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*Review*

# Trypsin polymorphism and modulation in *Penaeus vannamei* (Boone, 1931): a review

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**ABSTRACT.** The trypsin enzyme of white shrimp *Penaeus vannamei* is a polymorphic molecule, which longest isolated and sequenced cDNA encoded a pre-proenzyme of 255 amino acids. Three of the described sequences are translated in the digestive gland, and SDS-PAGE detects the isoforms. The three isoforms named C, B and A are distributed by an individual to originate three trypsin phenotypes (CBA, CB and CA) that have already been isolated and characterized by defining theirs biochemical and kinetical differences. The CBA phenotypes exerted higher hydrolytic capabilities than the others. The trypsin phenotypes are inherited in a Mendelian fashion, and external or internal factors do not modulate them. In commercial hatcheries in Sinaloa, México, the most abundant phenotype was CBA, followed by CB and CA. The following two hypotheses may explain this finding: phenotype CA is not chosen throughout the selection process of breeders because they do not fit the anatomical characteristics desired by professionals in aquaculture or it is possible that shrimps with the CA phenotype die during the early stages of development. Two experimental designs were developed trying to explain such hypotheses and, in both experiments, CA phenotype frequency declined to zero when shrimps reached around 5 g of body weight. So far, no evidence explains the mortality of juvenile shrimps with phenotype CA. Research is needed to explain why CA phenotypes disappeared, how the survival of this phenotype can be improved, and how to produce shrimps with the most hydrolytic capability in support of the shrimp aquaculture industry.

**Keywords:** *Penaeus vannamei*; trypsin; modulation; isotrypsins; phenotype

Life is an organized form of matter, including many chemical and physical processes. This organization allows living organisms to auto-regulate energy from food, materials for growth, reproduction, anatomic maintenance and survival in different environments (Barbieri, 2008). Different food items require structures and biochemical mechanisms in the organism to complete the process of nutrition. Herbivores and carnivores have specialized mechanisms to obtain, ingest, digest, absorb and excrete food components (Hochachka & Somero, 2002). This review aims to show the possibilities of improving shrimp aquaculture by understanding one of the most important enzymes in *Penaeus vannamei* digestion: trypsin and its isoforms.

The enzymatic activity and the strategic modulations that break down the food components are some of the most important aspects in nutritional processes, which occur before, during and after digestion (Molina *et al.*, 2000). Deficiencies or uncontrolled overproduction of enzymes may cause the death of the organisms (Secor, 2001).

Frequent and the non-frequent feeders are two groups of organisms that put apart two forms of digestive modulation (Secor, 2001). Non-frequent feeders up-regulate the enzyme production rate upon first feeding and then, down-regulate production upon completion of digestion (Secor, 2001; Soares *et al.*, 2011). Eventually, there are no enzymes synthesized in the digestive system. However, when the food is ingested, it promotes the synthesis of enzymes. Frequent feeders are always prepared to ingest and to digest food components, which imply a previous production of all enzymes involved in digestion and their storage as zymogens. Rats (Borta *et al.*, 2010), crabs (Kobayashi *et al.*, 2015) and shrimps (Sainz-Hernández *et al.*, 2004a).

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The trypsin enzyme (E.C. 3.4.21.4) is one of the most important enzymes in crustaceans (Dall, 1992). It has been studied in a variety of organisms having different feeding habits and has been found in all of them (Rypniewski *et al.*, 1994; Muhlia-Almazán *et al.*, 2008; Bougatef, 2013) digesting protein from food and activating other zymogens as chymotrypsinogen, pro-carboxypeptidases and pro-elastase (Eilertsen *et al.*, 2018), including trypsinogen (García-Moreno *et al.*, 1991).

Trypsin is a molecule present in heterotrophic species, not only as a single molecule type, but there is evidence that the trypsin gene was duplicated several times in the evolutionary process to generate polymorphism in the molecule (Roach *et al.*, 1997). This process, in theory, is advantageous for the development and prevalence of the species (Hochachka & Somero, 2002; Walsh & Stephan, 2002).

The white shrimp of the Pacific, *Penaeus vannamei* (Boone, 1931) has three trypsin isoforms expressed in the digestive gland (Sainz-Hernández *et al.*, 2004b), while other species as the insect *Aedes aegipty* (Linnaeus, 1762) has two isoforms (Noriega & Wells, 1999), up to ten trypsin genes exist in the mouse genome (Stevenson *et al.*, 1986), *Drosophila melanogaster* (Meigen, 1830) has four (Davis *et al.*, 1985) and *Anopheles gambiae* (Giles, 1902) mosquito has seven (Giannoni *et al.*, 2001).

The presence of several trypsin isoforms is not necessarily part of a permanent phenotype; some isoforms could outline an individual phenotype and other isoforms another phenotype, as in *P. vannamei*, where three trypsin isozymes originate three different phenotypes (Sainz-Hernández *et al.*, 2005). Similar characteristics occur in the lobster *Palinurus argus* (Latreille, 1804) with three isotrypsins and the same three phenotypes (Perera *et al.*, 2010). Differently, in *Salmo salar* (Linnaeus, 1758), the expression of different trypsin isozymes was observed to be influenced by water temperature during incubation and start of the feeding period (Rungruangsak-Torrissen *et al.*, 1998).

Kinetical and biochemical capabilities of trypsin vary widely among species and can be correlated with alimentary needs (Secor & Diamond, 2000; Pivnenko, 2004). Trypsin properties are studied looking for improvement in food digestion (Córdova-Murueta & García-Carreño, 2002) and in pursue of enzymes with qualities and capabilities to be integrated with biotechnological processes (Rodrigues Da Silva, 2017). Kinetical and biochemical capabilities of trypsin from *P. vannamei* are surprisingly higher than in other crustaceans (Sainz-Hernández *et al.*, 2004b). Shrimp performance with different trypsin phenotypes deserves our attention to direct its production to a more efficient catabolism, aimed at promoting a significant improvement in the shrimp aquaculture industry.

## Molecular cloning, sequence, and polymorphism of *Penaeus vannamei* trypsin

Utilizing two successive screenings of an amplified cDNA library from the digestive gland (DG) of *P. vannamei*, Klein *et al.* (1996) detected five cDNA encoding to five isoforms of trypsin; they were named variants 30, 39, 40, 21 and 42. The comparison of these cDNA sequences suggested a division of isotrypsins into two families (I and II), the first one formed by variants 30, 39 and 40, and the second one by variants 21 and 42. The amino acids composition of these two families varied in 25 positions, and within each family, the amino acid sequence differed in only six positions (Klein *et al.*, 1996).

A third family of trypsin sequences was detected and described in the genomic DNA from the spermatophore sac, and this sequence was not found in the cDNA library from the DG (Klein *et al.*, 1998). The third gene family has a different organization compared to that found in families I and II. However, its nucleotide sequence (without introns) is 93% identical to the trypsinogen sequence from the family I and 91% to the family II. Fifteen amino acids support evidence of an old evolutionary separation in the variable position of the trypsinogen families III, which did not match with the residues found at the same position in the cDNA variants. Alignment of sequences revealed that the *P. vannamei* trypsin was more related to the bovine trypsin than that of bacteria, and it had 74% similarity with trypsin from crayfish (Klein *et al.*, 1998).

The longest isolated and sequenced cDNA encoded a pre-proenzyme of 255 amino acids containing a putative precursor peptide of 14 residues (numbered -14 to -1) (chymotrypsin numbering system), a highly hydrophobic signal sequence of 14 amino acids (numbered -28 to -15), and the active enzyme (numbered 1 to 227). The three families include $Asp^{189}$ and $His^{57}$, $Asp^{102,}$ and $Ser^{195}$, which are implicated in the substrate-binding crevice and catalytic mechanism of serine proteases (Neurath *et al.*, 1967).

Trypsin from *P. vannamei* has eight Cys residues in conserved positions, which can form four disulfide bridges (Klein *et al.*, 1996); meanwhile, six Cys residues are found in crayfish, and twelve Cys residues are predominant in vertebrates trypsin (Fletcher *et al.*, 1987). The corresponding molecular weight of this enzyme is around 22,500 Da but differs from that obtained by SDS-PAGE, which register 33,000 Da (Klein *et al.*, 1998). Evidence that explains this

difference in molecular weight is the analysis for postraductional change in which the three *P. vannamei* trypsin isoforms are found to be glycosylated (Sainz-Hernández *et al.*, 2004b).

In vertebrates, trypsin genes have been found to contain four introns separating five exons (Craik *et al.*, 1984). In *P. vannamei* trypsin, two introns in a position quasi-conserved as the first two introns in the vertebrate were described. The first intron is located between the signal and the activating peptides, and the second interrupt the gene 170 nt downstream. The length of the three exons is identical for the three families, but the length of the introns is different (Klein *et al.*, 1998).

As the DNA sequences from family III were not found in the cDNA from the DG of *P. vannamei* under laboratory conditions (Klein *et al.*, 1996), several questions remain open such as: why were the corresponding cDNAs not detected by screening? Could it be because of the low expression in the DG or because this gene is expressed in tissues other than the DG as hemocyanin in *Macrobrachium nipponense*, which is mainly expressed in the digestive gland, but not in the muscle (Kong *et al.*, 2016). Although the gene is not found in adult *P. vannamei*, it may be present in other life stages as reported for the enzyme α-amylase, which occurs in the same *P. vannamei*, with an increase of α-amylase isoforms in adults (from 7 to 10 isoforms) revealed by SDS-PAGE (Castro *et al.*, 2012). Moreover, the last question is if the presence of this enzyme is a response to external factors as the expression of trypsin in the Atlantic salmon *Salmo salar*, which is related to temperature (Rungruangsak-Torrissen *et al.*, 1998). These questions have yet not been answered for *P. vannamei* trypsin.

**Trypsin zymogen**

Before 1996, the activation mechanism for trypsin zymogen by proteolytic cleavage was extensively studied in mammals (Case & Argent, 1993). However, no data were available about zymogens of crustacean trypsin. Whether a trypsinogen was synthesized in Penaeidae was an open question (Sellos & Van Wormhoudt, 1992). Klein *et al.* (1996) characterized the trypsin cDNA of *P. vannamei* DG and provided the first description of a putative trypsinogen sequence in crustaceans. It was a small sequence between the functional trypsin sequence and the signal sequence. The deduced peptide was composed of 14 amino acids with characteristics like the trypsin activation peptide (TAP).

Sainz-Hernández *et al.* (2004a), utilizing an immunological approach, speculated whether the TAP was synthesized attached to the stored trypsin, so it could be called trypsinogen. Two peptides were synthesized, according to the longest sequenced cDNA encoding the putative trypsinogen of the *P. vannamei* DG (Klein *et al.*, 1996): putative TAP, comprised 13 of the 14 amino acids, and an internal peptide (IP) that included 12 amino acids from an internal sequence corresponding to amino acid residues 135-146 of the functional sequence. Sequences were taken from SWISSPROT access code [TRYP-PENVA] or EMBL nucleotide sequence database, accession number Y15039, Y15040 and Y15041, and translated to amino acids to generate antibodies that recognized the trypsinogen molecule (Sainz-Hernández *et al.*, 2004a).

Western blots of denatured DG extract displayed three bands (Mr 29.7, 30.2 and 32.9) when the anti-TAP antibody was used, and two proteins were displayed when using anti-IP antibodies against the crude extract (Sainz-Hernández *et al.*, 2004a). The molecular mass of the two isoforms was the same as those of the two heavier proteins displayed by the anti-TAP antibodies (30.2 and 32.9 kDa). Isolated isotrypsins were analyzed by reducing electrophoresis (Sainz-Hernández *et al.*, 2004a). Results revealed that two isoforms matched the same molecular mass: two bands were visible, not three (Sainz-Hernández *et al.*, 2004a). The anti-TAP and anti-IP antibodies detected the same bands, suggesting that the trypsin activation peptide was part of the amino acid sequence of the *P. vannamei* trypsin.

The different cell types in the DG of *P. vannamei* have been identified by histology (Bell & Lightner, 1988). Analyzed shrimps presented reactive molecules with both anti-peptide antibodies in secretory B-cells of both fed and starved organisms (Sainz-Hernández *et al.*, 2004a). When animals were fed, it was possible to recognize food in the lumen. Some B-cells in both corresponding positions were stained with the antibodies, and the food in the lumen was also reactive (Bell & Lightner, 1988). Controls with antiserum blocked by the respective antigen (IP or TAP) showed no positive result. *P. vannamei* DG synthesizes and keeps trypsinogen stored ready to be secreted and activated when necessary (Sainz-Hernández *et al.*, 2004a). Trypsinogen purification was not possible, but eliciting antibodies from synthetic peptide were able to detect trypsinogen (Fig. 1).

The white shrimp is a frequent feeder, and its feeding habits follow a circadian pattern that in the wild depends on food availability and protection from predators (Hernandez-Cortes *et al.*, 1999). In aquaculture, however, it depends on cultivation practices. Sainz-Hernández *et al.* (2005) experimented with *P. vannamei*, which were fed once a day at 12:00 AM for one month. After that time they analyzed several DGs to follow the trypsin activity for 28 h. Results were comparable to those reported by Hernandez-Cortes *et al.* (1999)

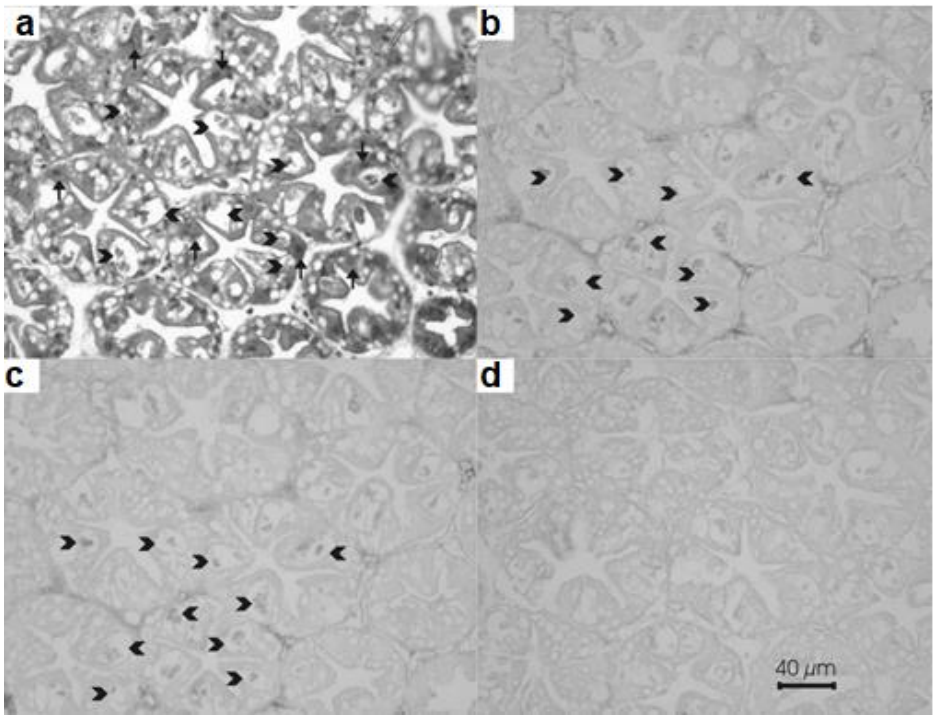


**Figure 1.** *Litopenaeus vannamei* midgut gland serial cuts. Samples were taken from starved organisms. a) Hematoxylin and eosin, B-cells (↑) and F cells (↑) are indicated, b) section treated with anti-TAP antibodies, c) section treated with anti-IP antibodies, positive B-cells are indicated, d) section treated with anti-IP antibodies blocked with its antigen (From Sainz-Hernández *et al*., 2004a).

with a trypsin and total protease activity increase four hours before the feeding time, indicating that: 1) proteases synthesis is modulated according to feeding frequency, and 2) that the species have a postraductional control to avoid auto-digestion. Organisms that store zymogens have the advantage of a faster response to feeding stimulus over sporadic feeders that have to transcribe or translate to get active enzymes (Secor, 2001).

**Biochemical characteristics**

The first effort to characterize *P. vannamei* trypsin was made by Klein *et al.* (1996). They isolated a pool of the three isoforms revealed by SDS-PAGE utilizing affinity chromatography and determined the molecular weight by reducing conditions, as ranging between 31 and 32 kDa; similar results were obtained by Sainz-Hernández *et al.* (2004b), who isolated the three trypsin isoforms individually and under non-reducing conditions; they defined an electrophoretic migration ranging between 21 and 23 kDa.

The first step to describe the characteristics of the isotrypsins was obtaining the N-terminal amino-acids sequence (Sainz-Hernández *et al.*, 2004b). Results indicated that they were different molecules and agreed with the deduced sequence found by Klein *et al.* (1996): trypsin A matched variant 30, trypsin B matched variant 39 or 40, and trypsin C matched variant 21. Considering that they were different molecules, they were characterized by molecular, biochemical and kinetic parameters (Sainz-Hernández *et al*., 2004b). The three isotrypsins were glycosylated and although other glycosylated trypsins have been described, such as trypsins in human seminal fluid (Yu *et al.*, 1994), in the DG of slipper lobster *Thenus orientalis* (Lund, 1793) (Johnston *et al.*, 1995) and in the blood-sucking fly *Stomoxys calcitrans* (Linnaeus, 1758) (Moffatt *et al.*, 1995), it is considered an unusual feature in serine proteases (Johnston *et al.*, 1995; Barrett *et al.*, 2012). Trypsin activity was higher than 80% when assayed at pH between 6-10 and temperatures between 40-70°C. Ranges of temperature and pH for maximum trypsin activity in other organisms are quite narrow: *Panulirus argus* (pH 6-8 and 37°C; Perera *et al*., 2012) and *Macrobrachium rosenbergii* (pH 7.5-8.5 and 50-60°C; Sriket *et al*., 2012); isotrypsins from *P. vannamei* were able to keep their activity in a wide range: more than 80% of activity was maintained at 40-70°C and pH 4-11 (Sainz-Hernández *et al.*, 2004b).

Giving support to the classification in two families, isoform C has higher physiological efficiency and specific activity, lower km. It requires higher concentrations of $Ca^{+2}$ to reach the same activity as isoforms A and B. The three isoforms from *P. vannamei* have, however, more efficient kinetic parameters than trypsins from other decapods (Sainz-Hernández *et al*., 2004a,b) such as *Uca pugilator* (Grant *et al.*, 1980), *Penaeus monodon* (Jiang *et al*., 1991) and *Thenus orientalis* (Johnston *et al*., 1995).

**Genotype and modulation**

Characteristics of the three isolated isoforms detected by SDS-PAGE from *P. vannamei* DG were named isoforms A, B and C (Sainz-Hernández *et al.*, 2004b). Isoforms are distributed by individual organisms in two or three isoforms to originate three phenotypes: CBA, CB and CA (Fig. 2) (Sainz-Hernández *et al.*, 2005).

Different positions have been stated about the presence of different phenotypes. For example, Le Moullac *et al.* (1997) observed that casein was an effective inductor of trypsin activity in *P. vannamei*, but not of changes in the trypsin phenotype. Muhlia-Almazán & García-Carreño (2002), however, observed changes of trypsin activity by the effect of molt or starvation, and registered changes in the number of trypsin bands revealed by SDS-PAGE. They suggested the presence of at least two genes encoding trypsin that started up or turned off an isotrypsin production as a response to feeding.

To give support to any of these possibilities, and to the fact that sequences of family III are not expressed in the DG of *P. vannamei* adults, isotrypsins were studied (Sainz-Hernández *et al.*, 2005) to determine if they were segregated throughout the next generation. Parents with a known trypsin phenotype were crossed to form a family; the offspring of each family was maintained in isolation from the others, and phenotypes were identified by SDS-PAGE as described by Ryman & Utter (1987). Isoenzymes A and B were evaluated by their segregation to find out if they were inherited in a Mendelian fashion; finally, the genotype from each family was defined (Table 1) (Sainz-Hernández *et al.*, 2005).

In analyzing the trypsin isoform and trypsin phenotypes, Sainz-Hernández *et al.* ( 2005) observed that isoenzyme C was present in all shrimps, 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, suggesting a polymorphic (two alleles) locus α. Analyzing the trypsin genotype frequencies at locus α in the offspring of the families (Table 1), all chi-square values from observed frequencies fitted the expected frequencies for Mendelian segregation for a locus with two alleles, indicating that trypsins were inherited in a Mendelian fashion, which implies that the phenotype was not modulated by environmental factors (Sainz-Hernández *et al.*, 2005).

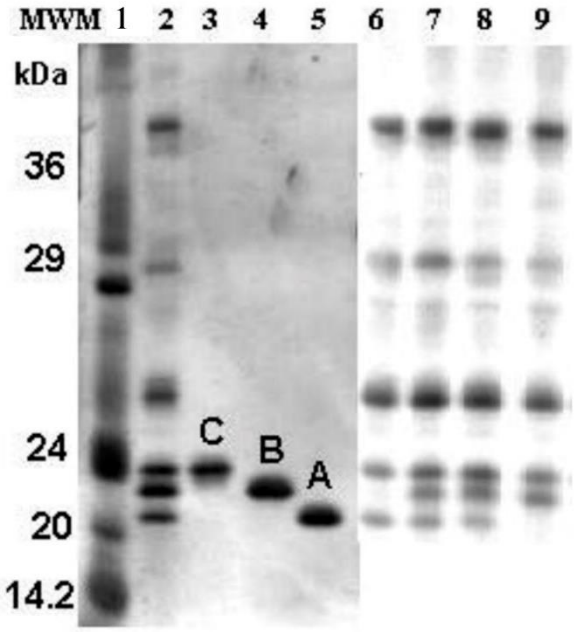


**Figure 2.** Trypsin purification and three different phenotypes from the digestive gland (DG) of *Litopenaeus vannamei*. Line 1: molecular weight markers; line 2: DG crude extract; lines 3- 5: isolated isozymes in non-reducing conditions; lines 6-9: three different phenotypes (Taken and modified from Sainz-Hernández *et al*., 2004 a,b).

Córdova-Murueta *et al.* (2003) found that trypsins present in DG extracts of *P. vannamei* were also present in feces, and analyzed if the phenotype in feces was affected by external and internal factors in the same organism over time. They concluded that the pattern of trypsin isoenzymes representing one of the three possible phenotypes did not change over time, food source or molt cycle, and always coincided with the trypsin phenotype observed in the DG of the same organism.

In another experiment, the phenotypic frequencies related to the different trypsin activities through a 28 h feeding cycle were analyzed by Sainz-Hernández *et al.* (2005). Several significant changes in the trypsin activity were observed during the experiment. However, when analyzing grouped trypsin phenotypes related to high or low trypsin activity, the three possible phenotypes were observed in precise proportions no matter the trypsin activity level or the moment of sampling, indicating that the phenotype was not affected by treatment or changes from low to high

**Table 1.** Trypsin genotype frequencies among 20 families of *Litopenaeus vannamei* and chi-square values ($X^2$) for expected Mendelian segregation. $X^2$ values ($P < 0.05$): 3.84 for df = 1 and 5.99 for df = 2 (From Sainz-Hernández *et al*., 2005).

| Family | Parental genotype | | Offspring count in each genotype class | | | Expected ratios | n | $X^2$ |
|---|---|---|---|---|---|---|---|---|
| | | | 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 |

trypsin activity. Changes in trypsin activity and trypsin mRNA by internal and external stimuli must be related to changes in the rate of synthesis of the established isoenzymes.

This finding suggests that the synthesis of any isoform is not turned on or off by the effects of external or internal factors. The trypsin isoforms that are synthesized by juvenile *P. vannamei* are the same synthesized by adult organisms. Moreover, there was no evidence that trypsin from family III was synthesized in the DG of *P. vannamei*.

**Population structure of trypsin patterns in aquaculture and natural environments**

Trypsin patterns in *P. vannamei* are present in natural and cultivated populations; however, their frequencies in juveniles and adults are not distributed according to the Mendelian rules (Rubio-Gastelum, 2010; Aguiñaga-Cruz *et al*., 2017). The description of trypsin phenotype frequencies in hatcheries is important for defining a management protocol if shrimp performance associated with trypsin phenotypes is to be determined. Producing pure phenotypes is necessary for identifying possible advantages in commercial aquaculture, as discussed by Sainz-Hernández & Cordova-Murueta (2009) and Perera *et al.* (2010), who worked with similar trypsin phenotypes in the spiny lobster *Panulirus argus*. The first study to describe the pattern frequencies in cultivated shrimp *P. vannamei* was carried out by Rubio-Gastelum (2010). In this work five commercial hatcheries were analyzed, four from Sinaloa, Mexico and one from Nayarit, Mexico. The results revealed a high frequency of CBA phenotype in all five hatcheries, followed by CB phenotype and in a lower frequency, the CA phenotype. Aguiñaga-Cruz *et al.* (2017) analyzed the genetic population structure of the locus α (isoforms AB) of digestive trypsin in 2,828 breeder shrimp of *P. vannamei* from 11 actively producing commercial hatcheries in the Sinaloa state. The trypsin phenotype frequencies were similar to those reported by Rubio-Gastelum (2010). The CBA phenotype was the most abundant, followed by the CB phenotype, and the least frequent phenotype was the CA. This dominance of CBA resulted in significant excess of heterozygosity and exhibited a Hardy-Weinberg imbalance in the hatcheries (Aguiñaga-Cruz *et al.*, 2017). The results of the inbreeding coefficient (FIS) analysis did not show evidence of endogamy but did show an excess of heterozygosity. The trypsin phenotypic structure, analyzed by pairwise comparisons among hatcheries according to the genetic distances (FST) analysis, showed a significant differentiation for most of the analyzed hatcheries, and four groups were defined by the unweighted pair-

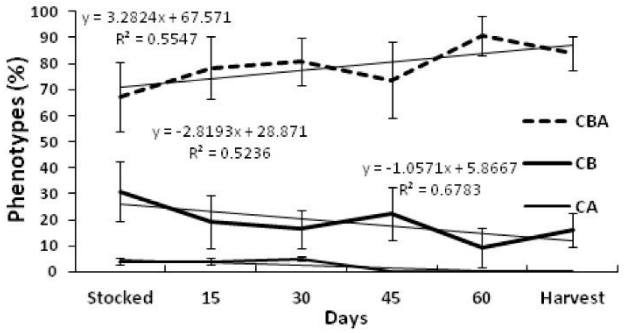


**Figure 3.** Frequency of trypsin phenotypes during 75 days of culture of *Litopenaeus vannamei* (From Aguiñaga-Cruz *et al.*, 2017).

group method with arithmetic mean (UPGMA) clustering analysis (Aguiñaga-Cruz *et al.*, 2017). The following two hypotheses may explain this finding: a) shrimps with the CBA phenotype are selected by breeders for commercial production because they fulfill the anatomical characteristics (size, age, weight, occurrence of deformities, color) (Racotta *et al.*, 2003), while the CA phenotype does not; and, alternatively, b) shrimps with the CA phenotype do not reach breeder size because of high mortality during the early stages of culture. Both hypotheses are highly relevant to the aquaculture industry. Aguiñaga-Cruz *et al.* (2017) conducted another experiment to clarify these hypotheses: the offspring from mates with the CBA-CBA phenotype which produce 50% CBA, 25% CB and 25% CA were stocked in nine ponds and analyzed to detect the period in which shrimps with the CA trypsin phenotype were absent. Under standard culture procedures, the results indicated that at the age of 20 days postlarvae, when they were stocked, the percentage of shrimps with the CA phenotype decreased to 5% and was absent when shrimps reached 5 g (Fig. 3). Shrimps with the CA phenotype inexplicably died, giving rise to the abundance of CBA shrimp, while specimens with the CB phenotype showed 80% survival. These results suggest that the population structure of trypsin phenotypes at the breeding age is possibly the result of natural selection rather than artificial selection by biologists in hatcheries. The economic consequences are relevant since the total shrimp population with the CA phenotype is assumed to be 25% of the total offspring, and, of this population, 80% dies in hatcheries while the remaining 20% perishes before being harvested. The hatcheries and shrimp workers' pay the expense of maintaining these shrimp throughout the culture process.

Despite those mentioned above, there was not enough evidence to conclude that natural selection was the cause of this distribution, and one open question remained whether the shrimp aquaculture is exerting pressure on the CA organisms causing their death or whether they die independent of the culture conditions. To answer this question, trypsin phenotype frequencies in 60 breeder shrimp of *P. vannamei* from a natural population collected in Babaraza Estuary, Guasave, Sinaloa, México, were analyzed (Sainz-Hernández, *pers. comm.*) to predict the trypsin phenotype frequency in the next generation. A total of 1,000 shrimps of the F1 generation of the same natural population was collected and analyzed. Results indicated that the F1 generation is out of the Hardy-Weinberg equilibrium and the mortality of shrimps with phenotype CA in the natural population was similar to that in cultivated *P. vannamei*. In conclusion, the process of aquaculture does not exert pressure on the CA phenotype. The mortality of cultivated *P. vannamei* is associated with other factors not yet understood and needs further attention. Currently, we know that a portion of that mortality occurs when shrimps inherit a trypsin co-dominant allele monomorphic called A, which acts as a lethal gene.

## Perspectives

Three trypsin characteristics from *P. vannamei* put in context the possibility of manipulating the production of the postlarvae to improve the shrimp aquaculture: the isoenzymes present different kinetics capabilities. It is possible to produce 100% of a particular trypsin pattern, and the patterns do not change by internal or external factors. Several studies have been conducted to 1) clarify the differential performance by the different patterns and 2) identify the strategies for producing those that are most convenient. Currently, we can assume that shrimps with phenotype CBA present better perspectives for aquaculture purposes, and shrimps with phenotype CA die during early stages of growth. Further research is needed to adequately explain the relationship between the mortality of shrimps with phenotype CA to propose alternatives to improve the survival of that phenotype. It will be important to establish a hatchery for research or commercial purposes, where it could be able to produce 100% of each trypsin phenotype or integrate a procedure to remove the CA phenotype in the commercial production.

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