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Differential gene expression in *Litopenaeus vannamei* shrimp in response to diet changes

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ABSTRACT

We evaluated changes in gene expression of *Litopenaeus vannamei* shrimp in response to a change in diet by comparing specimens which were fed with an animal protein diet against those which underwent a change from an animal protein diet to a plant protein diet. In addition, we also compared specimens which were fed with a plant protein diet with another group which underwent a change from plant to animal protein diet. Results showed that, in the case of hepatopancreas tissue, a total of six genes were differentially expressed and exhibited a high degree of homology with sequences which codify for proteins related to metabolism, transcription regulation, and rRNA subunits. In the case of muscle tissue, four differentially expressed sequences were recorded which codified for proteins associated to the immune system response, as well as cell structure and signaling. By means of RT-PCR, we also detected differentially expressed chitinase transcripts (ΔCt). We observed ΔCt expression values of 0.49 when comparing specimens fed a plant diet to those which underwent a change from plant to an animal diet, while a ΔCt value of 0.52 was observed when comparing animals fed with an animal diet to specimens which underwent a change from an animal to a plant protein diet. Differential gene expression was not observed in specimens which did not undergo a change in diet during the experiment.

1. Introduction

The feed industry related to shrimp farming is of great economic importance as it represents 60 to 80% of shrimp production costs, with proteins usually accounting for at least a third of the compounds found in commercial diets (FAO, 2006). However, the growth of the shrimp farm industry is strongly conditioned by the availability and quality of proteins produced from aquatic organisms, and effluents from shrimp farms have been shown to produce relevant environmental impacts. Current feeding methods take advantage of shrimp omnivorous habits by incorporating plant-based ingredients to their diet as an alternative source of protein which, in addition, may contribute to the reduction in environmental impacts derived from this industry (Kanazawa, 1989). As a result, current research is now focused on shrimp responses (e.g., metabolic, physiological) to phytochemical components present in the diet, as well as the identification of genes responsible for such changes at the molecular level. Results from these studies are beginning to shed some light on how specific nutrients and compounds produce specific changes at the molecular level, which in turn cause metabolic and physiological changes in shrimp. This information has triggered rapid advances in the shrimp feeding industry (Gillies and Faha, 2003; Match et al., 2005), which is now beginning to rely on an emergent research field known as "nutrigenomics" which seeks to understand how different components of the diet influence molecular mechanisms which in turn determine shrimp physiology, and in this way find strategies to optimize nutrient use and increase the quality of the final product (Bonneau and Laarveld, 1999; Paoloni-Giacobino et al., 2003).

Recent studies have reported that plant protein-based diets can cause significant changes in gene expression in fish (Gómez, 2007), because they are rich in bioactive compounds such as phytochemical agents, antioxidants, polyunsaturated fatty acids (PUFAs), vitamins and vitamin precursors. Nonetheless, genes that codify for proteins involved in metabolic pathways which are activated depending on the diet's protein source have not received much attention in shrimp. The same holds true for research on the physiological implications of changes in gene expression levels due to shifts in dietary protein source. The present work represents an explorative attempt to identify differentially expressed genes in shrimp as a result of changes in dietary protein source. Differentially expressed genes under such conditions may be

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linked to metabolic, physiological and biochemical processes which are relevant in order to achieve an increase in the efficiency and effectiveness of alternative shrimp feeding methods.

2. Materials and methods

2.1. Biological material and treatments

A total of 10,000 postlarvae of *Litopenaeus vannamei* were collected at La Paz, Baja California, Mexico, kept in 400-l tanks and fed with *Artemia salina*, microalgae and pellets until they reached the postlarva 25 stage (i.e., 25-day old specimens starting from the last metamorphic transformation). Subsequently, 200 juvenile specimens were randomly selected, distributed into separate tanks, and fed three times a day with two different diets for which vitamin, mineral and binder concentrations were held constant. One of the diets included squid flour at 30% and was named animal diet (A), while the second diet included a mix of potato, soy, wheat gluten and spirulina and was named plant diet (V) (see Table 1). Specimens were then divided into four treatment groups: VV and AA specimens were fed with plant or animal protein diets, respectively, throughout the experiment (54 days); AV specimens were fed with an animal protein diet during the first 36 days, after which they were switched to a plant protein diet until day 54; finally, VA specimens were fed with a plant protein diet the first 36 days, after which they were switched to the animal protein diet up until day 54. A total of 50 specimens were used per treatment group (divided into five tanks per treatment, and 10 specimens per tank). For all treatment groups abiotic conditions including photoperiod (12:12), salinity (28 ppm), temperature, pH, dissolved oxygen and [NH4]+ were held constant. Once specimens reached developmental stage C (following Aquacop et al., 1975), they were sacrificed, and the hepatopancreas (HP) and muscle tissues (MM) were extracted from each one. Both HP and MM samples were then frozen with liquid nitrogen and stored at −80 °C for subsequent molecular analyses. Molecular analyses were performed in triplicate.

2.2. RNA isolation, differential display (DD) and RT-PCR amplification

For each treatment group, 10 stage C specimens were randomly selected for RNA extraction. Total RNA was extracted and homogenized with nitrogen according to commercial Trizol® following the manufacturer's instructions (Invitrogen®, Carlsbad, CA). RNA replicates from specimens belonging to each treatment level were mixed which resulted in four samples per treatment group (AA, AV, VV or VA), which were used for subsequent differential display (DD) analysis (this was done for both muscle and hepatopancreas samples).

In order to perform the DD analysis, 2 mg of total RNA for each treatment (pooled RNA from all specimens of a given treatment) was incubated with the oligo-dT primer for 60 min at 42 °C. Subsequently, reverse transcriptase was denaturalized for 5 min at 70 °C. PCR amplification was performed with 1 μl of cDNA using six primer combinations (T1:P1, T2:P3, T4:P3, T7:P3, T1:P6 and T7:P6) from the commercial kit Delta® Differential Display (Clontech, Palo Alto, CA), and using the following conditions: 94 °C, 5 min; 40 °C, 5 min; 68 °C, 5 min; one cycle; then 94 °C, 2 min; 40 °C, 5 min; 68 °C, 5 min; two cycles; 94 °C, 1 min; 60 °C, 1 min; 68 °C, 2 min; 25 cycles. 5 μl of PCR products was fractioned by denaturing electrophoresis in a 5% polyacrylamide gel with 8 M urea using 0.5× TBE as buffer, at 70 W for 3 h. Gels were dyed with silver nitrate following the protocol of Bassam et al. (1991). Differentially expressed cDNA bands were retrieved, reamplified by PCR (we used the same set of primers used for Differential Display), reamplification binded to plasmid pGEM-T Easy® (Promega) following vector instructions, and then sequenced. Each nucleotide sequence and its corresponding amino acid sequence were compared with sequences from the GenBank database using BLASTX and BLASTN (default search values). Differential gene expression was assessed by comparing the expression pattern of the AA treatment group with the AV group, as well as comparing the VV group to the VA group.

The following conditions were used for RT-PCR quantification for every 50 μl of reaction: 25.5 μl of 2× Quantitec SYBR green promega, PCR master MIX, 1 μl of direct primer (Quit rev. 5′-ACCACCAAA-CACCTCAAC-3′), 1 μl of reverse primer (Quit forw. 5′-GCTTGGCTA-CAATGAGATC-3′), and 1 μl of cDNA template. RT-PCR values were also treated by the relative quantification method reported by Livak and Schmittgen (2001), for which we used the cycle threshold (Ct) of actin as a reference (Actin forward: 5′-cgcgacctcacagactacct-3′; Act Rev: 5′-gtggtcatctcctgctccaa-3′). The following conditions were used: cycle 1: 95 °C for 15 min, 95 °C for 30 s; cycle 2: 58 °C for 50 s, 70 °C for 2 min.

Table 1
Formula composition of the diets used for *L. vannamei* in the present study.

Ingredients	Animal protein diet (%)	Plant protein diet (%)
Soluble fish protein concentrate[a]	16	9
Soybean paste[b]	15	
Wheat meal	14	10.5
Dried squid	30	
Concentrated soybean protein		30
Concentrated starch		5
Wheat gluten		9
Spirulina		9
Cod-liver oil	3.5	5
Soy lecithin	2	2
Cholesterol	0.5	0.5
Starch	14.5	16.5
Vitamin C	0.0286	0.45
Mineral and vitamin premix[c]	2	1
Agglutinin	1	1
Zeolite (digestion marker)	1.5	1.5
Total	100	100

[a] 70% of protein, Apligén S.A, México.
[b] 48% protein, Nutrimin, S.A. México.
[c] DMS, Nutritional Products, México.

3. Results and discussion

3.1. Differential expression of hepatopancreas transcripts

A total of 52 bands exhibited differential expression due to a change in diet from an animal protein source to a plant protein source (AA vs. AV), while a total of 47 bands showed differential expression for specimens which changed from a plant protein diet to an animal protein diet (VV vs. VA) (Fig. 1). 33% of these bands were reamplified by PCR, and of these, 45% were sequenced and 33% were cloned. Finally, only six bands were sequenced with different sets of primers, one of which was the LV1 sequence obtained using primers P1–T1, which was up-regulated due to a change from animal protein to plant protein diet (AA specimens did not exhibit such response) (Table 2). This sequence corresponds to a gene which codifies for a transcription regulation protein involved in the inhibition of the STAT protein (Signal Transduction and Transcription Protein); the STAT protein is a transcription factor of the interferon system which is related to the immune system response, and has been suggested to play a relevant role in resistance against the white spot syndrome virus (WSSV) (Jin-Lu et al., 2007). Based on this result, we suggest that the LV1 sequence is at least partly responsible for an efficient immune system response associated to a change in diet from animal proteins to plant proteins. This finding agrees with a previous study which reported that diet changes enhance an organism's resistance to variation in environmental parameters, as well as improve the immune system response by promoting the agglutination titre of plasma and respiratory burst of haemocytes in *Penaeus stylirostris* (Chim et al., 2001). Similarly,