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Whiteleg shrimp (*Litopenaeus vannamei*, Boone, 1931) isotrypsins: Their genotype and modulation

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Abstract

Based on information about isotrypsins found by substrate–SDS-PAGE in the whiteleg shrimp *Litopenaeus vannamei* and their segregation, an explanation for the presence of three-trypsin phenotype was sought. Isotrypsins A, B, and C depend on two loci: locus β, which is homozygous, yielding isoenzyme C, and locus α, which is heterozygous, yielding isoenzymes A and B. Segregation in locus α in offsprings from 20 families was analyzed. Results demonstrated that isotrypsins were segregated according to Mendelian rules, and hence, external stimuli did not affect the phenotype. To corroborate this, the phenotype was evaluated in relation to changes in trypsin activity during a digestion process. Trypsin phenotypes found in shrimp feces were also analyzed. Results showed that phenotypes were conserved by individuals. Therefore, changes in trypsin activity found in previous investigations must be related to changes in the concentration of the established isotrypsins.

Keywords: Digestive enzymes; Enzyme regulation; *Litopenaeus vannamei*; Trypsin; Trypsin polymorphism; Whiteleg shrimp

1. Introduction

Recently, isotrypsins synthesized and secreted in the digestive gland of the whiteleg shrimp *Litopenaeus vannamei* were purified and characterized (Sainz et al., 2004), demonstrating for the first time by biochemical means that digestive trypsin is a polymorphic enzyme as anticipated by cDNA studies (Klein et al., 1996).

A zymogram method (García-Carreño and Haard, 1993) was used to evaluate composition, molecular weight, and type of proteases present in extracts from the digestive gland. From this, we observed that trypsin activity in enzyme extracts was performed by several isoenzymes (Hernández-Cortés et al., 1999; Sainz et al., 2004).

Analyzing the trypsin patterns found in this and previous work, we observed that *L. vannamei* specimens have two or three isotrypsins that generate three-

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trypsin phenotypes. Such isotrypsin arrangements were also observed in enzymes extracted from feces (Córdova-Murueta et al., 2003).

It was important to define how many gene loci and respective allele variants were involved (genotype). In previous studies with these trypsins, the genetic pattern or genotype could not be elucidated because the organisms evaluated were obtained from mixed families. The best way to define the genotype (loci and alleles) is by evaluating the segregation of different isoenzymes in offsprings of families (Hartl and Clark, 1997) to determine if there is Mendelian allele segregation. There are several studies of other animals and plants where the genetic pattern of enzymes was determined.

Evidence of changes in protease activity from alimentary stressors is found in the literature. For example, Lee et al. (1984) observed an increase in the activity of enzymes from the digestive system of *L. vannamei*, whereas in northern white shrimp (*Litopenaeus setiferus*), enzyme activities were reduced as a consequence of increases in protein concentration in feed. In *L. vannamei*, trypsin activity is affected by circadian rhythms (Hernández-Cortés et al., 1999) and alimentary stress is more striking than physiological factors in affecting the activity of digestive trypsin (Muhlia-Almazán and García-Carreño, 2002).

Until now, it was not clear whether this shrimp, under different conditions, regulated trypsin activity by controlling the synthesis rate of the isoenzymes present at the loci or alleles, or if they turn on or off one or more isoenzymes. Le-Moullac et al. (1996) observed that casein was an effective inductor of trypsin activity in *L. vannamei* and that the band pattern of trypsin isoenzymes did not change with the concentration of casein in food. Muhlia-Almazán and García-Carreño (2002) observed changes in the number of trypsin bands with time and suggested the presence of at least two genes encoding trypsin that started up or turned off an isoenzyme as a response to feeding. These alternative hypotheses to explain variations in trypsin activity were assessed in this study.

2. Materials and methods

2.1. Evaluation of trypsin phenotypes

To evaluate the distribution of trypsin phenotype by electrophoresis, 30 specimens (3 g each) from each of 20 reference families were analyzed as described by Ryman and Utter (1987). The reference families were originally produced to evaluate genetics characteristics in a breeding program described by Perez-Rostro and Ibarra (2003). Briefly, in the production of the families, a spermatophore of a male was obtained and artificially implanted in the female. The offsprings of each cross were maintained in isolation from the others. Because some of the parents were used as breeders during subsequent mating in the breeding program, they were not included in the study and their phenotypes were inferred from the phenotype observed in the progeny. The different offsprings were reared in flat-bottom tanks (400/m^3) at the CIBNOR facilities. The water was aerated through air stones; temperature and salinity were maintained at 28 °C and 37‰. Specimens were fed with microalgae during the larval stage, then *Artemia salina* and microalgae until PL 5. From PL 5 to PL 30 they were fed with *Artemia salina*, and then with a commercial food PI (Table 1) until the end of the experiment. For the analysis, specimens were decapitated, digestive glands excised, homogenized individually (1:3) (w/v) in dH_2O, centrifuged twice for 30 min at 10,000×*g*, and the supernatant separated from the extract. The protein concentration was measured by the method of Bradford (1976) adapted to micro-assay with bovine serum albumin as the standard. Absorbance was measured in a micro-plate reader at 595 nm (Bio-Rad 550).

Table 1
Chemical composition of feed according to AOAC (1990)

Feed	Moisture (%)	Protein[a] (%)	Lipids[a] (%)	Ash[a] (%)	Crude fiber[a] (%)	Energy (kJ g^{-1})
SC	6.5 ± 0.06	46.6 ± 0.1	7.2 ± 0.07	11.5 ± 0.02	1.9 ± 0.03	18.8 ± 0.04
PI	6.9 ± 0.06	36.8 ± 0.2	4.9 ± 0.04	7.4 ± 0.02	1.9 ± 0.09	19.4 ± 0.05

[a] Expressed as dry basis. Nitrogen-free extracts are determined as the difference from 100%.

2.2. Definition of genotypes and Mendelian segregation

Because isoenzyme C was present in all phenotypes and isoenzymes A and B were variable, we hypothesized that isoenzyme C was an allele of one locus, and isoenzymes A and B were alleles of another locus (Fig. 1). To assess this hypothesis, the genotype from offsprings of each family was defined. Isoenzymes A and B were assumed to be alleles belonging to one locus and the segregation was evaluated in each of the 20 offsprings to know if they were inherited in a Mendelian fashion. Observed phenotypic ratios in the progeny were compared by a chi-square test with expected segregation ratios of each specific parental pair phenotypes. The equation and the degrees of freedom (*df*), according to Hartl and Clark, 1997), were:

$$\chi^2 = \sum_{i=1}^{k} (O-E)^2/E \quad df = k-1$$

where O is the observed number of offspring in phenotypic class i, E is the expected number of offspring for that phenotypic class, and K is the number of phenotypic classes in that family.

2.3. Effect of feeding and trypsin activity on the phenotype

To evaluate the effect of different trypsin activities of the digestion process on the trypsin phenotype, 30 whiteleg shrimp *Litopenaeus vannamei* (18 g), obtained from commercial ponds, were reared in 3

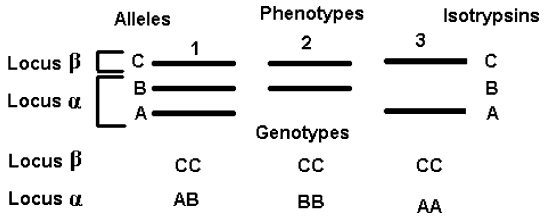

Fig. 1. Observed trypsin phenotype by substrate SDS-PAGE and inferred genotype in the digestive gland of subject specimens of the whiteleg shrimp *Litopenaeus vannamei*. In the left column the three alleles are called A, B, and C. In the three central columns are the phenotypes; 1, 2, and 3. The right column indicates the names of the three isotrypsins; A, B, and C according to Sainz et al. (2004).

Table 2
Selected period for taking samples during feeding

	Experimentally fed organisms	Controls (starved organisms)
Digestion	20 h after ingestion	20 h after ingestion
	Starting ingestion	24 h after ingestion
	2 min after ingestion	
	10 min after ingestion	
	Full digestive gland	
	Full intestine	
	1 feces pellet	
	(as long as intestine)	
	2 feces pellets	
	Empty intestine	28 h after ingestion
Post-digestion	6 h after ingestion	30 h after ingestion
	9 h after ingestion	33 h after ingestion
	12 h after ingestion	36 h after ingestion
Pre-digestion	15 h after ingestion	39 h after ingestion
	18 h after ingestion	42 h after ingestion
	21 h after ingestion	45 h after ingestion
	22 h after ingestion	
Digestion	24 h after ingestion	48 h after ingestion
	Starting ingestion	
	2 min after ingestion	
	10 min after ingestion	
	Full digestive gland	
	Full intestine	
	1 feces pellet	
	(as long as intestine)	

m^2 experimental ponds for two months. For the first month, they were acclimated to the laboratory condition and fed once a day between 12:00 and 16:00 during the month previous to the experiment. The tanks were siphoned to remove food and feces at 16:00. At the start of the experiment, 300 shrimp in intermolt (Chan et al., 1988) were selected. Two groups were assembled; one group was used as a starved control and the second one as the experimental group. Because the body of whiteleg shrimp is translucent, it was possible to observe when chyme entered the intestine, assuring that the specimens had ingested and digested food, and then producing feces. The digestive glands from groups of six specimens were sampled at different times during a 24-h feeding regimen (Table 2). The digestive gland extract and the phenotype were obtained as described in Subsection 2.1. Protease and trypsin activities were evaluated in vitro, using azocasein and BAPNA as substrates. Non-parametric analysis of variance, $\alpha = 0.05$; $n = 6$, specifically the Kruskal–

Wallis test (Sokal and Rohlf, 1981) was used to compare the mean of trypsin activity among the trials. STATISTICA 5.0™ (StatSoft, Inc., Tulsa, OK) analyzed the data. Trypsin activity in individuals was compared to the respective phenotype to evaluate if they were related.

2.4. Trypsin phenotypes in feces

To evaluate the effect of external (feeding) and internal (molting stage) factors on the trypsin phenotype in an individual, 11 specimens (8.0 g) were kept separated from each other in tanks of 0.5 m^2 at 20 °C and 35‰ salinity. Six specimens were fed with commercial feed SC (Table 1) throughout the experiment. Five specimens were fed with commercial feed SC until day 28 and then fed commercial food PI. Feces were collected daily for 51 days by siphoning the tanks for 2 h after feeding. Feces were gently rinsed with distilled water to eliminate salt, and placed in 1.5-mL test tubes. Trypsin extracts were obtained from feces according to Córdova-Murueta et al. (2003) after 200–300 μL dH_2O was added; the mixture was homogenized; then centrifuged for 10 min at 10,000×*g* at 4 °C. Supernatant was decanted and stored at −20 °C. On day 51, extracts from the specimens' digestive glands were also obtained. Samples were analyzed for protein content and trypsin activity. A constant amount of total protease activity was used to load substrate–PAGE gels for analysis of phenotypes.

2.5. In vitro enzyme activity

Total protease activity was evaluated using azocasein as the substrate as described by Córdova-Murueta et al. (2003). Trypsin activity was evaluated with 1 mM *N*α-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) in 20 mM Tris–HCl, 20 mM $CaCl_2$, pH 7.5 as the substrate, according to the method of Erlanger et al. (1961). The amount of *p*-nitroaniline liberated from BAPNA at pH 7.5 at 35 °C was quantified by recording absorbance at 410 nm (extinction coefficient = 8800 M^{-1} cm^{-1}). One enzyme unit was defined as the amount of enzyme that hydrolyzed 1 μmol of BAPNA per min under the conditions described above. Specific activity was expressed as enzyme units per mg protein.

2.6. Enzyme activity by substrate SDS-PAGE

Trypsins were identified as previously described by García-Carreño et al. (1993). From the same sample, 15-μg protein (Protein gel) or 2 mU of trypsin activity (enzyme activity gel) were mixed (1:2) (v/v) with non-reducing loading buffer (125 mM Tris–HCl, pH 6.8, 20% glycerol, 0.02% bromophenol blue) and analyzed by electrophoresis (12% acrylamide, 20 mA for 2 h at 4 °C). The first gel was stained with 0.05% Coomassie brilliant blue R-250 for at least 12 h, and the unbound dye washed out in 7.5% acetic acid and 5% methanol. Molecular mass standards (Sigma®) were loaded on each gel. After electrophoresis, molecular weight markers were cut apart from the second gel and stained with 0.05% Coomassie brilliant blue R-250 for at least 12 h and then de-stained. The remaining part of the gels were incubated for 30 min at 4 °C in 3% casein in buffer 20 mM of Tris–HCl at pH 8 and then incubated 90 min in casein solution at room temperature. Gels were washed with dH_2O, stained with 0.05% Coomassie brilliant blue R-250 for at least 12 h, and then de-stained. Clear bands on blue background indicated the presence of proteases from casein hydrolysis. Trypsins were identified by incubation of the extract with several trypsin inhibitors before electrophoresis.

3. Results

3.1. Trypsin genotypes

Litopenaeus vannamei trypsin isoenzymes were identified by mobility in substrate SDS-PAGE around 20 kDa. They were named isoenzymes A, B, and C, according to increasing molecular weight (Fig. 3). Evaluation of trypsin phenotypes by electrophoresis analysis of offspring from 20 families showed that the three isoenzymes were grouped into three genotypes (Fig. 1). Isoenzyme C was present in all specimens, regardless of family, which can be interpreted as a monomorphic (one allele) locus β. Isoenzymes A and B were present alone or together and generated two homozygotes and one heterozygote, which can be interpreted as a polymorphic (two alleles) locus α. Phenotype 1 expressed the three isoenzymes, imply-

Table 3
Trypsin genotypic frequencies among twenty families and chi-square values for expected Mendelian segregation

Family	Parental genotype[a]		Offspring count in each genotypic class			Expected ratios	n	χ^2
	"AB"		AA	AB	BB			
1	AB	BB		15	15	1:1	30	0
2	AB	AB	5	17	8	1:2:1	30	1.13
3	AB	"BB"		13	13	1:1	26	0
4	AB	"AB"	5	20	5	1:2:1	30	3.33
5	AB	"BB"		14	13	1:1	27	0.03
6	AB	"BB"		12	18	1:1	30	1.2
7	AA	"BB"		30		1	30	0
8	AA	"BB"		30		1	30	0
9	AA	"BB"		30		1	30	0
10	AA	"AB"	14	16		1:1	30	0.13
11	BB	"AB"		30		1	30	0
12	BB	"AB"		16	14	1:1	30	0.13
13	"AA"	"BB"		22		1	22	0
14	"AB"	"BB"		14	14	1:1	28	0
15	"AA"	"AB"		29		1	29	0
16	"AB"	"BB"		13	9	1:1	22	0.72
17	"BB"	"BB"			22	1	22	0
18	"AB"	"AB"	8	8	7	1:2:1	23	3.89
19	"AB"	"BB"		14	15	1:1	29	0.03
20	"BB"	"BB"			27	1	27	0

χ^2 values ($P<0.05$): 3.84 for $df=1$, and 5.99 for $df=2$.
[a] Genotypes in quotation marks were deduced from offspring.

ing that the compound genotype was the locus β homozygote and locus α heterozygote. Phenotype 2 expressed isoenzymes B and C; locus β homozygote and locus α B-homozygote. Phenotype 3 expressed isoenzymes A and C, locus β homozygote and locus α A-homozygote.

Analyzing the trypsin genotype frequencies at locus α in the offspring of the 20 families, all chi-square values from observed frequencies fitted the expected frequencies for Mendelian segregation for a locus with two alleles (Table 3), indicating that trypsins were inherited in a Mendelian fashion. In

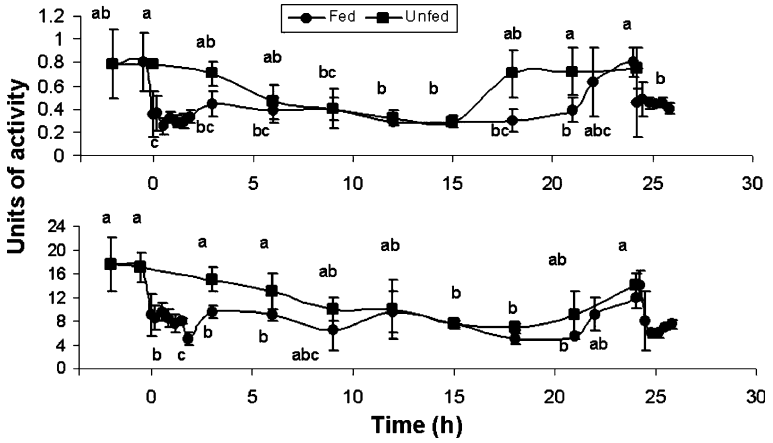

Fig. 2. Effect of fasting and feeding in trypsin (top) and total protease activity (bottom) in the digestive glands sampled during a feeding cycle. Different letters indicate statistical difference ($\alpha=0.05$; $n=6$). One unit of activity for synthetic substrate is the amount of enzyme (trypsin) needed to hydrolyze 1 μmol of substrate in 1 min. One unit of activity = absorbance at 366 nm min^{-1} mg $protein^{-1}$.

Table 4
Trypsin phenotype frequency (%) in the digestive gland of whiteleg shrimp *Litopenaeus vannamei* according to feeding regime

FEP			FFR		FAP		FEP	
Phe	Fed	Stv	Fed	Stv	Fed	Stv	Fed	Stv
1	60	53	40	35	33.3	63.2	47	ND
2	40	47	55	65	52.7	36.8	53	ND
3			5		15			ND

FEP=feeding periods, FFR=food and feces removal, FAP=fasting period, Phe=phenotype, Fed=fed organisms, Stv=starved organisms.

two families, where both parents were sampled, the genotypes agreed with the offspring segregation. In ten families, the female parent genotype was analyzed and the male phenotype was inferred; in eight families, both parents' genotypes were inferred from the offspring genotype.

3.2. Effect of a feeding cycle on trypsin activity and the phenotype

Phenotype frequencies were grouped according to trypsin activity differences through a 24-h feeding cycle (fasting period −3 to 0 h, feeding period 0 to 5 h, feed and feces removal period 5 to 13 h, fasting period 13 to 23 h, and second feeding period 24 to 26 h, see Fig. 2). Phenotypic frequencies of each group were compared to each other (Table 4). Analysis of proteolytic and trypsin activity in samples of digestive gland taken under feeding or starving conditions are shown in Fig. 2. Immediately after time zero (feeding time), differences between feeding and starving groups were measured. In fed specimens, proteolytic and trypsin activity were reduced significantly ($P<0.05$). Proteolytic and trypsin activities in unfed specimens diminished slowly and from 10 to 15 h later; activities reached the lowest values and nearly matched these biochemical activities of fed specimens. Between 4 and 6 h before the next feeding cycle, both activities increased significantly ($P<0.05$) to reach the high and similar levels that prevailed at time zero. Trypsin activity was related to the different feeding processes; declining when fed, and rising before the next feeding. However, the phenotype frequency observed in each group at feeding, before feeding, and during fasting did not show a dominant phenotype in any particular group. The three phenotypes were observed regardless of the treatment and moment of sampling.

3.3. Trypsin phenotypes in feces

The three-trypsin phenotypes were present in feces extracts of the 11 specimens evaluated. The number of specimens with phenotypes 1, 2, and 3 (Fig. 1) were 4, 6, and 1, respectively. Three selected zymograms, each representing a different phenotype, are shown in Fig. 3. Samples were taken daily, feces samples from days 9, 23, 30, 43, and 50, as well as digestive glands sampled on day 51 are shown. The number assigned to the organism is only for identification in the figure. All 11 organisms molted one or multiple times. The selected specimens molted as follows: shrimp I molted on day 26, shrimp II molted on days 18 and 33, and shrimp III molted on days 23, 37, and 51. In all sampled specimens, the phenotype was the same during the experiment, regardless of food composition

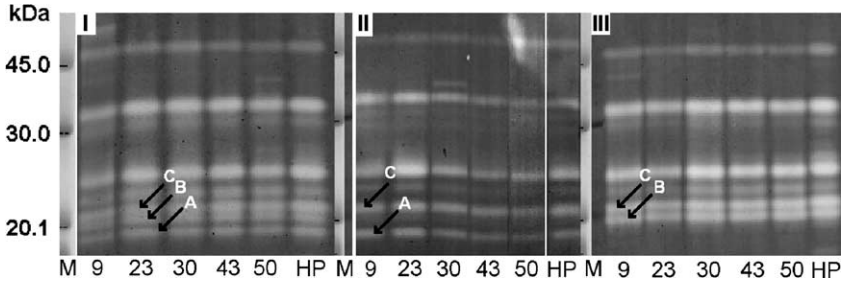

Fig. 3. Isotrypsin patterns found in feces of three selected specimens showing the three-trypsin phenotypes. Arrows point to trypsin bands A, B, and C. Zymograms I, II, and III exhibit specimens bearing phenotypes 1, 2, or 3. Lane M is the molecular weight markers; Lanes 9, 23, 30, 43, and 50 are samples taken at the same number of days from the beginning of the experiment. Lane HP is the extract from the digestive gland at the end of the experiment.

and molting pattern. Also, the phenotype was the same in feces as in the digestive gland tested at the end of the experiment.

4. Discussions

The digestive system of *L. vannamei* is of interest because this species embodies two concerns: shrimp are important organisms in the web food, and some penaeid shrimp are valuable seafood whose harvesting at sea reached its maximum long ago (Pauly et al., 2002). Aquafarming has become an important alternative. Therefore, as digestive system knowledge increases, understanding the role of nutrition can increase aquaculture production. Also, such knowledge will contribute to understanding the position of shrimp in the web food and environment.

The three-trypsin isoenzymes in the *L. vannamei* digestive gland have being observed in previous studies of the group in a variety of treatments (i.e. Córdova-Murueta et al., 2003) either in digestive gland extracts, gastric juice, and feces. And they perfectly match with those recently purified (Sainz et al., 2004). They bear different catalytic properties. They were previously identified by using cDNA techniques (Klein et al., 1996). However, it was not clearly understood how they are organized in the genome and how the process of synthesis is regulated. Molecular biology techniques, mostly those evaluating cDNA, are useful tools, but they lack the ability to explain what processes take place during mRNA maturation and translation. In other words, having a sequence does not necessarily imply having a functional protein in the cell. Recognizing the presence of mRNA does not guarantee the presence of the encoded protein because gene expression can be silenced and, in spite of the presence of mRNA, in some samples there is no protein synthesis (Dykxhoorn et al., 2003). Sainz et al. (2004) isolated and purified the real proteins from *L. vannamei* by using biochemical means. According to the amino acid sequence at the NH_2 terminal, there is no doubt about the relationship among the variants and these isozymes. Trypsin isoenzyme properties show that they are individual proteins.

The questions in this work were: (1) How many gene-loci and their respective alleles were involved in the trypsin isoenzymes observed by substrate SDS-PAGE? (2) Were the trypsin phenotypes affected by external or internal factors or was the presence of each isoenzyme unaffected and only the amount among the established trypsin changed?

Analysis of isoenzyme patterns from the offspring of 20 families analyzed by SDS-PAGE supported the hypothesis that trypsin isoenzymes were expressed at two loci: locus β, which was homozygous, yielding isoenzyme C, and locus α, yielding isoenzymes A and B. The physiologic interpretation, when a trait like the allozymes of trypsin from locus α is inherited under Mendelian rules, is that allozymes are not switched on or off by stimulus of external factors (Hartl and Clark, 1997). To corroborate this assumption, phenotypic frequencies related to the different trypsin activities through a process of feeding were compared. Several changes in both activities were observed during the experiment. However, when analyzing grouped trypsin phenotypes, the three possible phenotypes were observed in precise proportions no matter what the treatment was or the moment of sampling. This indicates that the phenotype was not affected by treatment or changes from low to high trypsin activity.

In a previous work, Córdova-Murueta et al. (2003) found that enzymes present in digestive gland extracts were also present in feces. Therefore, we evaluated whether the enzyme composition in feces was affected by external and internal factors in the same organism over the 51-day experiment. The pattern of trypsin isoenzymes representing one of the three possible phenotypes did not change over time, change in feed source, molt cycle, and always coincided with the trypsin phenotype observed in the digestive gland, indicating that the trypsin phenotype, observed by either non-reductive SDS-PAGE or substrate SDS-PAGE, was not affected by internal or external factors.

Previous investigations of molecular cloning and sequencing of trypsin cDNAs (Klein et al., 1996) and genomic organization and polymorphism of the trypsin multi-gene family in *L. vannamei* (Klein et al., 1998) concluded that there were six different trypsin sequences in the genome of *L. vannamei*. They grouped the sequences into three gene families; two of those three gene families were transcribed to mRNA in the digestive gland, suggesting that at least two of the genes were expressed in this organ. NH_2-terminal sequence analysis of the three purified

trypsins (Sainz et al., 2004) showed that they were three different isoenzymes of trypsin, and that they matched variants 30, 39, and 21 proposed by Klein et al. (1996). Our finding of two loci, one heterozygous and the other homozygous, agreed and complete the findings of Klein et al. (1998).

On the other hand, internal and external factors have been shown to influence the trypsin transcriptional activation system in *L. vannamei* (Klein et al., 1996; Le-Moullac et al., 1996; Muhlia-Almazán et al., 2003). Those studies showed relative increases in mRNA concentrations of total trypsins and non-specific mRNA coding for a specific isoenzymes. For this, it was impossible to conclude whether one isoenzyme synthesis was turned on or off by effectors.

Although the adaptive value of multi-gene trypsin is not clear, they may have evolved to provide a more efficient mechanism, i.e. for protein digestion in phytophagous species feeding on plants that contain proteinase inhibitors (Mitton and Grant, 1984; Ayala and Kigel, 1984; Broadway, 1997) or to provide better growth by having a specific phenotype (Bassompierre et al., 1998).

Regulators of gene expression have been studied for different metabolic processes. Macronutrients, such as cholesterol, glucose, and fatty acids, clearly have profound effects on gene expression. Nutrient interacts with the genome, along with other signaling networks, to allow integration of cellular control between food intake and internal regulatory mechanisms. It reflects an adaptive response to changes in type, quantity, and duration of nutrients ingested for growth (Jump and Clark, 1999), allowing cells to adjust to an on/off switch gene expression. However, this mechanism does not appear to exist for trypsin in the *L. vannamei* digestive gland, since only quantitative changes in the trypsin activity were observed.

5. Conclusion

Analysis of the isotrypsin pattern in the offspring by family and after challenges with internal or external factors allowed us to conclude that two gene-loci were involved in the synthesis of three-trypsin phenotypes in the *L. vannamei* digestive gland. The trypsin isoenzymes segregated in a Mendelian fashion, indicating that the trypsin phenotypes were not affected by internal or external factors. Their regulation was quantitative, but not qualitative. Changes in trypsin activity and in trypsin mRNA by internal and external stimuli must be related to changes in the rate of synthesis of the established isoenzymes.

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