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# Effects of Dietary Soy Protein Concentrate on Growth, Digestive Enzymes Activities and Target of Rapamycin Signaling Pathway Regulation in Juvenile Soft-Shelled Turtle, *Pelodiscus sinensis*

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## Abstract

**Soft-shelled turtle, *Pelodiscus sinensis* is important aquatic species in China, and searching for alternatives protein resources to fish meal (FM)-based feeds in feed has become urgent and important for its sustainability development. The present study was conducted to assess the effects of dietary soy protein concentrate (SPC) on growth, digestive enzymes and target of rapamycin (TOR) signaling pathway of juvenile *P. sinensis* (4.56 ± 0.09 g). SPC was applied to replace FM protein at 0%, 15%, 30% and 60% (designated as T0, T15, T30 and T60, respectively), and each diet was fed to triplicate groups. The results showed that there was no significant difference in growth performance and feed utilization except of the turtles fed with T60 diet, of which showed poorer daily weight gain and feed conversion rate. The pepsin/trypsin and $Na^+$-$K^+$ ATP-ase activities decreased dramatically when SPC level increased, and lipase activities in liver and intestinal tract also showed decline tendency. However, amylase activities were unaffected. No significant differences were observed in TOR, S6K1 and 4E-BP1 genes mRNA expression level of TOR signaling pathway among the treatments. However, the relative phosphorylated level of these proteins decreased significantly when SPC level increased. The present study indicated that high SPC substitution level would suppress digestive enzymes and TOR signaling pathway proteins phosphorylated level and eventually result in growth reduction of *P. sinensis*.**

## Keywords

**Soft-Shelled Turtle *P. sinensis*, Soy Protein Concentrate, Growth Performance, Digestive Enzymes,**

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## 1. Introduction

Soft-shelled turtle, *Pelodiscus sinensis* is an important aquatic species in Asian countries including China, Japan, Korea, Thailand, because of its nutritional and pharmic value for human being [1]. Traditionally, soft-shelled turtle is often fed a diet high in fish meal (FM) (often about 50%) [2]. However, due to increasing demand, limited supply, and the dramatic increase in FM prices, feed proteins and their inclusion in soft-shelled turtles diets has a significant impact on overall feed and culture costs. Soy protein concentrate (SPC), is considered as one of the most promising plant protein sources to replace FM in Aquatic feeds because it has high protein level (65% in average), relatively low anti-nutritional factors and is widely available [3]. Therefore, evaluating the potential of replacing FM protein by SCP in soft-shelled turtle feed would contribute to its sustainability development.

Digestion and absorption of nutrients directly depend on the activities of digestive-absorptive enzymes in the alimentary tract. Hence, measuring the activity of digestive enzymes provides insight into feed utilization and nutrient digestibility [4]. In the other aspect, molecular tools have been used in aquatic animals nutrition researches in recent years, revealing new and more specific aspects of its regulation. One pathway that has been the focus by many studies is the target of rapamycin (TOR) pathway [5]. This pathway has been described as an integration point of several inputs that modulate protein metabolism and growth, processes that involves signaling from nutrients such as amino acids, growth factors and energy status, and TOR protein was the central in TOR signaling pathway [5] [6]. In mammalian cells, TOR promotes cap-dependent protein synthesis through the phosphorylation and inactivation of its downstream effectors eukaryotic translation initiation factor 4E binding protein (4E-BP1) and S6 kinase protein (S6K1) [7] [8]. Studies on rainbow trout, *Oncorhynchus mykiss* [9]-[11], Jian carp, *Cyprinus carpio* var. Jian [12]-[14], cobia, *Rachycentron canadum* [15] and Chinese shrimp, *Fenneropenaeus chinensis* [16] have indicated that nutrition status could possibly regulate the TOR signaling pathway in aquatic animals as in mammals. Recently, the whole genome shotgun sequence of soft-shelled turtle has been projected by Wang *et al*. [17], and the TOR, S6K1 and 4E-BP1 gene sequences were reported, meanwhile, the potential of dietary FM protein replacing by SPC in soft-shelled turtle diet has been evaluated in our laboratory [18]. In view of the foregoing, the present research was designed to study the effects of dietary SPC on digestive enzyme activities and TOR signaling pathway regulation in juvenile soft-shelled turtle, for which could provide further theoretical evidence for SPC application in its commercial feeds.

## 2. Materials and Methods

### 2.1. Experimental Diets

The ingredient and proximate composition of the experimental diets are showed in **Table 1**. Four isonitrogenous and isoenergetic diets designated as T0, T15, T30 and T60 were formulated to contain graded levels of SPC, as a substitute for FM protein, at replacement levels of 0%, 15%, 30% and 60%. In preparing the experimental diets, all dry ingredients were finely ground and passed through a 100-μm mesh, weighed, mixed manually for 5 min and then transferred to a food mixer for another 20 min of mixing while corn oil was added slowly during the process. Subsequently, distilled water was added to achieve a proper consistency and the mixture homogenized and extruded through a food processor and a die size of 2.0-mm diameter. The noodle-like strands were then broken into pellets, sieved to remove pellets above 2 mm and then stored in a freezer at −20˚C until used. Representative triplicate samples of each experimental diet were taken for proximate analysis (**Table 1**).

### 2.2. Experimental Design

Juveniles soft-shelled turtle were obtained from Hangzhou Xiaoshan Tianfu Bio-Technique Co. Ltd. (Hangzhou, China) three days after hatching. Prior to the initiation of the experiment, the turtles were randomly stocked in their respective experimental container and acclimated to the laboratory condition for 14 days. After acclimation, 240 soft-shelled turtle (initial body weight 4.56 ± 0.09 g) juveniles were randomly distributed into twelve 350 L

**Table 1.** Ingredient formulation and proximate composition of the experimental diets (% dry matter basis)[1].

| Ingredients (%) | Diets | | | |
|---|---|---|---|---|
| | T0 | T15 | T30 | T60 |
| Fish meal (FM) | 50 | 42.5 | 35 | 20 |
| Soybean protein concentrate (SPC) | 0 | 7.7 | 15.4 | 30.8 |
| Fermented soybean meal | 10 | 10 | 10 | 10 |
| Brewers' yeast | 5 | 5 | 5 | 5 |
| Blood meal | 2 | 2 | 2 | 2 |
| Corn oil | 1.0 | 1.3 | 1.6 | 2.2 |
| *α*-starch | 23 | 23 | 23 | 23 |
| Zeolite | 2.3 | 1.8 | 1.3 | 0.3 |
| Vitamin mixture[2] | 2 | 2 | 2 | 2 |
| Mineral mixture[3] | 2 | 2 | 2 | 2 |
| Betaine | 0.2 | 0.2 | 0.2 | 0.2 |
| $NaH_2PO_4$ | 2.5 | 2.5 | 2.5 | 2.5 |
| Total | 100 | 100 | 100 | 100 |
| **Nutrient composition** | | | | |
| Crude protein | 41.9 | 42.5 | 41.8 | 42.8 |
| Met | 0.99 | 0.88 | 0.80 | 0.56 |
| Arg | 2.73 | 2.73 | 2.77 | 2.85 |
| Lys | 2.60 | 2.57 | 2.56 | 2.50 |
| His | 0.84 | 0.88 | 0.90 | 0.92 |
| Leu | 3.02 | 3.04 | 3.10 | 3.18 |
| Ile | 1.66 | 1.69 | 1.74 | 1.76 |
| Phe | 1.39 | 1.45 | 1.55 | 1.68 |
| Val | 1.86 | 1.90 | 1.94 | 1.98 |
| Thr | 1.63 | 1.61 | 1.63 | 1.64 |
| Crude lipid | 5.8 | 5.7 | 5.9 | 5.9 |
| Crude ash | 10.8 | 11.1 | 10.9 | 11.2 |
| Gross energy ($kJ \cdot g^{-1}$ diet) | 13.11 | 13.08 | 13.04 | 13.12 |

[1]Values for the proximate composition of diets are means of triplicate analyses; [2]Vitamin premix ($mg \cdot kg^{-1}$ diet): retinyl acetate, 40; cholecalciferol, 0.1; a-tocopheryl acetate, 80; menadione, 15; niacin, 168; riboflavin, 22; pyridoxine HCl, 40; thiamin mononitrate, 45; D-Ca pantothenate, 102, ascorbic acid, 500, biotin, 0.4; folic acid, 10; vitamin $B_{12}$, 0.04; and inositol, 450; [3]Mineral premix ($g \cdot kg^{-1}$ of premix): $Na_2SiO_3$, 0.4; $CaCO_3$, 350; $NaH_2PO_4 \cdot H_2O$, 200; $KH_2PO_4$, 200; $MgSO_4 \cdot 7H_2O$, 10; $MnSO_4 \cdot H_2O$, 2; $CuCl_2 \cdot 2H_2O$, 1; $ZnSO_4 \cdot 7H_2O$, 2; $FeSO_4 \cdot 7H_2O$, 2; NaCl, 12; KI, 0.1; $CoCl_2 \cdot 6H_2O$, 0.1; $Na_2MoO_4 \cdot 2H_2O$, 0.5; $AlCl_3 \cdot 6H_2O$, 1 and KF, 1.

plastic containers with 20 turtles per container. Each of the experimental diet was fed to three replicate groups of turtles. During the experimental period, the mean water temperature, pH and $NH_3$–N were 29.2 ± 1.0˚C, 7.40 - 7.55 and 0.01 ± 0.005 $mg \cdot L^{-1}$, respectively. Dissolved oxygen concentrations were maintained above 5.0 $mg \cdot L^{-1}$ throughout the feeding trial with the use of air stones for continuous aeration. The turtles were fed 3% of their body weight twice daily at 8:00 and 17:00 for eight weeks. The containers were cleaned twice per week.

## 2.3. Dietary Nutrient Composition Analysis

Proximate analysis of diets samples were performed according to methods of the Association of Official Ana-

lytical Chemists (AOAC, 1995) [19]. The essential amino acids (EAAs) content of experimental diets were analysed by automatic amino acid analyzer (Hitachi 835-50, Tokyo, Japan) equipped with a column (Hitachi custom ion exchange resin No. 2619). Representative triplicate samples of each experimental diet were taken for proximate analysis (**Table 1**).

## 2.4. Digestive Enzymes Analysis

At the termination of the feeding trial, the turtles were fasted for 24 h and then harvested and weighed individually. The liver, stomach and intestinal tract samples were obtained from five turtles per containers. Each section was rinsed with ice-cold distilled water to remove the eventual remaining gut contents. All digestive organ samples were placed in tubes and immediately frozen at −70˚C until enzyme assay. Activities of the pepsin/protease, lipase and amylase in the homogenate were measured as the change in absorbance using a spectrophotometer (UV-2802S, Unico, Shanghai, China) using assay kits (Jiancheng Biotech. Co., Nanjing, China) according to the manufacturer's instructions.

## 2.5. Real-Time PCR Analysis

Total RNA was extracted from experimental soft-shelled turtles (five turtles per containers) using the TRIzol Reagent (Invitrogen, USA) and RNase-Free DNase I (Qiagen, Germany) from homogenization of liver following manufacturer recommendations. RNA was quantified using Ul-trospec 2000 UV/Visible Spectrophotometer System (Amersham Pharmacia Biotech, Buckinghamshire, UK). Assessment of RNA quality was performed by electrophoresis on a 1.2% formaldehyde agarose gel containing ethidium bromide. Only the RNA samples with 260/280 ratios of 1.8 - 2.0 indicating a satisfactory purity were used for further investigation. 1 ul of total RNA (500 ng of RNA) was subjected to reverse transcribed to cDNA by PrimerScript® 1st Strand cDNA Synthesis Kit using Oligo-dT primer (Takara, Japan) in 10 µl volume according to reagent's instructions.

Real-time PCRs were performed for TOR (NCBI Reference Sequence: XM_006127040.1), S6K1 (NCBI Reference Sequence: XM_006138647.1) and 4E-BP1 (NCBI Reference Sequence: XM_006134043.1) according to standard protocols with the specific primers which designed with Primer Premier software (Premier Biosoft International, Palo Alto, CA, USA) based on published sequences of soft-shelled turtle from NCBI (**Table 2**). And 18S ribosomal RNA of soft-shelled turtle was used as housekeeping gene. Real-time PCR was performed on the BIO-RAD IQTM5 Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) with Power SYBR® Master Mix (Invitrogen). The total volume of the PCR reaction was 25 µL, containing 0.5 µL of each primer (10 µM), 1.0 µL of cDNA, 12.5 µL of Power SYBR® Master Mix and 10.5 µL of sterile double distilled water ($ddH_2O$). Following amplication conditions were used in the present trial: 1 min at 95˚C, 40 cycles of 10 s at 95˚C (denaturation), and 25 s at 64˚C (annealing + extension). Melting curve analysis was performed by running a gradient from 95˚C to 55˚C (gradient 0.3˚C) to confirm the presence of single PCR products. Standard curves were made with five different dilutions (in triplicate) of the cDNA samples and amplification efficiency was analyzed according to the following equation $E = 10^{(-1/Slope)} - 1$. The expression levels of the analytical genes were calculated by $2^{-\Delta\Delta CT}$ method, and the value stood for n-fold difference relative to the calibrator.

## 2.6. Western Blot Analysis for Phosphorylated Proteins

Liver tissues were homogenized and dissolved with RIPA buffer [150 mM NaCl, 1% Triton X-100, 1% sodium

**Table 2.** Real-Time PCR primers and conditions.

| Gene | Genbank Accession | 5'-3' Forward Primer | Size(bp) | Annealing (˚C) |
|---|---|---|---|---|
| mTOR | XM_006127040.1 | CCTTCATTGGGGATGGCTTAGT<br>GCGTGGGTACATCCAGAGTTTC | 129 | 63 |
| S6K1 | XM_006138647.1 | GGTGCTTCAGCCAGTGCATCAA<br>GATGCCTCTCCGCAAACTGTCA | 101 | 63 |
| 4EBP1 | XM_006134043.1 | GGCATAGGTGAGGAAGCTCAGTT<br>CCATCCTCCACCGGACAAACA | 77 | 63 |
| 18s | JX481969 | GGGGATGCGTGCATTTATCAGA<br>TGCGATCGGCCCGAGGTTAT | 85 | 63 |

deoxycholate, 0.1% SDS, 50 mM Tris (pH 7.4)] containing 1 M Phenylmethanesulfonyl fluoride (PMSF). The breif process of Western blot analysis for phosphorylated protein as follows: protein concentrations were determined with the BCA Protein Assay Kit. Samples (60 μg, 10 - 15 μl) were transferred onto polyvinylidene fluoride membranes (Millipore) after electrophoresing on 10% sodium dodecyl sulfate-polyacrylamide gel for 5 h. The blots were then blocked with 5% casein in Tween 20 in Tris-buffered saline (TTBS) for 1 h, rinsed three times for 5 min each time with TTBS, and then incubated with antibodies in 3% casein TTBS at 4˚C overnight. The membranes were then rinsed with TTBS four times for 5 min each time and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce, USA) in 2% casein TTBS for 1 h at room temperature. Each membrane was washed 5 times for 5 min each time in TTBS and then detected with SuperSignal® West Dura Extended Duration Substrate (Thermo Pierce Chemical, Rockford, IL, USA). Approximately 1 mL of the enhanced chemiluminescence (ECL) working solution was prepared for the membranes prior to their transfer and incubation. After 1 min, excess ECL working solution was removed, and the membranes were sealed with cling film. The X-ray film was placed inside a darkroom and exposed 10 min before development and fixation, and the density of each band was quantified thrice using the densitometry function of Bandscan 5.0 software (Glyko, Novato, CA, USA). Densitometry is reported using the integral optical density value (IOD). The results were represented in the form of IOD ratio of the target protein to *β*-actin. Phosphorylated mTOR (Ser2448, D9C2, XP® Rabbit mAb, #5536), S6K1 (Thr389, 1A5 Mouse mAb #9206) and 4EBP1 (Thr37, 236B4 Rabbit mAb #2855) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA), and the experiment was repeated 3 times for each sample.

### 2.7. Statistical Analyses

All data were presented as means ± SE and subjected to one way analysis of variance (ANOVA) to test the effects of experimental diets using SPSS software (ver16.0; SPSS, Chicago, IL, USA). Tukey's multiple range test and the critical ranges were used to test differences among treatment means ($n = 3$). The difference was regarded as significant when $P < 0.05$.

## 3. Results

The effects of dietary SPC substitution on growth the soft-shelled turtle juveniles were showed in **Table 3**. There was no significant difference on survival among the treatments ($P > 0.05$). The turtles fed with the control diet showed the best daily weight gain (DWG), and the turtles fed with T15 and T30 diets had comparable growth with the control group ($P > 0.05$). However, when the turtles fed with the diet with 60% FM protein replaced by SPC, the growth decreased significantly and lower than any other groups ($P < 0.05$). The poorest of feed utilization (FCR) and nitrogen retention efficiency (NPE) were also observed in T60 group ($P < 0.05$) (**Table 3**).

Pepsin activities in stomach, as well as trypsin activities in liver and intestinal tract all significantly decreasing tendency when the turtles fed diets with increasing SPC level ($P < 0.05$) (**Table 4**). Lipase activity in stomach were unaffected by dietary treatments ($P > 0.05$), in contrast, the activities in liver and intestinal tract tended to decrease when dietary SPC substitution level increased ($P < 0.05$). Amylase activities kept relatively constant

**Table 3.** Effects of dietary SPC on growth and feed utilization of juvenile *P. sinensis*[a].

| Ingredients | Diets | | | |
|---|---|---|---|---|
| | T0 | T15 | T30 | T60 |
| Survival (%)[b] | 100.0 ± 0.0 | 98.3 ± 1.7 | 98.3 ± 1.7 | 100.0 ± 0.0 |
| Daily weight gain (DWG, g)[c] | 0.32 ± 0.01[a] | 0.31 ± 0.01[a] | 0.29 ± 0.01[a] | 0.24 ± 0.01[b] |
| Feed conversion rate (FCR)[d] | 1.10 ± 0.01[a] | 1.15 ± 0.02[a] | 1.16 ± 0.01[a] | 1.38 ± 0.04[b] |
| N retention efficiency (NPE)[e] | 34.02 ± 0.31[a] | 33.18 ± 0.03[a] | 32.11 ± 0.51[a] | 23.99 ± 0.23[b] |

[a]Values with different superscripts are significantly different ($P < 0.05$); [b]Survival (%) = 100 × final soft-helled turtle number/initial soft - shelled turtle number; [d]Daily weight gain (DWG, g) = (average final body weight the average - average initial body weight/ number of days of the experiment.); [e]Feed conversion rate (FCR) = dry diet fed in g/wet weight gain in g; [f]N retention efficiency (NPE) = 100 × (N gain/N intake).

level among dietary treatments in all digestive tissues in the present study ($P > 0.05$). The turtles fed the T0 diet had higher $Na^+$-$K^+$ ATP-ase activity in intestinal tract than the data found in any other groups ($P < 0.05$), but among the SPC replacement groups, no significant differences were observed ($P > 0.05$) (**Table 4**).

The TOR mRNA expression level in liver showed a slight declince trend with increasing dietary SPC level without significantly differences ($P > 0.05$) (**Figure 1(a)**). S6K1 (**Figure 1(b)**) and 4E-BP1 (**Figure 1(c)**) mRNA relative expression levels were also unaffected by dietary treatments ($P > 0.05$). Western blotting detection result of TOR signaling pathway proteins phosphorylated was showed in **Figure 2**, and the IOD ratio result was presented in **Table 5**. The relative protein phosphorylated level of mTOR in liver significantly decreased in T60 group in response to the high SPC level ($P < 0.05$), but there was no statistical difference between the turtles fed with other diets ($P > 0.05$). For its downstream effectors, S6K1 and 4E-BP1 proteins phosphorylated level showed similar trend and the IOD ratios were significantly decreased when dietary SPC level increased ($P < 0.05$).

## 4. Discussion

As reported in some previous studies that high SPC substitution for FM would cause negative effects on aquatic animals [20]-[22], the data observed herein also demonstrated that the suppression of growth was observed when the FM protein was 60% replaced by SPC in soft-shelled turtle. Feed and protein utilization efficiency by

**Table 4.** Effects of dietary SPC on digestive enzyme activities of juvenile *P. sinensis*[1].

| | Diets | | | |
|---|---|---|---|---|
| | **T0** | **T15** | **T30** | **T60** |
| Stomach | | | | |
| Pepsin (U/mg prot) | 13.99 ± 0.83$^a$ | 11.40 ± 0.99$^a$ | 9.24 ± 1.08$^b$ | 5.01 ± 1.33$^c$ |
| Lipase (U/g prot) | 211.67 ± 75.44 | 243.67 ± 22.32 | 266.48 ± 40.83 | 144.61 ± 14.76 |
| Amylase (U/mg prot) | 2.66 ± 0.88 | 2.09 ± 0.16 | 2.84 ± 0.38 | 1.69 ± 0.15 |
| Liver | | | | |
| Trypsin (U/mg prot) | 4028.7 ± 179.9$^a$ | 3169.2 ± 122.5$^b$ | 3044.0 ± 160.8$^b$ | 1522.7 ± 191.1$^c$ |
| Lipase (U/g prot) | 461.67 ± 27.99$^a$ | 436.12 ± 53.83$^a$ | 435.48 ± 45.39$^a$ | 242.76 ± 53.88$^b$ |
| Amylase (U/mg prot) | 1.08 ± 0.40 | 1.30 ± 0.17 | 1.85 ± 1.04 | 0.98 ± 0.28 |
| Intestinal tract | | | | |
| Trypsin (U/mg prot) | 2851.9 ± 155.9$^a$ | 2716.3 ± 52.8$^a$ | 1927.7 ± 85.0$^b$ | 1771.4 ± 112.6$^b$ |
| Lipase (U/g prot) | 813.81 ± 13.15$^a$ | 669.89 ± 24.78$^{ab}$ | 458.57 ± 32.09$^b$ | 294.79 ± 15.54$^c$ |
| Amylase (U/mg prot) | 2.74 ± 0.33 | 2.68 ± 0.68 | 2.22 ± 0.31 | 2.54 ± 0.52 |
| $Na^+$-$K^+$ ATP-ase (U/mg prot) | 0.89 ± 0.05$^a$ | 0.69 ± 0.04$^b$ | 0.62 ± 0.06$^b$ | 0.55 ± 0.05$^b$ |

[1]Values with different superscripts are significantly different ($P < 0.05$).

**Table 5.** Effects of dietary SPC on the IOD ratio of the target protein to *β*-actin in liver of juvenile *P. sinensis*[1].

| | Diets | | | |
|---|---|---|---|---|
| | **T0** | **T15** | **T30** | **T60** |
| TOR | 28.48 ± 4.37$^a$ | 26.03 ± 0.44$^a$ | 24.16 ± 1.74$^a$ | 14.08 ± 1.99$^b$ |
| S6K1 | 26.03 ± 1.66$^a$ | 23.76 ± 1.11$^a$ | 22.65 ± 2.22$^b$ | 12.27 ± 1.52$^c$ |
| 4EBP1 | 24.40 ± 1.58$^a$ | 22.42 ± 0.74$^a$ | 21.29 ± 1.51$^b$ | 8.29 ± 1.01$^c$ |

[1]Values with different superscripts are significantly different ($P < 0.05$).

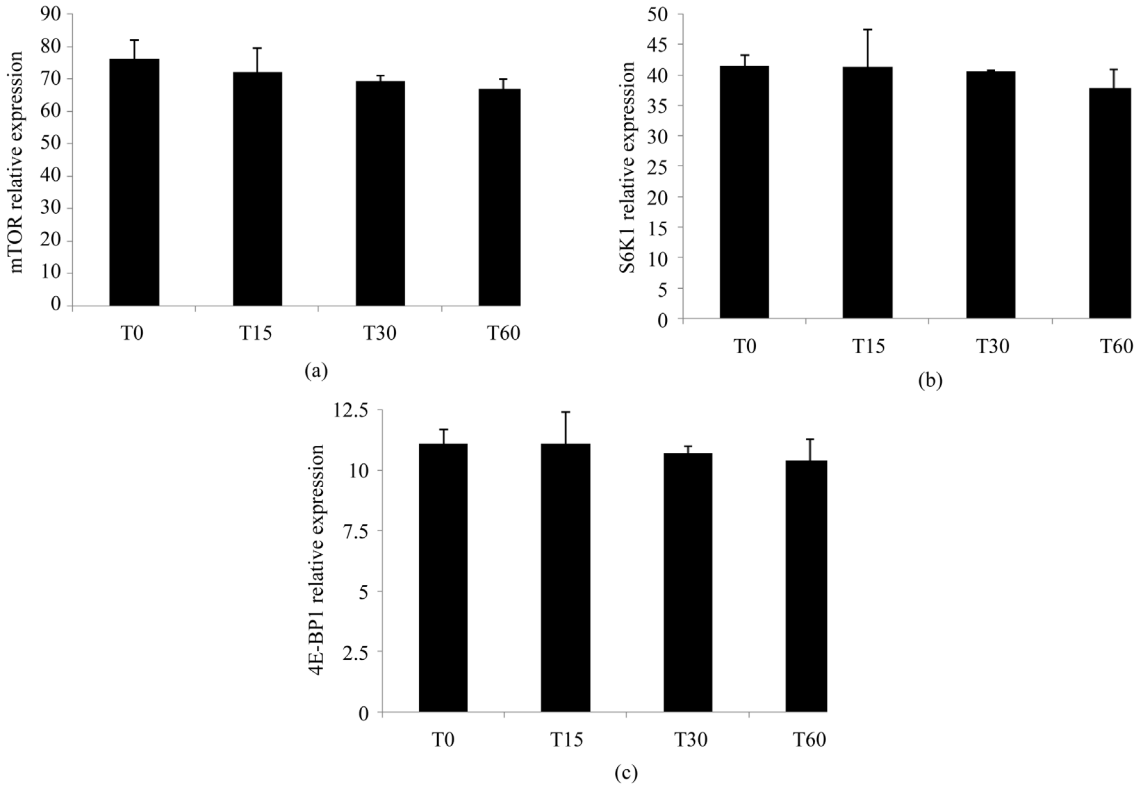


**Figure 1.** (a) Effects of dietary SPC on hepatic TOR mRNA relative expression of juvenile *P. sinensis*; (b) Effects of dietary SPC on hepatic S6K1 mRNA relative expression of juvenile *P. sinensis*; (c) Effects of dietary SPC on hepatic 4E-BP1 mRNA relative expression of juvenile *P. sinensis.*

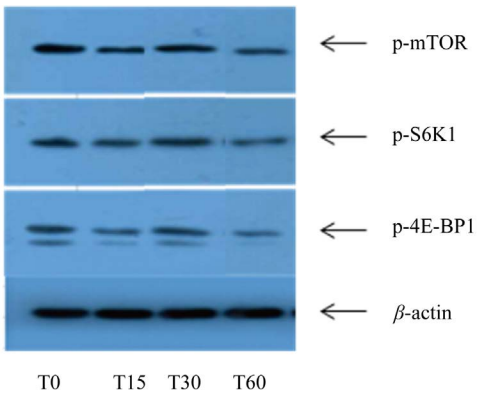


**Figure 2.** The Western blot analysis for hepatic TOR signaling pathway key proteins phosphorylation in response to dietary SPC in juvenile *P. sinensis*.

the turtles followed a pattern similar to that of growth. It has been known that aquatic animals weight gain is associated with the digestive and absorptive capacities and body proteins accretion [23]. However, to our knowledge, no study has addressed the effects of dietary SPC level on digestive enzymes activities and signaling molecules of TOR signaling pathway in aquatic animals, and the possible correlation in soft-shelled turtle was investigated in the current study.

Aquatic animals are suggested to have the capacity to adapt their digestive physiology in response to changes in their nutrient requirements or dietary profile to some extent. The pepsin/trypsin activities, which generally

reflect dietary protein digestibility and amino acid availability with various protein ingredients [24], showed significantly decreasing tendency with increasing dietary SPC level in all these digestive tissues of the turtles. Similarly, reports from freshwater crayfish, *Cherax quadricarinatus* [25]; European sea bass, *Dicentrarchus labrax* [26]; Japanese flounder, *Paralichthys olivaceus* [27]; and common carp, *Cyprinus carpio* L [28] also showed that pepsin/trypsin could adjust to different protein resources. According to Fontagné *et al*. [29], diet with SPC, fish are in a different postprandial stage, lipid digestion is affected. The lipase activities in present study were found to vary differently in responding to dietary SPC level, and the turtles fed diet with high SPC level showed relatively lower enzyme activity. In the present study, amylase activity was not related to the dietary SPC variation, these foundings were according to the results observed in common carp, in which a significant decrease of trypsin specific activity was found in the larvae fed the high SPC diet, whereas amylase specific activity was not affected [30]. $Na^+/K^+$-ATPase, an intestinal membrane-bound enzyme that actively transports $Na^+$ out of and $K^+$ into cells [31], played an important role in absorption of most of the amino acid and glucose. In the current trial, the turtles fed diets with SPC had lower activities of $Na^+/K^+$-ATPase compared with the FM control group. Generally, digestive enzyme activities of the turtles showed decreasing tendency with increasing dietary SPC level, and the variation was correlated with changes in body weight in the current study. Similar results were observed in Atlantic salmon, *Salmo salar* L. [32] and tilapia, *Oreochromis niloticus* × *O. aureus* [33].

The growth of aquatic animals is mainly the result of protein synthesis and deposition [34], and the regulation of protein synthesis is achieved by alterations in peptide chain initiation through changes in the rate of translation of mRNA [35]. The nutrient-sensitive TOR pathway has been reported to play a key role in protein synthesis [36]. 4E-BP1 and S6K1, are well-known targets of TOR kinase and regulation of protein synthesis. In rainbow trout, hepatic TOR gene expression decreased significantly when fish fed with the plant protein diets with lowest level of branched-chain amino acid [37]. Studies conducted on Jian carp have demonstrated that dietary tryptophan, threonine and isoleucine levels could regulate the expression of TOR signaling pathway genes, respectively [12]-[14]. Lansard *et al*. [10] reported that SGR significantly decreased in fish fed diets with fish meal and fish oil replaced with plant ingredients, while no differences were observed in the activation of the TOR. In the present research, the relative expression levels of TOR signaling pathway genes in soft-shelled turtle also showed no significant differences among the dietary treatments. The contradictory findings may contribute to species specific, or related with different amino acid-sensing mechanism. The EAAs content in each experimental diet (**Table 1**) were showed that the experimental diets were nearly identical for EAAs concentration except for 41% decreasing in methionine content occurred in T60 diet than T0 diet. Methionine is well known for its effects on protein synthesis, acts as a sulphur source for synthesis of other sulphur-containing compounds and as a methyl donor for methylation reactions [38]. Dietary methionine level is likely to be a major factor in TOR pathway regulation in the present study, of which is valuable for further investigation. Interestingly, different with the results of mRNA expression, down-regulation effects of mTOR, S6K1 and 4E-BP1 protein phosphorylation status were observed when soft-shelled turtles fed diet with increasing SPC level. Similarly, dietary amino acid level was able to regulate TOR signaling in muscle cell line of rainbow trout as measured by changes in the phosphorylation status of TOR, S6K1 and 4E-BP1 [39]. In zebrafish *Danio rerio*, L-leucine was reported to increase the phosphorylation levels of 4E-BP1 and activated the TOR signaling pathway under Diamond-Blackfan anemia [40]. In the past decade, gene expression patterns analysis was widely applied to study the relationship between dietary nutrition and fish growth, however, typically ~30% to even ~85% of the variation in protein levels can be attributed to variation in mRNA expression, the other 15% to 70% of the variation is explained by post-transcriptional and post-translation regulation [41], and the phosphorylation state was considered as a convenient measure of the activity of the TOR pathway [42]. Previous researches have demonstrated that refeeding can enhance the phosphorylated TOR protein level in liver of rainbow trout [9] [40], insulin and amino acid supplementation can also increase the phosphorylated TOR protein in liver of rainbow trout [11]. It is speculated that the SPC replacement in diet may not influence the TOR signalling pathway proteins of soft-shelled turtle at the transcriptional level, but regulate proteins phosphorylation. Nevertheless, this hypothesis is need for further investigation adding insulin and EAAs such as methionine in feed formula to run as positive control of the phosphorylated TOR proteins.

## 5. Conclusion

In the present study, the soft-shelled turtles fed the diet with 60% of FM protein replaced by SPC showed

significant decreasing in growth and feed utilization, and the suppression of pepsin/trypsin, lipase and $Na^+$-$K^+$ ATP-ase activities as well as hepatic TOR signalling pathway proteins phosphorylation status may be the reasons for the phenomenon. The precise mechanisms whereby dietary SPC regulates TOR signalling pathway in this species warrant further study.

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