# PURIFICATION AND CHARACTERIZATION OF CHYMOTRYPSIN FROM *PENAEUS VANNAMEI* (CRUSTACEA: DECAPODA)

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## ABSTRACT

*The purification and characterization of a chymotrypsin from the hepatopancreas of the white shrimp* Penaeus vannamei *is described. Only one chymotrypsin was detected in contrast to other shrimp that have two major forms.* P. vannamei *chymotrypsin has a molecular mass of 33.2 kDa and a* pI *of 3.1. The molecular mass is high relative to other penaeid chymotrypsins. The proteinase is acid labile and exhibits optimum activity at pH 8. The enzyme is thermostable both at 25 and 37C. It is a serine proteinase. Phenylmethylsulphonyl fluoride and soybean trypsin inhibitor blocked the activity of the enzyme, and it was not affected by chymotrypsin inhibitors such as tosyl-Phe$CH_2Cl$ or N-carbobenzoxy-L-Phe-$CH_2Cl$. Protein profiles of the hepatopancreas from two populations varied*

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*and this is suspected to be caused by differences in the expression of chymotrypsin in the white shrimp.*

## INTRODUCTION

The deterioration of fishery products by proteases is of major concern (An *et al.* 1994; Rodger *et al.* 1984). Proteases from lobster and shrimp are involved in the black spot or melanosis phenomena of their meat after storage (Wang *et al.* 1992; Gopakuma 1990). Proteases from hepatopancreas are also implicated in the autolysis of krill (Kawamura *et al.* 1984) and the mushy texture in crayfish meat (Kim *et al.* 1992). Protease characterization is therefore important to maximize seafood quality.

Trypsin and chymotrypsin are the most abundant proteases in the digestive system of aquatic organisms. Most knowledge of chymotrypsin is based on mammalian sources, although research on the enzymes from other groups of organisms is now available. Chymotrypsins have been purified from cod (Bjarni and Bjarnason 1991), scallop (Le Chevalier *et al.* 1995), abalone (Groppe and Morse 1993), and lugworm (Eberhardt 1992). The catalytic properties of these enzymes, such as hydrolysis of synthetic substrates and the effects of some inhibitors, are similar to those of mammals.

In crustaceans, protein digestion is influenced by the anatomy of the digestive system, which is composed of a foregut, a midgut, and a hindgut (Ceccaldi 1989). The hepatopancreas or midgut gland is an organ that combines functions of the mammalian liver and pancreas and that produces digestive proteases (Glass *et al.* 1989). There is no evidence of acid digestion of protein in penaeids. According to Osness (1985), serine proteases are less specific for synthetic substrates and are able to hydrolyze native protein including collagen (Iida *et al.* 1991). The broader specificity replaces the need for certain enzymes such as elastase and acid proteases.

Crustacean chymotrypsin hydrolyzes synthetic substrates in a narrow range of specificity and seems to possess unique catalytic properties (Tsai *et al.* 1986). This paper presents the isolation, purification and properties of chymotrypsin from the white shrimp, *Penaeus vannamei*.

## MATERIALS AND METHODS

### Reagents

Succinyl-$(Ala)_2$-Pro-Phe-nitroanilide, SAAPFNA; tosyl-Lys-chloromethyl ketone, TLCK; tosyl-Phe-chloromethyl ketone, TPCK; N-carbobenzoxy Phe-

chloromethyl ketone, ZPCK; soybean trypsin inhibitor, SBTI; 1,10-phenanthroline; ethylenedinitrilotetraacetic acid disodium salt, EDTA; phenylmethanesulfonyl fluoride, PMSF; and sodium dodecyl sulfate, SDS, were purchased from Sigma Chemical Co. (St. Louis, MO).

## Shrimp Hepatopancreas Extraction

*Penaeus vannemei* were obtained from the CIBNOR shrimp farm and kept alive until the process began. The hepatopancreas was dissected from the decapitated animals and homogenized in cold water (1:1.5, w/v), then centrifuged at 4500 g for 30 min to eliminate lipids and tissue debris. The aqueous supernatant, called the crude extract, was freeze-dried.

## Assay of Enzyme Activity

The substrate stock, 1.75 mM SAAPFNA, was prepared in sterilized 50 mM TRIS buffer, containing 20 mM $CaCl_2$, pH 7.5. This stock was stable up to 2 months and was diluted when assays were done. The substrate stock solution was diluted five times in the same buffer for detection during purification, SAAPFNA final concentration in the reaction mixture was 0.05 mM. For characterization, the substrate stock was diluted 2.5 times in the same buffer and SAAPFNA concentration was 0.1 mM. One hundred microliters of diluted substrate were added to 590 $\mu$L of buffer. The reaction was started when 10 $\mu$L of enzyme preparation was added. The final volume of the reaction mixture was 700 $\mu$L. Absorbance was recorded continuously at 410 nm. Reaction was considered linear if the change in absorbance per minute was below 0.1/min at 410 nm (Geiger 1984); otherwise samples were diluted and a factor was applied to calculate the activity. The absorbance was recorded continuously at 410 nm for 3 min for characterization assays and 1 min for the purification process. Kinetic constants were determined by Lineweaver-Burk method with SAAPFNA concentration of 0.1 to 0.2 mM.

## Enzyme Purification

Freeze-dried hepatopancreas extract was dissolved in 20 mM $NaH_2PO_4$, containing 20 mM NaCl, pH 7.0. The sample was dialyzed in this buffer for 8 h with several changes of it. The extract was loaded onto a Q-Sepharose fast flow column, previously equilibrated with 20 mM $NaH_2PO_4$, containing 20 mM NaCl, pH 7.0. The column was eluted using a linear gradient of NaCl from 0.2 to 0.6 M. All steps were done at 4C. Fractions with chymotrypsin activity were pooled and concentrated in an Amicon PM 10 and loaded onto a phenyl-Sepharose column previously equilibrated with 50 mM TRIS, containing 0.5 M $(NH_4)_2SO_4$,

pH 7.5. Chymotrypsin was eluted by adding ethylene glycol and by lowering ammonium sulfate concentration in a linear gradient. Equilibration and elution were done at 25C. Fractions with chymotrypsin activity were pooled and concentrated by using an Amicon PM10.

Preparative electrophoresis was run in a 1.5 mm, 7% acrylamide gel. Duplicate samples were diluted 1:2 with sample buffer according to manufacturer's standard instruction (Bio-Rad). Total protein added was 0.04 mg. Gels were electrophoresed at constant current of 15 mA per slab for 2.5 h. The gel was cut and one sample was stained with Coomassie blue. When the protein was visualized in the stained gel, the corresponding protein in the unstained gel was cut. This acrylamide slide was introduced to Bio-Rad electroelution system for 4 h using 50 mM ammonium bicarbonate as running buffer at constant current of 20 mA. Protein eluted from the gel was recovered in a PM10 membrane. Protein concentration was determined according to Bradford (1976) or Whitaker and Granum (1980) when protein concentration was low or high, respectively.

Purified enzyme was electrophoresed in duplicate through a discontinuous gel (4% stacking gel and 12.5% separating gel) at 15 mA per gel (1.45 h) in a minigel apparatus at 5C in a refrigerated circulating bath. Protein purity was confirmed by silver staining of the gel. The molecular weight of the enzyme was estimated using standard molecular weight markers from Sigma (St. Louis, MO).

## Enzyme Characterization

The isoelectric point was determined with a Pharmacia Phast system (Pharmacia). One hundred nanograms of purified protein was loaded in a precast gel of pH 3 to 9 (Pharmacia). A first step of 16 volts/h was followed with 410 volts/h. Protein was silver stained. Isoelectric point was calculated according to the migration of known isoelectric markers.

The purified chymotrypsin was sent for amino terminal protein analysis at Uppsala Genetical Center. Protein was subjected to Edman degradation and amino acids were determined in a HPLC (C-18) system.

The effect of inhibitors on purified chymotrypsin were evaluated according to Garcia-Carreño (1992). Serine proteases inhibitors, chelators, and chymotrypsin inhibitors were used. The effect of pH on chymotrypsin activity was determined with buffered solutions at pH 3 to 11 in universal buffer containing the same mixture of salts (Stauffer 1989) and using 0.1 mM SAAPFNA as the substrate. pH stability of the enzyme was studied by incubating the purified chymotrypsin in buffered solutions at defined pH values for defined intervals. Thermostability was studied by incubating the chymotrypsin at defined temperatures for 60 min. The concentration of pure chymotrypsin in these assays was 0.15 $\mu$g. Each experiment was done in triplicate.

## RESULTS

### Purification

Ion-exchange chromatography served as the first step of purification. One peak of chymotrypsin activity was recovered from the Q-Sepharose fast-flow column at a concentration of 0.4 M NaCl. Hydrophobic-interaction chromatography was used as the second step of purification. Chymotrypsin activity eluted from the phenyl-Sepharose column when the gradient reached a concentration of 32.5% ethylene glycol and 0.25 M ammonium sulfate (Fig. 1). The enzyme was electroeluted from preparative polyacrylamide gels. Recovered protein was assayed for purity in SDS-PAGE and stained with silver (Fig. 2a). A single band was observed on the gel corresponding to *P. vannamei* chymotrypsin. The low yield may have been caused by inefficient electroelution from the gel or loss of protein

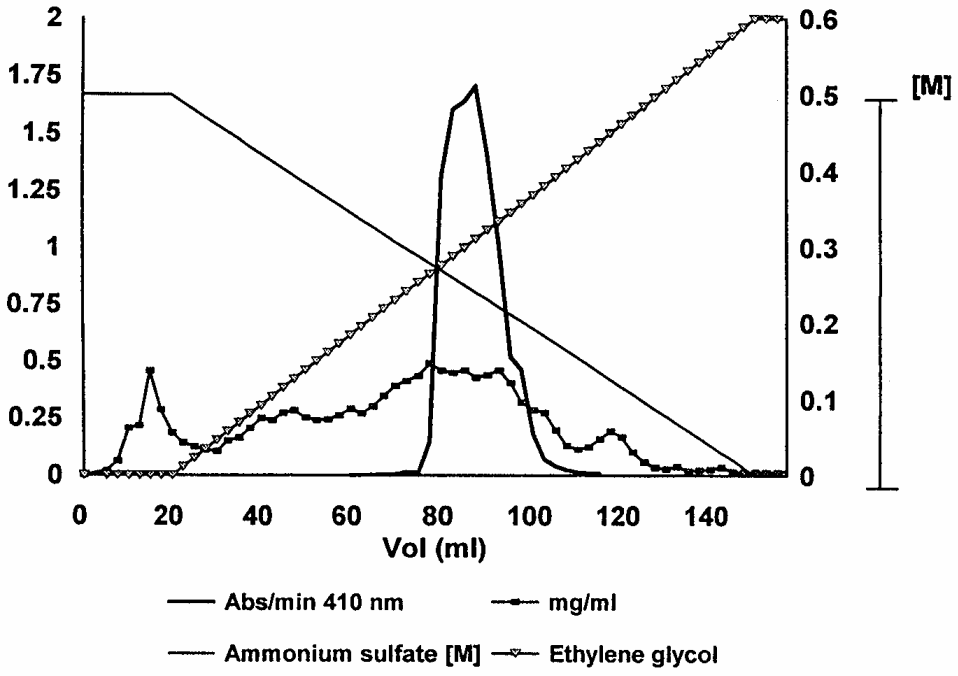


FIG. 1. PURIFICATION OF THE *PENAEUS VANNAMEI* CHYMOTRYPSIN AFTER Q-SEPHAROSE FAST FLOW CHROMATOGRAPHY

Phenyl Sepharose was used as ligand in 8.08 mL column. Fractions of 2.5 mL were collected and flow rate of 30 mL/h was used. Protein concentration is expressed as mg/mL. Activity is expressed by the $\Delta A/\Delta t$ of hydrolysis of SAAPFNA. Protein was eluted by a simultaneous gradient that increase the percentage of ethylene glycol and decrease ammonium sulfate concentration.

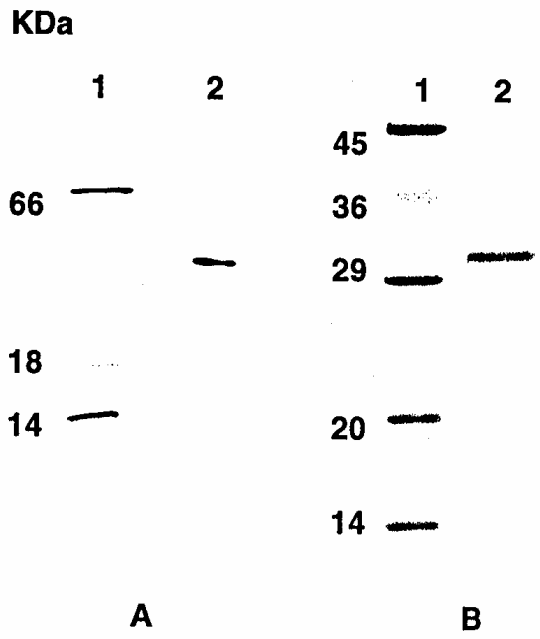


FIG. 2. PURIFIED SHRIMP CHYMOTRYPSIN
A, silver stained 12.5% SDS-PAGE and, B. Coomassie blue stained gel. Lanes: 1, molecular mass markers; 2, *P. vannamei* chymotrypsin. Chymotrypsin purity is shown by silver staining and molecular mass was calculated according to the migration of the markers.

in the filter (Table 1). In all purification steps, a single form of the chymotrypsin was found. The protein patterns of fractions recovered from the different purification steps are shown in Fig. 3.

## Molecular Properties

A molecular mass of 33.2 kDa was calculated (Fig. 2b). Treatment with the reducing agents DTT or beta-mercaptoethanol showed that this enzyme is a monomer. The enzyme had an isoelectric point of 3.1 and a single band was present (Fig. 4). The N-terminal sequence of 30 amino acids was compared to other chymotrypsin and to related serine proteinases (Fig. 5). The N-terminus was identical to the one reported previously (Van Wormhoudt *et al.* 1992) and was 93% homologous to crab serine collagenase (Tsu and Craik 1996), 30% homologous with hornworm chymotrypsin (Peterson 1995), and 10.9% with abalone chymotrypsin (Tsai *et al.* 1991).

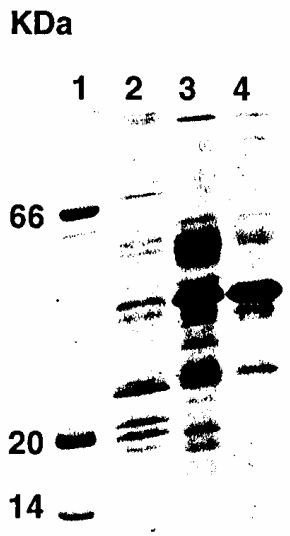


FIG. 3. SDS-PAGE PROTEIN PATTERNS OF *P. VANNAMEI* FRACTIONS
Lanes: 1, molecular mass markers; 2, hepatopancreas extract; 3, ion-exchange fractions; 4, hydrophobic chromatography fractions.

## Enzymatic Properties

The pH profile was a bell-shaped curve with 50% activity at pH values of 6.6 and 10.1 (Fig. 6). Total inactivation of the enzyme occurred at 25C immediately following mixing of enzyme with the buffer. Alkaline activities above pH 11 were not evaluated because the substrate hydrolyzed spontaneously. The stability of chymotrypsin was tested over a pH range of 4 to 12 (Fig. 7), the chymotrypsin was stable at pH 8 for 40 min.

A thermostability of chymotrypsin profile is shown in Fig. 8. The chymotrypsin retained 84% activity after 60 min at 50C and was stable for 60 min at 37C.

Kinetic values are shown in Table 2. The Km for SAAPFNA was 1.6 mM, similar to those found in *Penaeus monodon* (Tsai *et al.* 1991). The ratio kcat/Km, was also similar to the *P. monodon* enzyme. Km values of *P. monodon* and *P. vannamei* were higher than for abalone (Groppe and Morse 1993) and bovine chymotrypsin (Del Mar *et al.* 1979), but catalytic efficiencies were lower.

The effect of inhibitors and chelators are shown in Table 3. *P. vannamei* chymotrypsin activity was completely inhibited by 2 mM PMSF or 5 $\mu$M SBTI after 1 h, which is similar to bovine chymotrypsin. However, neither 0.2 mM

TABLE 1.
PURIFICATION OF SHRIMP CHYMOTRYPSIN

| Fraction | Protein (mg) | Total activity (U) | Specific activity (U/mg) | Fold | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 2060 | 8001 | 3.9 | 1 | 100 |
| Anion exchange | 104 | 4045 | 38.8 | 10 | 50 |
| Hydrophobic interaction | 13.5 | 2947 | 218.3 | 56 | 36.8 |
| Electroelution | 0. 03 | 14 | 435.7 | 112 | 0.18 |

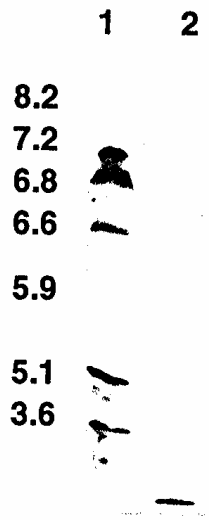


FIG. 4. ISOELECTRIC POINT OF *P. VANNAMEI* CHYMOTRYPSIN
Lanes: 1, standards; 2, *P. vannamei* chymotrypsin. Isoelectrofocusing was done over a pH range of 3 to 9.

| | 10 | 20 | 30 |
|---|---|---|---|
| *Penaeus vannamei* [a] | IVG GVE ATP HSW PHQ AAL FID DMY F*G GSL | | |
| *Penaeus vannamei* [b] | IVG GVE ATP HSW PHQ AAL FID DMY FCG GSL | | |
| Crab collagenase [c] | IVG GVE AVP NSW PHQ AAL FID DMY FCG GSL | | |
| *Manduca sexta* [d] | IVG GSS SSV GQF PYQ AGL VIT LPR GTA ACG | | |
| Abalone chymotrysin [e] | IVG GSN AAA GEF PWQ GSL QVR SGT SWF HIC | | |

FIG. 5. COMPARISON OF THE N-TERMINAL SEQUENCE OF THE CHYMOTRYPSIN OF *PENAEUS VANNAMEI* AND RELATED ENZYMES
Positions that are occupied by different amino acid residues of shrimp chymotrypsin are indicated in bold. a, this work; b, Sellos and Van Wormhoudt (1992); c, Tsu and Craik (1996); d, Peterson *et al.* (1995) and, e, Groppe and Morse (1993). Asterisk indicates not determined.

TPCK nor 0.5 mM ZPCK, both known as mammalian chymotrypsin inhibitors, affected the shrimp enzyme. The chelator EDTA and the trypsin inhibitor TLCK had negligible effects. The chelator 1,10-phenanthroline, at 4 mM, reduced the activity to 14%, and is consistent with the influence of divalent ions such as $Ca^{2+}$ in the activity of chymotrypsins (Bjarni and Bjarnason 1991).

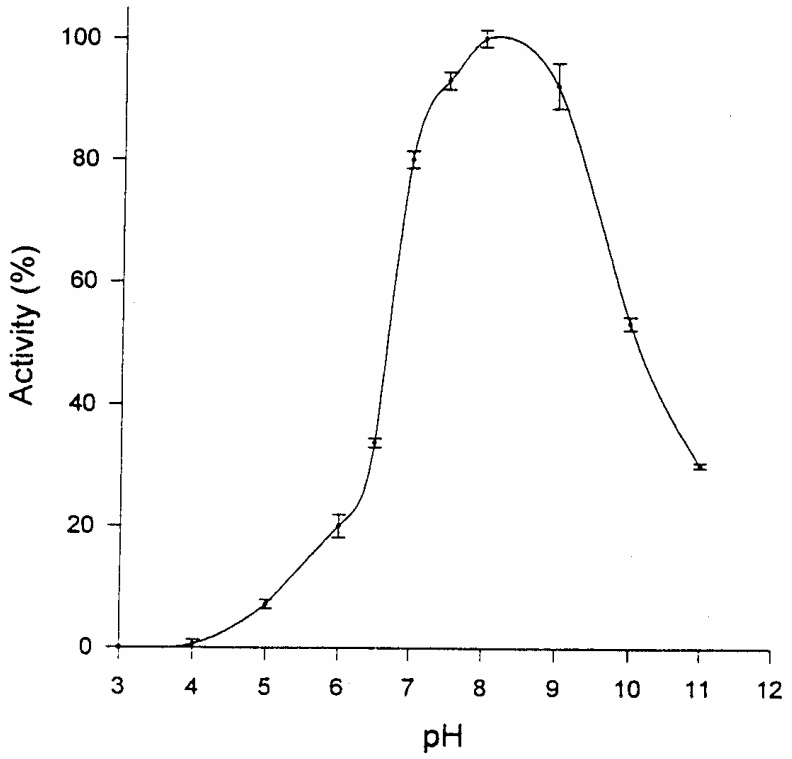


FIG. 6. EFFECT OF pH ON SAAPFNA HYDROLYSIS BY *P. VANNAMEI* CHYMOTRYPSIN

Assays were done by dissolving the substrate at different pH values. Results are expressed as the percentage of maximum activity. Data was the average of triplicate experiments. Standard deviations are indicated.

## DISCUSSION

The chymotrypsin from the hepatopancreas of *Penaeus vannamei* was purified upon homogenization by ion-exchange and hydrophobic chromatography. In the present work, we purified and studied the molecular and enzymatic properties of chymotrypsin from *P. vannamei*.

Early work on this shrimp species chymotrypsin (Van Wormhoudt *et al.* 1992) showed that the enzyme had a molecular weight of 25 kDa by SDS-PAGE. The molecular mass deduced from amino acid sequence of the cDNA (Sellos and Van Wormhoudt 1992) was 23.8 kDa and the deduced *pI* was 4.46. The chymotrypsin purified in this study had molecular mass of 33.2 kDa and a *pI* of 3.1. The

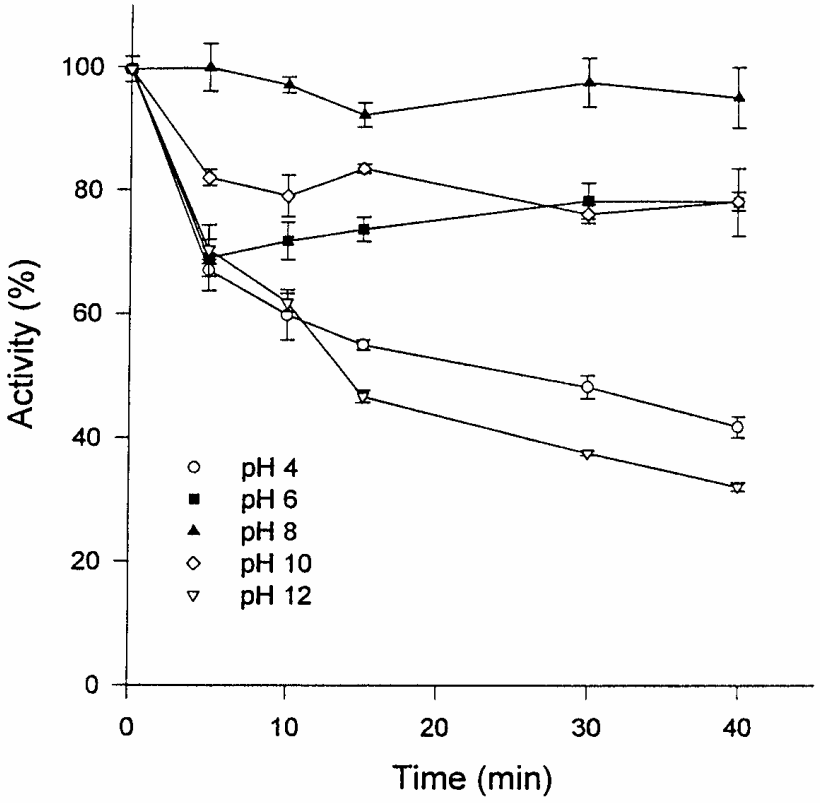


FIG. 7. pH STABILITY OF *P. VANNAMEI* CHYMOTRYPSIN
Assays were done by incubating the enzyme at different pH values for 40 min. Samples at defined intervals and assayed with SAAPFNA at 25C. Results are expressed as residual activity. Data was the average of triplicate experiments. Standard deviations are indicated.

observed differences between the deduced and calculated molecular weights of shrimp chymotrypsin might be due to glycosylation. An acid *pI* was also found for *P. monodon* (Tsai *et al.* 1991) chymotrypsins, with molecular masses of 27 and 26 kDa.

Kinetic and enzymatic studies of this enzyme will be necessary for understanding the digestive function of this shrimp. This proteinase hydrolyzed SAAPFNA and therefore hydrolyzed the peptide bond at the carboxyl side of phenylalanine. However, the *P. vannamei* chymotrypsin exhibited a high Km value towards SAAPFNA, as did *P. monodon*. Lower Km values toward this substrate were found for the bovine (Del Mar *et al.* 1979), cod (Kristjansson *et al.* 1995), and abalone chymotrypsins (Groppe and Morse 1993) forms. Substrates, such as ben-

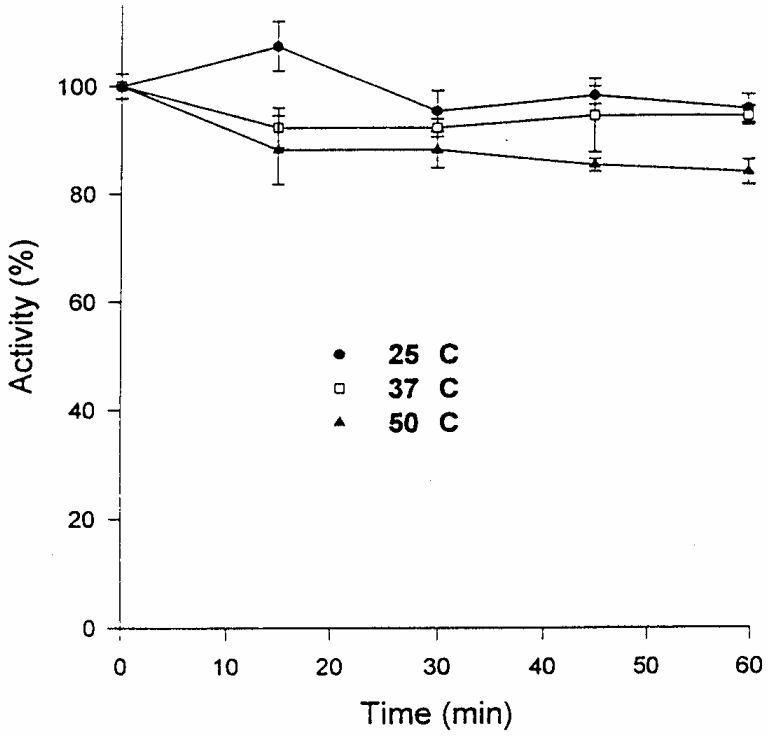


FIG. 8. THERMOSTABILITY OF *P. VANNAMEI* CHYMOTRYPSIN
Assays were done by incubating the enzyme for 60 min at 25, 37, and 50C at pH 7.5. Samples were taken every 15 min and assayed with SAAPFNA. Results are expressed as percentage of residual activity. Data was the average of triplicate experiments. Standard deviations are indicated.

zoyl or acetyl esters coupled to tyrosine, were not hydrolyzed by *P. vannamei* chymotrypsin.

The pH optimum of *P. vannamei* chymotrypsin was similar to other invertebrate and vertebrate chymotrypsins, including *Penaeus indicus* trypsin (Honjo *et al.* 1990) and serine collagenolytic protease of the greenshore crab, *Carcinus maenas* (Roy *et al.* 1996). In general, proteinases from crustaceans have maximum activities from neutral to alkaline pH, similar to mammalian enzymes but, unlike the mammalian enzymes, they are acid labile. The effect of pH shows that white shrimp chymotrypsin was more stable at alkaline than acid conditions. This result relates to the pH of the intestine (pH 7.4) and the absence of acid degradation of proteins by shrimp.

The acid-labile properties and mild activity above room temperature make shrimp chymotrypsin a candidate for food technology applications. Protein

TABLE 2.
MOLECULAR AND ENZYMATIC PROPERTIES OF CHYMOTRYPSINS

| Species | Forms | MW (kDa) | pI | *Km* (mM) | *Kcat* ($s^{-1}$) | *Kcat/Km* $10^3$ | pH max activity |
|---|---|---|---|---|---|---|---|
| *Pecten maximus* [1] Scallop | 3 | 32.0 | ---- | ---- | ---- | ---- | 8/8.5 |
| *Haliotis rufescens* [2] Abalone | 1 | 28.0 | ---- | 0.01 | 32.0 | 4600 | 8/8.5 |
| *Arenicola marina* [3] Marine lugworm | 2 | 24.2 | 3.5/5 | ---- | ---- | ---- | 8 |
| *Penaeus monodon* [4] Shrimp | 2 | 27/26 | 3.2/3.3 | 8.9/0.56 | 250/7.8 | 28/14 | ---- |
| *Penaeus vannamei* [5] Shrimp | 2 | 25/25 | ---- | ---- | ---- | --- | ---- |
| ***Penaeus vannamei**** **Shrimp** | 1 | **33.2** | **3.1** | **1.6** | **15.5** | **10** | **8** |
| *Manduca sexta*[6] Tobacco hornworm | 1 | 24 | ---- | ---- | ---- | ---- | 10.5-11 |
| *Gadus morhua*[7] Cod | 2 | 26.0 | 6.2/5.8 | 0.09 | 65.8 | 731.1 | 8 |
| Bovine[8, 9] | 1 | 25.0 | 9.1 | 0.08 | 26.0 | 330 | 7.5 |

Kinetic values were obtained with SAAPFNA
*This work; 1, Le Chevalier et al. (1995); 2, Groppe and Morse (1993); 3, Eberhardt (1992); 4, Tsai et al. (1991); 5, Van Wormhoudt et al. (1992); 6, Peterson et al. (1995); 7, Kristjansson et al. (1995); 8, Del Mar et al. (1979) and 9, Laskowski. (1955).

TABLE 3.
DIFFERENTIAL EFFECTS OF PROTEASE INHIBITORS ON SHRIMP AND BOVINE CHYMOTRYPSIN

| Compound | Concentration (mM) | Target protease | *P. vannamei* | Bovine chymotrypsin |
|---|---|---|---|---|
| PMSF | 2 | Serine | 99* | 92 |
| SBTI | 5 µM | Serine | 100 | 100 |
| TPCK | 0.2 | Chymotrypsin | 0 | 98 |
| ZPCK | 0.5 | Chymotrypsin | 0 | 93 |
| TLCK | 0.2 | Trypsin | 0 | 93 (bovine trypsin) |
| 1,10-Phenanthroline | 4 | Metallo | 14 | 10 |
| EDTA | 0.4 | Metallo | 0 | 0 |

*Activity is expressed as percentage of inhibition. Each compound and the enzyme were incubated for one hour at 25 °C. Aliquots of this mixture were assayed with SAAPFNA.

modification by controlled hydrolysis enhanced the functionality of raw protein (Panyam and Kilara 1996). Food-grade proteases are needed to work at different temperature optima. Inactivation of enzymes to achieve the desired degree of hydrolysis is done by lowering the pH of the reaction. Further studies in this direction are needed to establish the conditions for protein hydrolysis for possible application of shrimp chymotrypsin.

The effects of PMSF and STBI establishes that this chymotrypsin is a serine protease. These results are similar to those obtained with bovine chymotrypsin. However, the effects of TPCK and ZPCK differ from the mammalian and other invertebrates enzymes which are inhibited by chloromethyl ketone derivatives. Shrimp chymotrypsin also bound to *p*-aminobenzamidine-Sepharose, a ligand that is a competitive inhibitor of trypsin, and was eluted with butylamide. The ability of chymotrypsin to bind to *p*-aminobenzamidine illustrates the broader specificity of crustacean proteinases. Preliminary assays with a soybean-trypsin agarose column showed that white shrimp chymotrypsin was retained very tightly by this affinity matrix and it was not possible to release an active enzyme, which is similar to crayfish trypsin (Zwilling and Neurath 1981). Our results show the active site of decapod chymotrpsin has different catalytic properties than chymotrypsins from other organisms.

The idea that chymotrypsin is absent in all crustaceans is now known to be incorrect (Glass and Stark 1994). Garcia-Carreño *et al.* (1994) demonstrated chymotrypsin can be detected even in crude extracts from the hepatopancreas of decapod langostilla (*Pleuroncodes planipes*). This is confirmed in this report.

In this study, no evidence of a chymotrypsinogen was found. Chymotrypsin and trypsin were found in their active forms when activity was tested in hepatopancreas extracts. Sellos and Van Wormhoudt (1992) found a putative zymogen in *P. vannamei* of 27.2 kDa for chymotrypsin. Nevertheless, chymotrypsin and tryspin from *P. vannamei* and *P. californiensis* (data not shown) were found in their active forms by us. Further studies are needed to explain activation mechanisms of the zymogens. Furthermore, the protein pattern from the hepatopancreas reported previously (Van Wormhoudt *et al.* 1995) and the pattern we found are different. Other work reported that different species of the *Penaeus* genus exhibited different hepatopancreatic protein patterns (Galgani 1988). The protein pattern found in this work showed that *P. vannamei*, as named as others (Sellos and Wormhoudt 1992) and by us in this report are different species. Some differences may be caused by different geographical sites of collection. However, 30 amino acids at the amino terminus of the chymotrypsin in contrast to previous work that reported two forms (Sellos and Wormhoudt 1992). Both gene expression and functionality of forms will be required to explain why more than one form of chymotrypsin is found among some invertebrates and vertebrates, and not others.

It was not surprising to find differences among vertebrate and invertebrate chymotrypsins. However, it was unexpected to find that crustacean chymotrypsins seem to evolve in a special pattern. Molecular biology studies could provide answers to these questions.

## ACKNOWLEDGMENTS

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