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Penaeus vannamei isotrypsins: purification and characterization

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Abstract

Three isotrypsins from digestive gland of *Penaeus vannamei* were purified and characterized by molecular, biochemical and kinetic parameters. Purified isotrypsins A, B, and C are glycoproteins with molecular masses between 30.2 and 32.9 kDa, and, therefore similar to other trypsins. The isoelectric points are anionic and different among the three isotrypsins: pH 3.5 for isotrypsin A, pH 3.0 for isotrypsin B, and pH 4.5 for isotrypsin C. Differences in the NH_2-terminal amino acid sequences allowed us to define three different protein entities that match isotrypsins previously deduced by cDNA. Isoform C has higher physiological efficiency and specific activity, lower K_m, and requires higher concentrations of Ca^{+2} to reach the same activity as the other two isotrypsins.

Keywords: Invertebrate trypsin; *Penaeus vannamei*; Isoenzymes

1. Introduction

Protein digestion in the white shrimp *Penaeus vannamei* (Bonne, 1931) relies on the concerted action of a protease complex with trypsin as the most abundant enzyme. Trypsin (EC 3.4.21.4) is a serine proteinase that hydrolyzes peptide bonds at the carboxylic side of positive charged amino acid residues within a protein. It is also responsible for activation of zymogens. Trypsins, like other digestive enzymes, are key elements because they act as mediators between the intake of food and nutrient assimilation (Dittrich, 1992).

Trypsin activity in the white shrimp was first reported by Lee and Lawrence, 1982. In previous studies, enzyme extracts from the digestive gland of the white shrimp presented three isoforms of trypsin (Klein et al., 1996; Le Moullac et al., 1996; Ezquerra et al., 1997; Muhlia-Almazán et al., 2003). However, when samples of individuals are analyzed by substrate-SDS-PAGE, two or three isoforms may be present (Córdova-Murueta et al., 2003).

To define characteristics of the three isoforms and whether some adaptive advantage is gained by having two or three isotrypsins, the three isoenzymes were purified to homogeneity and characterized by kinetics parameters, isoelectric point, the effect of ions and inhibitors on activity, and glycosylation.

2. Materials and methods

2.1. Purification

The digestive gland (DG) was collected from white shrimp (*P. vannamei*) reared in experimental ponds at CIBNOR. Shrimp were decapitated and the DG rapidly removed and kept at −20 °C. A pool of DG was homogenized (1:2) in distilled water in a Warring blender. Homogenates were centrifuged three times for 30 min at 10 000×*g* and the aqueous extract was separated from lipids and sediment. The extract was filtered through a 0.45-μm membrane to obtain soluble protein.

Protein concentration was measured by the method of Bradford (1976), with bovine serum albumin as the standard. To purify isoenzymes, 150 μg of the enzyme extract was loaded in five wells of non-reductive-PAGE plates. After separation, lanes were cut apart and labeled for further identification. From each lane a one-fifth piece was cut out longitudinally, and stained with Coomassie blue until protein bands were evident. The remaining pieces of the gel were kept at 10 °C. Strips with stained bands were matched with the remaining lanes to identify and separate the corresponding trypsin bands. Trypsin

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bands were previously identified by substrate-SDS-PAGE (García-Carreño et al., 1993). Each separated band was placed in 1.7 ml tubes containing 100 μl 20 mM TRIS–HCl, pH 7.5. Tubes were incubated at 10 °C overnight. To confirm trypsin activity, 5 μl of samples were assayed, as described below. To confirm the purity of the protein, each eluted band was analyzed by electrophoresis in non-reductive conditions, and stained with silver stain (Amersham Pharmacia Biotech, 1999). To obtain enough sample for further analysis, several separations were done and those bands perfectly separated from the others were grouped in one of three fractions: low (trypsin A), medium (trypsin B) and high M_r (trypsin C). Fractions were desalted passing the fractions through a chromatographic column containing Sephadex G-25.

2.2. Analysis of molecular characteristics

Trypsin was analyzed by electrophoresis under non-reductive and reductive conditions. Vertical 12.5% polyacrylamide (w/v) slab gels were used for electrophoresis. For reducing conditions, samples were diluted (1:2) with Laemmli sample buffer, 0.125 M TRIS–HCl, 4% SDS, 20% v/v glycerol, 0.02% bromophenol blue, pH 6.8, 5% mercaptoethanol and heated for 2 min at 100 °C (Laemmli, 1970). For non-reducing conditions, samples were mixed in the same buffer without mercaptoethanol and SDS and were not boiled. Separation was performed for 2 h at 20 mA constant current and 4 °C. To reveal protein bands, gels were stained for 2 h with 0.1% Coomassie blue (R-250) in 7.5% acetic acid and 5% methanol at RT and destained in 7.5% acetic acid and 5% methanol.

Analysis of the NH_2-terminal sequence of the three purified trypsins was done with a gas-phase sequencer (Model 470A, Applied Biosystems), using the program designed by the manufacturer.

Isoelectric points (IP) of the purified trypsins were evaluated by analytical electrofocusing in thin-layer polyacrylamide flat gel (LKB Ampholine PAGE plate), containing an ampholine with a pH range of 3–9. An isoelectric focusing calibration kit containing 11 standard proteins was used (SIGMA). Protein bands were silver stained.

Trypsins were analyzed for glycosylation, both in non-reduced and reduced forms. In triplicate, polyvinylidene fluoride (PVDF) membranes were soaked for 20 s in methanol; 1 μg of each non-reduced and reduced trypsin was loaded directly and dried at RT (Harlow and Lane, 1988). Loaded strips were tested with Schiff reagent to detect carbohydrates covalently attached to trypsins. Membranes were incubated for 20 min in 15% isopropanol and 10% acetic acid, and incubated 20 min in 10% acetic acid. Membranes were immersed in 0.5% periodic acid for 2 h in the dark at 4 °C, incubated for 30 min in 0.5% sodium arsenite, 5% acetic acid, then incubated twice for 20 min in 0.1% sodium arsenite, 5% acetic acid, and finally incubated for 10 min in 5% acetic acid. Membranes were transferred into Schiff reagent and incubated overnight in the dark at 4 °C (Clark, 1973).

The type of carbohydrate was evaluated using eight fluorescein-labeled lectins (Table 1). In triplicate, PVDF membranes were immersed in methanol for 20 s and dried at RT; 1 μg of reduced and non-reduced trypsins were dot-blotted and dried at RT (Harlow and Lane, 1988). Strips were individually incubated for 2 h in the dark at RT with fluorescein-labeled lectins, and then washed three times for 20 min with phosphate buffer. Fluorescein-labeled dots were observed under UV light.

Table 1
Presence of glycosides in purified isotrypsins (A, B, C) from *P. vannamei*. Glycosides were identified by using specific lectins. (+++)=strong reaction, (+)=weak reaction, (−)=no reaction

Lectin: origin and carbohydrate specificity	Reaction of lectins		
	A	B	C
(PWM) *Phytolaca americana* *N*-acetyl, β-D-glucosamine-oligomers	−	−	−
(Jacalin) *Artocarpus integrifolia* β-D-Gal (1-3) D-Gal Nac	−	−	−
(PHA) *Phaseolus vulgaris* aglutinin	+	+	+
(WGA) *Triticum vulgaris* *N*-acetyl-β-D glucosaminyl residues *N*-acetyl-β-D-glucosamine olygomers	+++	+++	+++
Con-A	+	+	+
(LCA) *Lens culinaris* α-D-manosyl and α-D-glucosyl residues	−	−	−
(SBA) Soybeans *N*-acetyl-D-galactosamine	−	−	−
(WAP) *Wisteria floribunda* *N*-acetyl-D-galactosamine	+++	+++	+++

2.3. Analysis of biochemical and kinetic characteristics

Trypsins were evaluated for amidase activity using 1 mM benzoyl-arginine *p*-nitroanilide (BAPNA) in 20 mM TRIS–HCl, 20 mM $CaCl_2$, and pH 7.5 as the substrate (Erlanger et al., 1961). The amount of *p*-nitroaniline liberated from BAPNA at 35 °C was quantified by the increase in absorbance at 410 nm (extinction coefficient=8800 M^{-1} cm^{-1}). One enzyme unit was defined as the amount of enzyme that hydrolyzes 1 μM BAPNA per min under the conditions described above. Specific activity was expressed as enzyme units per mg protein.

To evaluate the effect of temperature on amidase activity, BAPNA solutions were equilibrated at temperatures ranging from 10 to 80 °C and added to 2 μg of enzyme. The reaction was incubated at the same temperature for 20 min and the trypsin activity evaluated.

For pH stability, 2 μg trypsin in 10 μl were diluted (1:3) with universal buffer (Stauffer, 1989); 20 ml stock solution (57 mM H_3BO_3, 36 mM citric acid, 28 mM NaH_2PO_4, 310 ml 1 N NaOH with dH_2O to make 1 l) were mixed with enough 1 N HCl to yield the suitable pH and diluted to 100 ml. For this experiment, different pH values, ranging from pH 3 to 12 were included. After incubation for 1 h at 20 °C, pH was increased to pH 7.5 by the addition of 750 μl of substrate solution in 50 mM TRIS–HCl, pH 7.5, and trypsin activity was measured.

The effect of Ca^{2+} on the enzymes was examined, by assaying the activity with 0, 10, 30, 50 and 100 mM $CaCl_2$ in the substrate solution.

The effect of specific inhibitors on enzymes was evaluated. Equal volumes of inhibitors and enzyme solution (0.2 mg/ml) were incubated for 1 h at 20 °C, before residual trypsin activity was measured. In this study, 10 mM tosyl-L-lysine chloromethyl ketone (TLCK) for serine proteases, 5 mM tosyl-L-phenylalanine chloromethyl ketone (TPCK) and 5 mM carbobenzoxy-phenyl chloromethyl ketone (ZPCK) for chymotrypsin, and 0.25 mM soybean trypsin inhibitor (SBTI) and 100 mM phenylmethylsulfonyl fluoride (PMSF) for trypsin were used, including a control without inhibitors.

Statistical analysis for biochemical characteristics of the three isoforms with nonparametric analysis of variance was done (Kruskal–Wallis test) (α=0.05) (Sokal and Rohlf, 1981). The STATISTICA 5.0 computer program was used for analyses. Kinetic parameters of the three *P. vannamei* isotrypsins were evaluated by Lineweaver–Burk plots (Copeland, 2000). Concentration of BAPNA substrate varied from 0.0375 to 7.0 μM.

3. Results

Isotrypsins from *P. vannamei* digestive gland, named trypsin A, B and C, are shown in Fig. 1. Isotrypsins A, B, and C yielded three bands, after PAGE under non-reducing conditions, with M_rs of 21, 22 and 23, respectively. SDS-PAGE under reducing conditions showed trypsin C to have a molecular mass of 30.2 kDa, while isotrypsins A and B had molecular masses of 32.9 kDa. The outline and the results of the purification of the three isotrypsins are summarized in Table 2. From 1 g of digestive gland, 0.5 mg pure trypsin was recovered. The specific activity (Abs_{410}/min/mg) of isoform C is eight times higher than isoform B, and 2.3 times higher than isoform A. Amino acid substitutions in the three isoforms at the NH_2 terminal were conservative and semi-conservative (Table 3). Trypsins A and B differed at position eight, where trypsin A has a threonine residue and trypsin B a lysine residue, both hydrophilic ones, the last one positively charged. Isotrypsin C differed from isoforms A and B at position 6 and 7, having glutamate and valine, keeping the character in both positions. By using IEF gels pH 3–9, all the isoenzymes migrated to the anode. Isotrypsins A, B and C had IPs of 3.5, 3 and 4.5, respectively.

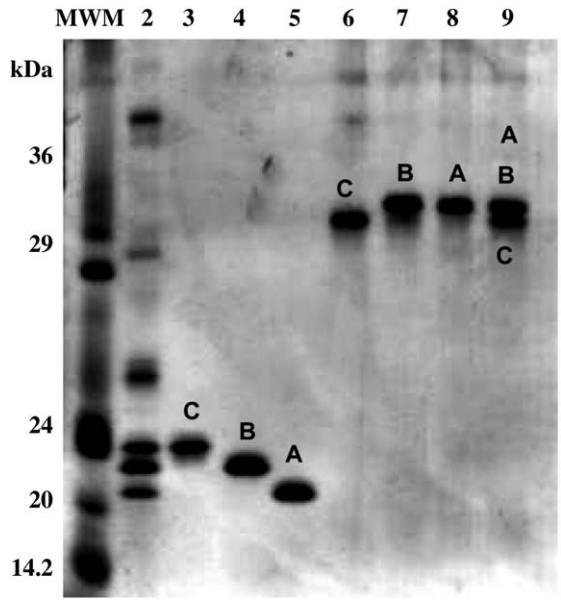

Fig. 1. Purified trypsins from digestive gland (DG) of *P. vannamei*. *Footnote*: lane 1=Molecular weight markers; 2=DG crude extract; 3, 4, 5=trypsins A, B, C in non-reducing conditions; 6, 7, 8=trypsins A, B, C in reducing conditions; 9=mixed pure trypsins. Molecular masses and M_r were calculated in these samples.

Isotrypsins were analyzed for carbohydrate moiety composition. Reaction was positive in all the non-reduced and reduced isoforms with Schiff reagent. By using fluorescein labeled lectins to identify the type of carbohydrate bound to isotrypsins, both glucosamine residues and polymers of galactosamine were identified (Table 1).

3.1. Study of biochemical and kinetic characteristics

The effect of temperature on enzyme activity was evaluated by assaying activity in a range from 10 to 80 °C (Fig. 2a). The maximum activity under the assayed conditions took place at 60 °C. Between 10 and 20 °C; activity was

Table 2
Summary of purification procedure of isotrypsins from digestive gland of *P. vannamei*

Extract	Protein (mg)	Total units (ΔAbs_{410}/min)	Specific activity (ΔAbs_{410}/min/mg)	Purification factor	Yield (%)
Crude hydroelution	80	51.8	0.64	1	100
Trypsin A	0.013	4.8	369	576	9.3
Trypsin B	0.015	1.6	106	165	3
Trypsin C	0.011	9.37	851	1330	18

less than 20%. Above 60 °C, activity fell abruptly and was negligible at 80 °C.

The effect of pH on stability of the three trypsins is presented in Fig. 2b. No significant differences ($P>0.05$) were observed. Activity was higher than 80% when assayed at pH 6–10. At pH 3, 4, and 12, trypsin activity was reduced more than 50%. Concentration of Ca^{2+} affected the activity of the three isotrypsins. Highest activity was recorded between 30 and 50 mM $CaCl_2$ (Fig. 3a). Lowest activity occurred at 100 mM $CaCl_2$. In controls and 10 mM $CaCl_2$, isoform C showed significantly less activity ($P<0.05$) than isoform A and B. The effect of protease inhibitors on the activity of the three isotrypsins is given in Fig. 3b. TLCK, SBTI and PMSF inhibited the three trypsins by 80–90%. No significant difference ($P>0.05$) among trypsins was observed with these inhibitors. Activity of trypsins treated with ZPCK and TPCK was not different ($P>0.05$) than controls without the inhibitors. K_m, K_{cat} and K_{cat}/K_m values for the three trypsins are shown in Table 4. When comparing kinetic values among enzymes from different organisms, *P. vannamei* had the lowest K_m (μM), and highest physiological efficiency. Isoform C showed the smallest K_m, and had almost twice the physiologic efficiency compared to isoforms A and B.

4. Discussion

Substrate-SDS-PAGE (García-Carreño et al., 1993) allowed visualization of isotrypsins (Lemos et al., 2002; Córdova-Murueta et al., 2003). Different electrophoretic migration called for an explanation since Klein et al. (1996) defined that the corresponding molecular mass of the deduced sequences were of 22 500 Da, differing from that obtained by SDS-PAGE. They suggested the possibility of glycosylation based on previous studies in serine proteinases (Kimoto et al., 1983) and trypsin from the blue crab (*Callinectes sapidus*; Dendiger and O'Connor, 1990). Changes in relative mass between non-reduced and reduced isotrypsins have been observed in other organisms. For example, an increase of mass in a trypsin in human seminal fluid (Yu et al., 1994), porcine digestive trypsin (Jeohn et al., 1995), and carp (*Cyprinus carpio*) digestive trypsin (Cao et al., 2000) was found. Although, *P. vannamei* trypsins are glycoproteins, differences in trypsins found in this research could not be explained totally by glycosylation, but by other intrinsic characteristics that need to be addressed.

Glycosylation, as in *P. vannamei* isotrypsins, is considered an unusual feature in serine proteases (Johnston et al., 1995; Barrett et al., 1998). Other glycosylated trypsins have been described such as trypsins in human seminal fluid (Yu et al., 1994), the slipper lobster (*Thenus orientalis*; Johnston et al., 1995) and the blood-sucking fly (*Stomoxys calcitrans*; Moffat et al., 1995). The function of glycosylation is to tag the enzyme for biological recognition of structures other than substrate (Nelson and Cox, 2000), usually to generate an allosteric function or for auto-catalytic protection. Because in our study analysis for glycosylation was only qualitative, differences in galactosamine and glucosamine content among isoenzymes were not obtained. Therefore, it is not possible to discuss differences among the trypsin activities due to the effect of glycosylation.

The six variants of trypsin described by Klein et al. (1996, 1998) have differences in the amino acid sequence where glycosylation occur. Glycosides bind to proteins at Thr, Ser, Asn and Leu residues (Nelson and Cox, 2000). Isoform A has one Thr residue more than isoform B.

Table 3
NH_2-terminal sequences of pure isotrypsins from *P. vannamei*. Solid letters are the substitution

Trypsin	Variant*	NH_2-terminal sequence													
		2		4		6		8		10		12		14	
A	30	I	V	G	G	T	D	A	T	P	G	E	L	P	Y
B	39/40	I	V	G	G	T	D	A	K	P	G	E	L	P	Y
C	21	I	V	G	G	T	E	V	T	P	G	E	L	P	Y
	42	E	V	T											
	Try Pv III	I	V	G	G	S	D	A	T	P	G	E	L	P	Y

* cDNA variants according to Klein et al. (1996, 1998).

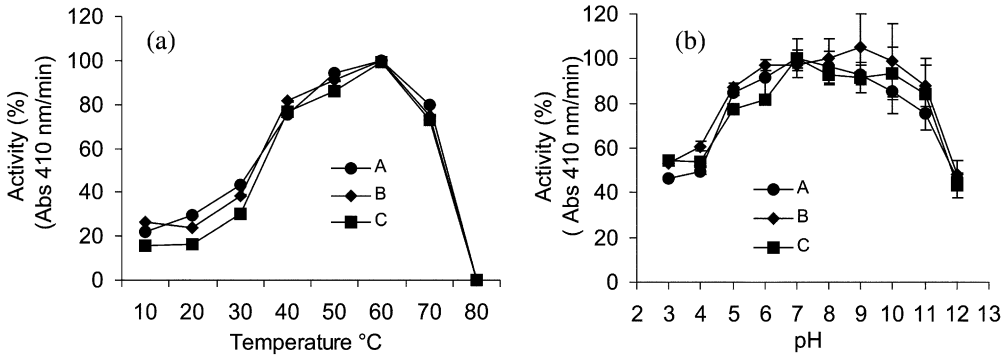

Fig. 2. Effect of temperature (a) and pH (b) on *P. vannamei* isotrypsin activities. *Footnote*: No statistical differences were found among isotrypsins. (α=0.05), (S.E.M., n=3).

Isoform C has one Asn and two Ser more and one Leu and two Thr less than variants A and B. The content of glycoside-binding amino acids matches results from SDS-PAGE, in which isoforms A and B have the same molecular mass and are different from isoform C. However, complexity of a glycoprotein and the subtle differences between isoforms are worthy of future investigation.

According to the nucleotide sequences reported by Klein et al. (1996, 1998) obtained from cDNA analysis, six variants of trypsin exist in the genome of *P. vannamei*: 30, 39, 40, 21, 42 and Try pv III. According to the number of nucleotide substitutions, variants were grouped into family 1 (30, 39 and 40), family 2 (21 and 42), and family 3 (Try pv III). Variants from family 1 differ in 5–7 nucleotides, compared with family 2 variants differing in 41 nucleotides. However, the enzymes were never isolated and characterized. According to the amino terminal sequence found in the present study, trypsin A match's variant 30, trypsin B matches variant 39 or 40, and trypsin C matches variant 21.

According to the IP of the three purified isotrypsins, they are anionic forms. The IPs from *P. vannamei* isotrypsins are similar to those found in other marine organisms, such as digestive trypsin A and B from anchovy *Engraulis engrasicholus*, which have IPs of 4.6 and 4.9, respectively (Martínez et al., 1988), and two trypsins from the tiger shrimp *P. monodon* with IPs of 2.1 and 2.4 (Lu et al., 1990). Differences in IPs among the three isotrypsins from *P. vannamei* support the amino acid terminal sequence data, indicating that they are in fact different isoforms.

Besides other factors, activity and stability of an enzyme are functions of temperature and pH (Copeland, 2000). In *P. vannamei*, 80% or more of trypsin activity is maintained over a broad range of temperature (40–70 °C). Temperatures for maximum trypsin activity in other organisms are quite narrow; trypsin from the digestive tract of *P. monodon* exhibited maximum activity between 55 and 65 °C (Jiang et al., 1991), trypsin from the crustacean *Triops* sp. reached maximum activity between 50 and 60 °C (Maeda-Martínez et al., 2000), and in the sand crab (*Portunus pelagicus*) the maximum was at 50 °C (Zwilling and Neurath, 1981). Digestive trypsin of *Clibanarius striolatus*, *Uca urillei*, *Pagarus bernhardus* and *Euphasia superba* had their highest activity at 53 °C, 52 °C, 48 °C and 53 °C, respectively (Dittrich, 1992).

The pH range over which a native enzyme will be stable varies from one protein to another. Trypsins from *P. vanna-*

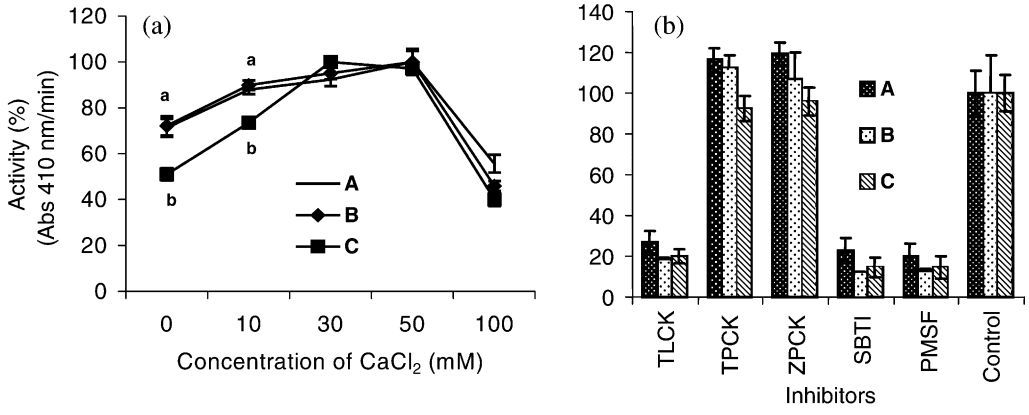

Fig. 3. Effect of $CaCl_2$ concentration (a) and inhibitors (b) on *P. vannamei* isotrypsin activities. *Footnote*: Statistical differences were significant when letters are different (α=0.05), (S.E.M., n=3).

Table 4
Comparison of kinetic values of several trypsins

Enzyme	K_m (mM)	K_{cat} (S^{-1})	K_{cat}/K_m (S^{-1} mM^{-1})	Reference
trypsin A	0.000304	0.433	1424	
trypsin B	0.000342	0.37	1081.8	
trypsin C	0.000272	0.583	2142.3	
U. pugilator	0.0069	2.0	290	Grant et al. (1980)
C. carpio	0.039	3.10	79.5	Cao et al. (2000)
Penaeus monodon	N.D.	N.D.	20.4	Jiang et al. (1991)
Euphausia superba				
Enzyme II	0.04	0.74	18.5	Osnes and Mohr, 1985
T. orientalis	0.093	0.91	9.7	Johnston et al. (1995)
E. encrasicholus	0.66	3.2	4.84	Martínez et al. (1988)
C. fumiferana	0.025	N.D.	N.D.	Milne and Kaplan, 1993
G. ogac	1.25	N.D.	N.D.	Simpson and Haard, 1984

N.D.=non-available.

mei keep more than 80% activity over a pH range between 6 and 11. The pH for maximum activity of trypsins in other organisms, such as in crayfish is between 7 and 8 (Dionysius et al., 1993). Highest hydrolysis ability of three trypsins from the digestive gland of *P. monodon* is between pH 7 and 8 (Jiang et al., 1991). Maeda-Martínez et al. (2000) evaluated the influence of pH on two trypsins from two morphotypes of *Triops* sp., showing maximum activity at pH 8. Trypsins from organisms other than crustaceans also have maximum activity in the alkaline range of the pH scale: *Lygus lineolaris* (Hemiptera), pH 10 (Zeng et al., 2002); pancreatic trypsin from chicken, pH 8 (Guyonnet et al., 1999); carp, *C. carpio*, pH 9 (Cao et al., 2000).

Concentration of Ca^{2+} differently affects the *P. vannamei* isotrypsins. Isotrypsin C needs higher Ca^{2+} concentrations to reach the same activity as isotrypsins A and B. The activity of the three isotrypsins is the same at 30 mM Ca^{2+}. In the presence of Ca^{2+}, trypsins undergo a conformational change giving stability to the enzyme (Walsh, 1970), as occurs in ostrich trypsin activity, which is enhanced with $CaCl_2$ at concentrations between 10 and 100 mM. Calcium binds to Asp or Glu residues at specific trypsin positions (Rypniewski et al., 1994). The three trypsins from *P. vannamei* have Glu available for Ca^{2+} binding in positions 70, 80 and 230. At positions 21 and 62, isotrypsin A has Asp and Glu, and isotrypsin B has Glu and Asp. Isotrypsin C differs from trypsin A and B at position 68, where a Ser is present. These substitutions can be responsible for differences in Ca^{2+} binding and hence in trypsin activity. From these data, it appears that 30 mM $CaCl_2$ used in the substrate solution is quite adequate to ensure maximum activity of the three trypsins and facilitates the specific activity measurements.

The pure shrimp trypsins show properties of serine proteases. They are inhibited by PMSF, which is consistent with the presence of serine and histidine residues at the catalytic site. Inhibition by TLCK indicates that these serine proteases are indeed trypsins. ZPCK and TPCK did not affect activity of the purified isoforms, confirming their identity (Whitaker, 1994).

K_m values of the three isotrypsins from *P. vannamei* are all quite different, and the values are smaller than trypsins from different organisms. The lowest K_m is observed in trypsin C; the K_m average of the three trypsins is 5566 times smaller than Greenland cod (*Gadus ogac*) and 2157 times smaller than trypsin B from the European anchovy (*Engraulis encrasicholus*). Smaller differences were found in trypsins from spruce budworm (*Chroristoneura fumifera*) and fiddler crab (*Uca pugilator*) only 81 and 22.5 times smaller, respectively. Data show that isotrypsins from *P. vannamei* have higher affinity for the substrate BAPNA than other trypsins of marine origin. Physiological efficiency (K_{cat}/K_m), for isotrypsin C is as much as two-fold higher than isotrypsins A and B, which is probably, related to the amino acid substitutions in this enzyme. Although the amino acids at the active site, the specific pocket, Cys bridges, and other important residues are conserved, isoform C presents higher specificity and conversion rate of substrate to product. Average physiological efficiency of the three trypsins is 1549. It is 5.2 times higher than the physiological efficiency of trypsin from *U. pugilator* and 19.5 fold higher than enzymes from *C. carpio*. Since physiological efficiency is an indicator of enzyme competence, we can reasonably speculate that physiological efficiency compensates for the short residence time for food in the digestive tract.

Regarding the question, what is the contribution of each of the isotrypsins in food protein digestion in the white shrimp? We can only speculate that because they are present, forming different phenotypes (data to be published elsewhere), organisms presenting particular phenotypes may benefit adaptable characteristics for acclimatizing to the environment. If this is found to be true, knowing the phenotypes will aid in selection of families for better performance in aquaculture. At least in salmon, trypsin phenotypes have special roles in food protein digestion (Rungruangsak and Male, 2000). Moreover, growth has been correlated with isotrypsin phenotype patterns due to the differences in digestion in vitro for the same dietary

proteins (Bassompierre et al., 1998). In this work we showed that trypsin isoforms in *P. vannamei* have different kinetic properties, and thus, we suggest that phenotype could be related to better protein digestion. This hypothesis has to be proven in future studies.

In summary, three of six trypsin sequences deduced by Klein et al. (1996, 1998) are translated in the digestive gland of *P. vannamei*. Differences in amino acid sequences are reflected in variations of the studied parameters, in which isoforms A and B are closely related and less connected to isoform C. Differences in amino acid sequences among the three isoforms suggest an early separation of isotrypsin C from isotrypsins A and B. We are gaining knowledge for understanding physiological regulation of digestive proteases in penaeids. Besides the information in the present paper, we have demonstrated unequivocally that trypsins are synthesized as zymogens (Sainz et al., 2004). The picture points to the fact that shrimp digestive enzymes are regulated similar to frequent feeders, in contrast to that of sporadic feeders. Besides physiological functioning, characteristics of the three isotrypsins make them suitable biological reagents for biotechnology purposes.

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