw material to decrease the cost though it might not be as preferable as fish meal or soybean meal. This new material should be able to be produced locally, inexpensive and provide high nutrition. Certain plant materials offer the most promising alternative aqua feed ingredients and in fact locally produced materials have already been used in Thailand.

Thailand is an agricultural country with a lot of plants in nature. The moringa (*Moringa oleifera*) is a fast-growing plant widely available in tropics and subtropics with several economic-important industrial and medicinal uses, and is a native food in Southeast Asia. The moringa leaves have been known to be effective for certain medicinal purposes. Based on a number of reports of the nutritional or medicinal values of a natural product, there are a staggering number of purveyors of “healthful” food who are now promoting moringa as a panacea. In addition, the products from the moringa hold considerable potential for becoming animal and fish feed ingredients because of their high nutritional quality. However, there is no information regarding the utilization

of moringa in fish feed. An alternative is to use moringa leaves to replace soybean meal since they provide 260.0 g kg^{-1} protein (Makkar and Becker, 1996). EAA composition in moringa leaves is sulfur amino acid such as methionine, cystine, tryptophan (Makkar and Becker, 1996), as required by EAA for aquatic animals (WHO, 1985). These amino acids, however, are very low in soybean meal. It is found that Methionine acid allows protein synthesis as well as being a reactant for homocysteine cystine carnitine creatine and choline. To some degree, most plant proteins contain some anti nutritional factors that vary with the processing, type and quality of the plant protein. Formulators should keep these concerns in mind so as to find the correct quality and type of plant protein for their purposes. Proteins of animal origin are generally more digestible than those of plant origin. Some technological treatments applied to plant proteins bring about a marked improvement in Apparent Digestibility Coefficient (ADC) by destroying antinutritional factors (Guillaume *et al.*, 2001). This study offers an alternative of utilizing moringa leaves as a protein source to replace soybean meal in carp diet and add value to raw materials. The aims of this study were to investigate protease activity and the in vitro digestibility, and the effect of supplemented moringa leaves in fancy carp on growth performance in fancy carp.

Materials and Methods

Fish, diets and feeding protocol

Mixed sexes of fancy carp were maintained on a commercial diet of gold fish with 300.0 g kg^{-1} protein for four weeks prior to feeding the three experimental diets. Each treatment with an initial mean wet weight of 18.08 ± 0.26 g per fish, were

randomly distributed to each of 500-liter tanks with flow-through water. The diets were substituted by moringa leaves, and protein in soybean is replaced as follows:

1. Control diet; without moringa leaves
2. Diet substituted by moringa leaves with 200 g kg^{-1} replacement of soybean protein
3. Diet substituted by moringa leaves with 500 g kg^{-1} replacement of soybean protein

Three isonitrogenouse and isoenergetic diets were formulated to contain approximately 350 g kg^{-1} protein and 13.12 KJ g^{-1} and to meet the known nutrient requirement of carp. All diets were supplemented with L-Methionine, so the balance of this amino acid in the diets was similar in all case.

The daily feed was done by hand-fed method to apparent satiation twice a day (09.00 and 16.00), 6 days a week for six weeks. Total feed was recorded weekly. Fish from each tank was weighed to measure growth at the end of the experiment at six weeks and growth performance calculated.

Proximate analysis

Proximate analysis of diets were analyzed as follows: dry matter after drying in an oven at 105 ºC until constant weight; ash content by incineration in a muffle furnace at 600 ºC for 6 h; crude protein (N x 6.25) by Kjeldahl method after acid digestion; lipid by petroleum erher extraction in a Soxlet apparatus by AOAC (1990) method. The amino acids of fish carcass and diets were analysed with an ultra fast liquid chromatography (UFLC), Shimadzu system (Shimadzu, Kyoto, Japan). (as shown in **Table 1**).

Crude enzyme preparations

The upper, lower and whole intestine were homogenized (1:2 w/v) with 50 mM Tris – HCl buffer at pH 7.5 (Fisher Scientific, USA) in an ice water bath, using a tissue

96 homogenizer. The preparation was centrifuged at 10,000 x g for 15 min at 4^{o}C. The
97 floating lipid fraction was removed and the aqueous supernatant was recovered and kept
98 at -20^{o}C until analysis completed (Gimenez *et al.*, 1999).

99 **Protease activity**

100 Protease activity was monitored in triplicate by measuring the increase in
101 cleavage of short chain polypeptide (Bezerra *et al.*, 2005). The total protease activity
102 was determined by using 1 g L^{-1} (w/v) azocasein (Sigma- Aldrich, USA). The substrate
103 (500 µl) was incubated with crude extract (20 µl) and buffer solution (200 µl) for 60
104 min at 30^{o}C. Then, 500 µl of 200 g L^{-1} (w/v) trichloroacetic acid (Sigma- Aldrich, USA)
105 was added to stop the reaction. After 15 min, centrifugation was carried out at 10,000 g
106 for 10 min. The supernatant (1.0 ml) was added to 1 M NaOH (1.5 ml; Qrec, New
107 Zealand) and the absorbance was measured at 440 nm against a blank similarly prepared
108 but without the crude extract sample. The protease specific activity was expressed as
109 unit of change in absorbance per min per mg protein of the enzyme extract (ΔAbs min^{-1}
110 mg $protein^{-1}$).

111 **Protein concentration**

112 Protein concentration was determined by using bovine serum albumin (Sigma-
113 Aldrich, USA) as a standard (Lowry *et al.*, 1951).

114 ***In vitro* protein digestibility**

115 Three experimental diets were measured in triplicate by the cleavage peptides
116 (Gimenez *et al*., 1999). Twenty milligrams of each diet was added with 12 ml of 50mM
117 phosphate buffer (pH 7.5) and incubated overnight at 30^{o}C. *In vitro* digestion was
118 started by adding 500 µl of the crude enzyme extract and incubated for 24 h at 30^{o}C.
119 After digestion, 1 ml of each digested mixture was determined of cleavage peptides by

measuring the absorption at 750 nm and converted in to mg protein using a standard curve (Lowry *et al*., 1951).

Pepsin digestibility test

To assess the quality of experimental diets, digestible crude protein was determined by pepsin digestibility test (AOAC, 1990), using pepsin (Sigma- Aldrich, USA).

Statistical analysis

Mean value and standard deviation (S.D.) were calculated from the results. One way analysis of variance (ANOVA) was applied for comparison of the mean values, $P < 0.05$ was established as significant.

Results

Total protease activity

Among these three parts of intestinal tract in initial fish, total protease activity was significantly ($p<0.05$) higher in upper tract (1.77 ± 0.06 U mg protein^{-1} min^{-1}), followed by whole and lower tract (1.48 ± 0.15 and 1.15 ± 0.15 U mg protein^{-1} min^{-1}) (**Table 2**).

Digestive protease activity of fish after the end of experiment is displayed in **Figure 1,** which showed higher activity of upper tract followed by whole and lower tract. Total protease activity of upper tract was significantly ($p<0.05$) higher in fish fed with diet substituted by moringa leaves to replace protein in soybean at 50 g kg^{-1} (1.50 ± 0.11 U mg protein^{-1}min $^{-1}$).

***In vitro* protein digestibility and Pepsin digestibility study**

The study of *in vitro* protein digestibility by crude enzyme extract from intestine of carp and pepsin digestibility on three experimental diets were shown in **Table 3**. The results showed no difference ($p>0.05$) in *in vitro* protein digestibility assay and pepsin digestibility study of all experimental diets.

Growth performance and feed utilization

Growth parameters of fancy carp were shown in **Table 4**. The results indicated that there were no significant differences in weight gain (WG) and average daily gain (ADG) ($p>0.05$). Nevertheless, the higher WG and ADG were found in fish fed with the diets supplemented with moringa leaves to replace protein in soybean at 200 g kg^{-1} and control diet. WG ranged from 22.93 to 16.70 grm, while ADG ranged from 0.81 to 0.56 g/fish/day. In terms of feed utilization, the data showed that there were also no significant differences in feed conversion ratio (FCR) and protein efficiency ratio (PER) ($p>0.05$) among all groups. FCR ranged from 0.72 to 0.43 and PER ranged from 0.59 to 0.41. All fish grew normally, and no specific signs of disease were observed. No mortality occurred throughout the experiment.

Amino acid composition in fish flesh

The amino acid composition of fish at the end of experiment is given in **Table 5**. With higher inclusion of moringa leaf meal in the diets, the amino acid content remained constant in all experimental groups. All groups showed no statistical difference ($p>0.05$) in muscle tissue amino acid content when compared to fish fed with the reference diet.

Discussion

Nowadays, plant sources have been used to replace the protein in fishmeal and soybean meal, either partially or totally. Practical fish feed has been an area of focus in

168 aquaculture nutrition research recently (Gomes *et al.*, 1995; Hossain *et al.*, 2001; Ogunji
169 and Wirth, 2001; Siddhuraju and Becker, 2003). Moringa leaf has been widely studied
170 as an alternative protein source in fish diet and seems to be a promising protein source.
171 Moringa leaf can partially replace conventional diets without any depression in growth
172 performance of Nile tilapia (*Oreochromis niloticus* L.) (Richter *et al.*, 2003; Afuang
173 *et al.*, 2003). In the present study, total protease activity from intestine of different parts
174 of carp was investigated, as presented in Table 1. The activity of protease in upper tract
175 was higher than those in the other parts ($p<0.05$) and higher in fish fed with diet
176 substituted by moringa leaves that replace protein in soybean at 500 g kg^{-1} (1.50 ± 0.11
177 U mg $protein^{-1}min^{-1}$). The results agreed with a report which also found that the
178 protease activity of three carps were higher in rohu (1.219 ± 0.059 U mg $protein^{-1}$ min^{-1})
179 followed by silver carp (1.084 ± 0.061 U mg $protein^{-1}$ min^{-1}), and catla (0.193 ± 0.006 U
180 mg $protein^{-1}min^{-1}$) (Kumar *et al.*, 2007). The digestive protease activity was different in
181 other species (Chakrabarti and Sharma, 2005), which may greatly depend upon their
182 digestive capability, feeding habits and environment. Regarding food quality, a
183 complete diet with essential amino acids, fatty acids and vitamins is required for high
184 growth rate in fish including available proteins in their diet by increasing consumption
185 rate and enzyme production (Hofer, 1982). From the results, the activities of protease,
186 which are essential for the utilization of protein from feed, contribute to high growth
187 rate in fish.

188 *In vitro* methods of evaluating protein digestibility are important as they are
189 rapid, less expensive, and allow close observation of the dynamics of the breakdown of
190 protein by using only small amounts of raw materials (Grabner, 1985). Thus,
191 characterization of digestive proteases is essential along with the quantitative

192 estimations for the better understanding of digestive capability of the cultured species
193 and for assessing protein ingredients in feed formulations (Moyano *et al.*, 1996). None
194 of the diets adversely affected the *in vitro* protein digestibility and pepsin digestibility
195 compared to the control diet without moringa leaves, but the diets supplemented with
196 moringa leaves seemed to offer lower digestibility compared to the control diet. Plant
197 ingredients (bean meal, groundnut oilcake and sunflower oilcake) can efficiently
198 substitute fishmeal at 250 g kg^{-1} in African catfish diets, and there were no significant
199 differences in protein ADCs (88–90) with increased levels of dietary plant-based protein
200 in diets (Nyina *et al.*, 2010). The ADC in protein of plant leaf ingredients was
201 determined and barnyard grass and dried maize leaves were found not only to offer poor
202 digest but also yield negative impact on the digestibility of the reference diet. On the
203 contrary, fresh maize leaves were well digested for grass carp; with percentage 60.9,
204 70.5 and 84.7, ADC respectively in protein compared to 94.1 in control diet. This
205 indicated that dry plant materials seem to be low digestible and could even inhibit fish
206 utilization of other nutrients contained in diet (Dongmeza *et al.*, 2010).

207 In this study, neither growth nor feed conversion efficiency were affected
208 significantly by dietary treatment for all treatments diets. Growth parameters, namely
209 weight gain, feed conversion ratio and survival were similar ($p>0.05$). This agrees with
210 the study that shows no effects of dietary supplement of methanol-extracted leaf meal
211 containing 11, 22 and 33 g kg^{-1} found on the growth of Nile tilapia (*Oreochromis*
212 *niloticus* L.) (Afuang *et al.*, 2003). Tilapia fed with raw moringa leaf meal revealed that
213 10% of replacement of fishmeal-based dietary protein did not cause any adverse effect
214 on growth performance (Richter *et al.*, 2003). Most published research on the use of
215 plant protein as a substitute of SBM in fish feeds has focused on the inclusion of palm

kernel meal (Ng and Chen, 2002), cotton seed meal (Yue and Zhou, 2008) and Faba beans (Azaza *et al*., 2009) with the goal to increase inclusion of sustainable plant-based diet for fish and all results show that dietary protein source from plant origins did not affect growth and survival of fish. The amino acid compositions in all experimental diets of this present study were generally similar, but substantial differences existed in methionine content. This was because lower level of methionine in diets supplemented with moringa leaf (diets 2 and 3) were more limited in this amino acid than in the control diet. In the present study, low dietary levels of methionine have been shown to suppress growth and feed utilization. Methionine content of the experimental diets supplemented with moringa leaf gradually decreased at 14.96 and 37.79 g kg^{-1} compared to the control diet. Methionine is generally limiting amino acid and methionine deficiency, frequently causing reduced growth (Jackson *et al*., 1982; Gaber, 2006).

This indicates that methionine deficiency may be one of the reasons responsible for the lower growth performance and poorer diet utilization of the groups fed the diets supplemented with moringa leaves. Similar to a study report of low dietary levels of methionine, growth of juvenile hybrid striped bass and increased mortality has been shown (Keembiyehetty and Gatlin, 1993). However, essential amino acid (EAA) composition in moringa leaves is sulfur amino acid such as methionine, cystine and tryptophan[1] which should be used as supplementation only (Goff and Gatlin, 2004).

Conclusions

In conclusion, this study indicated that moringa leaves used as a plant protein sources for replacing soybean meal could support the growth, adversely affected and

digestibility of fancy carp. Thus, moringa leaf could replace possibly up to 200 g kg^{-1} of protein in soybean and become an alternative plant protein source in fish diet to lower the production cost of fish diets and add value to a plant origin.

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360 **Table 1** Ingredients and chemical composition of experimental diets

Ingredient (g Kg^{-1})	Protein replacement in soybean meal by moringa leaves (g kg^{-1})		
	0	200	500
Fish meal	320	320	320
Soybean meal	230	184	115
Moringa leaves	0	88	220
Wheat Flour	120	120	120
Cellulose	160	117.6	54.1
Fish oil	35	35	35
Soybean oil	35	35	35
Guar gum	10	10	10
Dicalcuim phosphate	20	20	20
Premix	70	70	70
L-Methionine	0	4	9
Total	1000	1000	1000
Nutrient composition by analysis (g kg^{-1} dry weight on basis)			
Protein	35.63 ± 1.95	34.67 ± 0.03	35.12 ± 1.07
Fat	9.39 ± 0.01	9.42 ± 0.04	9.37 ± 0.14
Fiber	2.11 ± 0.78	2.10 ± 0.17	2.18 ± 0.25
Dry matter	67. 60 ± 0.23	68.04 ± 0.28	67.68 ± 0.25
Ash	11.79 ± 0.09	11.52 ± 0.05	11.93 ± 0.45
Amino acid composition (g kg^{-1}dry weight on basis)			
Histidine	2.50 ± 0.01	2.58 ± 0.02	2.74 ± 0.01
Arginine	20.38 ± 0.01	18.50 ± 0.01	15.86 ± 0.10
Asparagine	3.28 ± 0.03	3.83 ± 0.02	5.88 ± 0.03
Glutamic acid	3.19 ± 0.01	3.44 ± 0.04	4.45 ± 0.04
Alanine	4.36 ± 0.09	4.08 ± 0.01	4.03 ± 0.01
Proline	2.52± 0.02	3.30 ± 0.04	3.87 ± 0.05
Methionine	1.27 ± 0.03	1.08 ± 0.01	0.79 ±0.01
Valine	3.33 ± 0.03	3.30 ± 0.03	3.52 ± 0.04
Trytophane	n.d.	n.d.	n.d.
Leucine	9.33 ± 0.06	9.30 ± 0.04	9.53 ± 0.04
Lysine	6.11 ± 0.02	9.08 ± 0.01	10.53 ± 0.02
Cysteine	10.06 ± 0.10	8.62 ± 0.03	8.86 ± 0.07

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362

363 **Table 2** Protein content and total protease activities of digestive tract of initial fish

Digestive tract	Protein content ($mg\ ml^{-1}$)	Total protease activities ($U\ mg\ protein^{-1} min^{-1}$)
Whole tract	1.17 ± 0.04	1.48 ± 0.15^{a}
Upper tract	1.15 ± 0.03	1.77 ± 0.06^{b}
Lower tract	1.13 ± 0.03	1.17 ± 0.15^{c}
P – value	0.0749	0.0059

364 Values were means of triplicate analyses. Mean value within the columns with different
365 letters were significantly different at $p<0.05$.

366

367 **Table 3** *In vitro* protein and pepsin digestibility study in experimental diets

Digestibility (%)	Protein replacement in soybean meal by moringa leaves ($g\ kg^{-1}$)			P - value
	0	200	500	
In vitro protein digestibility	65.65 ± 2.92	66.93 ± 2.54	64.63 ± 5.41	0.6571
Pepsin digestibility	76.18 ± 1.35	75.02 ± 4.34	74.74 ± 0.97	0.8958

368 Values were means of triplicate analyses. Mean value within the row with different
369 letters were significantly different at $p<0.05$.

370

Table 4 Growth performance and feed utilization of fancy carp fed with experimental diets supplemented with moringa leaves at terminal period

Parameters	Protein replacement in soybean meal by moringa leaves (g kg^{-1})			P - value
	0	200	500	
WG	22.93 ± 3.71	24.16 ± 3.16	16.70 ± 0.73	0.144
ADG	0.77 ± 0.12	0.81 ± 0.11	0.56 ± 0.73	0.137
SR	100	100	100	-
FCR	0.72 ± 0.40	0.62 ± 0.23	0.43 ± 0.01	0.583
PER	0.54 ± 0.12	0.59 ± 0.17	0.41 ± 0.01	0.403

Mean with the different letters in same row are significantly different at $p < 0.05$.

Note: WG: Weight gain (g) ADG: Average daily gain (g/fish/day)

FCR: Feed conversion ratio (FCR) SR: Survival (%)

Table 5 Amino acid composition (g kg^{-1} dry weight on basis) in muscle of fancy carp at terminal of the experiment

Amino acid	Protein replacement in soybean meal by moringa leaves (g kg^{-1})			P-value
	0	200	500	
Histidine	8.82 ± 0.08	8.58 ± 0.08	9.27 ± 1.55	0.200
Arginine	27.94 ± 4.35	25.28 ± 5.71	26.11 ± 5.15	0.924
Asparagine	3.08 ± 0.59	2.72 ± 0.40	3.04 ± 0.25	0.209
Glutamic acid	2.81 ± 0.34	2.82 ± 0.58	3.17 ± 1.85	0.908
Alanine	5.28 ± 0.89	4.58 ± 0.06	4.43 ± 0.86	0.069
Proline	3.00 ± 0.53	2.71 ± 0.30	2.80 ± 0.51	0.465
Methionine	3.21 ± 1.81	2.84 ± 0.59	2.94 ± 0.22	0.136
Valine	2.97 ± 0.86	2.81 ± 0.35	2.12 ± 0.57	0.180
Trytophane	n.d.	n.d.	n.d.	-
Leucine	4.43 ± 1.94	3.85 ± 1.28	3.14 ± 0.61	0.377
Lysine	1.31 ± 0.31	0.97 ± 0.44	0.93 ± 0.35	0.393
Cysteine	2.69 ± 0.44	0.45 ± 0.32	0.99 ± 0.38	0.003

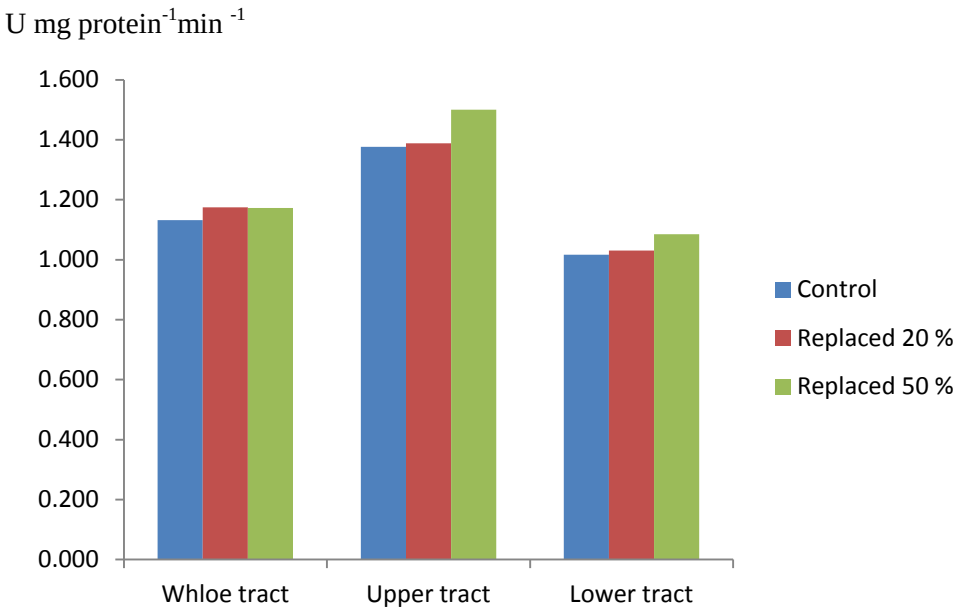

Figure 1 Total protease activities of digestive tract at terminal of experiment