

# Digestive Endo-Proteases from the Midgut Glands of the Indian White Shrimp, *Penaeus Indicus* (Decapoda: Penaeidae) from Kenya

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**Abstract**—In order to provide information on the digestive capacity of marine crustacea of mariculture potential in Kenya with a view to aiding the development of suitable feeds to support the industry, a biochemical survey was made of enzymes of extracellular digestion in the Indian White shrimp, *Penaeus indicus*. Results showed midgut gland endo-proteases in wild adult shrimp from the Kenya coast to have optima between pH 7.2 and 8.5 (Trypsin pH 7.5-8.0, Chymotrypsin pH 7.2-7.8, Elastase pH 6.8-8.5) with maximum specific activities of 101-408, 37-516, 70-90 Units mg protein<sup>-1</sup> min<sup>-1</sup> for trypsin, chymotrypsin and elastase respectively. There was no pepsin. The North Sea Norway lobster, *Nephrops norvegicus*, was investigated to a lesser extent and found to have much lower trypsin activity than the shrimp and no chymotrypsin. In addition to the cited serine endo-proteases, significant activity in the shrimp was thought to originate from non-serine proteases. This situation may differ from other shrimps in which serine endo-protease activity, especially trypsin, is dominant. Diphenylcarbamyl chloride (DPCC) and 2-Nitro-4-Carboxyphenyl N,N-Diphenylcarbamate (NCDC) inhibited chymotrypsin but not trypsin, Soybean Trypsin Inhibitor (SBTI), Bowman-Birk Chymotrypsin-Trypsin Inhibitor (BBSTCI), N-Tosyl-L-Phenylalanine Chloromethyl Ketone (TPCK), 4-(2-Aminoethyl)-Benzenesulfonyl fluoride Hydrochloride (AEBSF) and N-Tosyl-L-Lysine Chloromethyl Ketone/1-Chloro-3-Tosylamido-7-Amino-L-2-Heptanone Hydrochloride (TLCK) inhibited both, while Phenyl Methanesulfonyl Fluoride/ Phenylmethyl Sulfonyl Fluoride/  $\alpha$ -Toluenesulfonyl Fluoride (PMSF) and Ovomuroid Trypsin Inhibitor (Ovomucoid/ OTI) precipitated shrimp homogenate. The effect of the former was inferred from the action of AEBSF which together with TLCK inhibited shrimp trypsin more than chymotrypsin. In contrast, TPCK inhibited shrimp chymotrypsin more than trypsin.

These results indicate that relying on imported commercial feeds, usually developed for other species or strains of farmed shrimp in other parts of the world, may not only be too uneconomical but may not provide adequate nutrition to local animals if not efficiently digested. There is, therefore, greater need and urgency to establish detailed enzymic profiles and digestive capacities of locally cultured fin and shellfish. Such studies should parallel those prospecting for suitable feed ingredients while developing local capacity for feed technology.

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## INTRODUCTION

Declining shrimp fishery yields and increasing demand has stimulated the expansion of shrimp

farming in the last 40 years (Weidner & Rosenberry, 1992). However, Africa has lagged behind in the exploitation of shrimp resources when over 35.2 million Africans rely on fisheries

for a livelihood (Seki & Bonzon, 1993). Other than increasing local protein reserves, shrimp farming offers a way of exercising greater control over shrimp production. Greater income is possible over a long period with proper management to avoid environmental disasters experienced in some Latin American countries in the initial years (Currie, 1994). Because shrimp farming also enables a wider spread of the production base, employs more people directly and indirectly through associated industries, it can be a long term economic diversifier, especially since most African countries often rely on single-item sources for foreign exchange: cash crops, minerals and tourism or combinations. Unfortunately, the benefits of shrimp farming may not be realised as the indigenous technical expertise and the necessary research base required are not well developed. Few African countries (e.g. Madagascar, Mozambique, Guinea and Kenya) have attempted commercial shrimp farming with limited success. Species utilized include *Penaeus indicus*, *P. semisulcatus*, *P. monodon*, *Metapenaeus monoceros*, *P. notialis* and *Parapenaeopsis atlantica* (Garcia & Le Reste, 1981). However, there are no local, commercially available, specific artificial feeds for these species. The present study was undertaken to provide information on the digestive enzymes of *P. indicus* for the future development of appropriate feeds for its culture in Kenya where it constitutes 95% of the shrimp catch. Activities and pH-profiles of midgut gland enzymes important in digestion were investigated. Accounts of carbohydrase and general proteolytic activities were given in Omondi and Stark (1995, 1996, 2001). The present paper concentrates on the pH-response of endo-protease activities of wild shrimp from Kenya. The North Sea Norway lobster, *Nephrops norvegicus*, was used in some assays to indicate the likely situation in spiny lobsters in Kenya.

## MATERIALS AND METHODS

### Materials

#### *Sample collection*

The over 100 wild adult *P. indicus* used in this study were caught by local fishermen from the areas

around Tsunza beach and Port Reitz on the Kenya coast. Fifty adult *Nephrops* were obtained from the Firth of the Forth estuary on the east coast of Scotland. The animals were frozen in liquid nitrogen and then stored in a deep freezer at -22.4°C until assayed. Some lobsters were kept for several months in recirculating sea water (35 ppt) aquaria held at 15°C and fed on a shrimp diet (Omondi & Stark, 1995) indicating that they could be easily cultured.

### Chemicals

Most chemicals (analytical grade), inhibitors and all commercial enzymes were from SAF (Sigma-Aldrich-Fluka, Dorset, UK). 'Soluble' casein, HCl, NaOH, K<sub>2</sub>CO<sub>3</sub>, KHCO<sub>3</sub>, TCA (Trichloroacetic acid), TEA (triethanolamine) and Triton X-100 (Triton) were from BDH (British Drug Houses/Merck Ltd, Leicestershire, UK) while Calbiochem-Novabiochem (UK) Ltd, Nottingham, UK supplied AEBSE.

### Methods

#### *Preparation of Enzyme Extracts*

Homogenate was prepared as in Omondi & Stark (1995) but samples from *N. norvegicus* were diluted 1/5-1/10 w/v because of very low enzyme activities. After centrifugation, fat mats were removed by glass wool filtration. Enzyme extract was prepared by filtering homogenate supernatant (38,000 x g) through Whatman glass micro fibres (1µM, BDH).

#### *Estimation of Enzyme Activities*

Enzyme numbers are as given in Webb (1992). Protein, pH, and absorbance measurements as well as buffers and statistical analyses were as described in Omondi & Stark (1995).

#### *Total Protease Activity*

Casein (Dabrowski & Glogowski, 1977), azocoll (Galgani & Nagayama, 1987, Glass, 1989), Congo red fibrin (Congo red) (Glass, 1989) and Sulfanilamide azocasein (Charney & Tomarelli, 1947, Galgani & Nagayama, 1986, Galgani & Nagayama, 1987) were used as substrates (Omondi

& Stark, 2001). These were taken to represent 100% proteolytic activity.

### ***Endo-protease Activities***

#### **Pepsin-like activity (EC 3.4.23.1)**

This was estimated using N-Acetyl-Phenylalanyl-3,5-L-diiodotyrosine (Ace-Phe-Dit) (Ryle, 1984). The assay (digest) mixture (DM), containing 0.5 ml of extract ( $1/_{2000}$ , w/v), substrate (0.25 ml, 0.001 M in 0.01 M NaOH) and HCl (0.25 ml, 0.05 M), was incubated at 30°C for  $1/_{4}$  - 1 h. Sodium acetate (4.0 M)-buffered ninhydrin reagent (1.0 ml) was then added to the DM which was then placed in a boiling water bath for 15 minutes. After cooling (in tap water, for 10 minutes) and adding 5 ml ethanol (60%), 570 nm absorbances were read against distilled water reagent blanks and corrected with extract blanks (ninhydrin reagent added after incubation but before addition of substrate and HCl). Amino groups in the midgut gland/hepatopancreas (H/P) even after considerable dilution resulted in very high blank values that also indicated the sensitivity of the assay. Sigma pepsin revealed that most hydrolysis occurred within the first 15 minutes of incubation. A standard incubation time of 20 minutes was therefore adopted. Calibration graphs were prepared using Dit in HCl (0.05 M). Results were expressed as moles (U) Dit released milligrammes of H/P protein<sup>-1</sup> min<sup>-1</sup>. The method was validated using Sigma pepsin (Porcine stomach mucosal pepsin, 3200-4300U (A280nmHb 0.001 mg protein<sup>-1</sup> min<sup>-1</sup>) (160-320U ml<sup>-1</sup>).

#### **Trypsin-like activity (EC. 3.4.21.4)**

Two substrates, Tosyl Arginine Methyl Ester/Toluene Sulphonyl-L-Arginine Methyl Ester (TAME) (Hummel, 1959, Glass, 1989) and Benzoyl-DL-arginine NA hydrochloride (BapNA) (Erlanger *et al*, 1961) were used to determine trypsin-like activity. In the assay for TAME trypsin activity, digest mixtures (DMs) contained TAME (0.01 M, 0.9 ml), buffer (2.8 ml, 0.015 M CaCl<sub>2</sub>) and extract (0.3 ml,  $1/_{250}$  w/v). They were incubated in silica quartz cuvettes (Hellma® Far-UV, 1 cm light path, Merck/BDH) for 10 minutes at 30°C and the change in absorbance at 247 nm followed in a Philips PU 8730 UV/Vis Scanning spectrophotometer (Philips, Holland) against

substrate blanks (TAME 0.9 ml, buffer 2.8 ml, distilled water 0.3 ml) at each pH. The incubation temperature was controlled by means of a Philips PUC 300 Cell Temperature Controller and a Philips PUPH1 Cell Holder. This equipment was used in all initial velocity (rate) measurements. The initial reaction rate followed zero order kinetics. Sigma trypsin (45-2500µg ml<sup>-1</sup>) (Bovine pancreatic Trypsin Type III, 10200-10400 BAEE U ( $\Delta A_{253nm}^{0.001}$ ) mg protein<sup>-1</sup> min<sup>-1</sup>) was used as a check. Results were expressed as change ( $\Delta$ ) in absorbance (A) mg protein<sup>-1</sup> min<sup>-1</sup> and converted into International Enzyme Units (I.E.U =  $\Delta 0.001A$  mg<sup>-1</sup> min<sup>-1</sup>). This unit was used to express total protease activities with casein, azocasein, azocoll and Congo red as well as endo- and exo-protease activities. In pepsin assays, the unit was also expressed as moles of product (Dit).

To corroborate the results of the TAME assay, the chromogenic substrate BApNA (0.001 M) was used and the increase in absorbance over a 10-minute period measured at 410 nm. The digest mixture (DM) consisted of substrate (2.0 ml), Tris buffer (1.3-1.4 ml) and extract (0.1-0.2 ml;  $1/_{5}$  [lobster],  $1/_{40}$  [shrimp] w/v). The final CaCl<sub>2</sub> concentration (0.02 M) was slightly higher than that used with TAME (0.015 M). The use of TEA buffer (0.2 M, CaCl<sub>2</sub> 0.02 M, pH 7.8) and a more concentrated substrate solution (0.004 M) was tried (405 nm, Geiger & Fritz, 1984) and abandoned because BApNA formed a milky-white suspension in water that stuck to the glass container. There was also considerable non-enzymatic substrate autolysis. As the BApNA Km for trypsin activity in the H/P of *P. indicus* is 0.0003 M (Honjo *et al*, 1990), there was excess substrate in the present study. Following the method of Erlanger *et al* (1961), BApNA was completely dissolved in a small quantity (1.0-2.0 ml) of DMSO (Dimethyl sulfoxide) before the addition of buffer or distilled water to make a clear solution which kept well for at least one week at 30°C. Self-hydrolysis set in earlier and to a greater extent in buffer (pH 7.7-8.1) than in water (pH 5.6-6.2).

#### **Chymotrypsin-like activity (EC. 3.4.21.1)**

Products of BTEE (N-Benzoyl-L-tyrosine ethyl ester) hydrolysis were assayed at 253 nm (Hummel, 1959, Glass, 1989). The digest mixture

(DM) was made up of substrate (0.0011 M, 1.4 ml), buffer (1.5 ml) and extract (0.3-0.5 ml,  $1/_{40}$ - $1/_{80}$  w/v [shrimp]). As with TAME, use of high substrate volumes resulted in high initial absorbances that decreased the sensitivity of the method by obscuring any minor changes in absorbance during the 10min incubation at 30°C.

SAPNA (SAAPPNA/N-Succinyl-(Ala)<sub>2</sub>-Pro-Phe-p-NA) (0.001M) was used according to Geiger (1984a) but p-nitroaniline (p-NA) release was measured at 410 nm instead of 405 nm. The DM had Tris (2.9 ml, 0.1 M, 0.005% Triton w/v and CaCl<sub>2</sub> 0.02 M), substrate (0.001 M, 0.5 ml) and extract (0.1 ml,  $1/_{40}$ - $1/_{80}$  [shrimp],  $1/_{5}$  [lobster]) and was incubated at 30°C for 10 minutes.

#### **Elastolytic activity (EC 3.4.21.36/EC.3.4.21.37)**

Two synthetic peptide and two natural chromogenic substrates were used to measure elastolytic activity. Triton (0.05 %) was included in all buffers to reduce enzyme adsorption to the inner walls of tubes and cuvettes (Geiger, 1984a). Plastic cuvettes and polycarbonate centrifuge tubes as described in Omondi & Stark (1995) were also used to further reduce adsorption. Digest mixtures (DMs) were incubated in a water bath shaker (200 strokes/min, Grant Instruments (Cambridge) Ltd, Barrington, England, UK) and centrifuged (5 min, 2000 g) before reading absorbances.

The release of p-NA from SAApNA (N-Succinyl-L-(alanyl)<sub>2</sub>-L-Alanine-p-Nitroanilide), was followed at 410 nm (Bieth *et al*, 1974, Geiger, 1984b). The final DM volume was 3.5 ml (substrate 0.1 ml, extract 0.5 ml, buffer 2.9 ml). The final substrate concentration (0.0004 M) was lower than that of others (0.001 M). Incubation was for 10 minutes but much higher rates were observed after 24 h than the 4 h used by others (Glass, 1989).

N-CBZ-L-Ala-pNPE(N-Carbobenzoxycarbonyl-L-alanine p-nitrophenyl ester) (1.0 ml, 0.009M, 0.003M DM) was suspended with extract (0.5 ml) and buffer (2.0 ml), incubated for Ω-1 h and p-NP released measured at 420 nm (Geneste & Bender, 1969). In fixed time assays, K<sub>2</sub>CO<sub>3</sub> or KHCO<sub>3</sub> (1.0 ml, 0.1 M) was added to supernatant after centrifugation of the DM to enhance the colour of product but in initial velocity measurements, it was not possible to add the alkaline solution.

Orcein (Elastin Orcein) and Elastin (Elastin Congo red) (0.005 g ml<sup>-1</sup> DM) were incubated with extract (0.5 ml) and buffer 3.5 ml for 3-4 h and the colour of supernatants read at 590 nm (Sacchar *et al*, 1955) and 495 nm (Naughton & Sanger, 1961) respectively.

#### **Enzyme Inhibition Studies**

The following inhibitors were used in the present study :

**AEBSF** - 4-(2-Aminoethyl)-Benzenesulfonyl fluoride Hydrochloride

**BBSTCI** - Bowman-Birk Chymotrypsin-Trypsin Inhibitor

**DPCC** - Diphenylcarbonyl chloride

**NCDC** - 2-Nitro-4-Carboxyphenyl N,N-Diphenylcarbamate

**OTI** - Ovomuroid Trypsin Inhibitor

**PMSF** - Phenyl Methanesulfonyl Fluoride/Phenylmethyl Sulfonyl Fluoride/α-Toluenesulfonyl Fluoride

**SBTI** - Soybean Trypsin Inhibitor

**TLCK** - Nα-Tosyl-L-Lysine Chloromethyl Ketone/1-Chloro-3-Tosylamido-7-Amino-L-2-Heptanone Hydrochloride

**TPCK** - N-Tosyl-L-Phenylalanine Chloromethyl Ketone

To distinguish between different activities, inhibitors were pre-incubated with extracts before assays. AEBSF, SBTI, BBSCTI and OTI were dissolved in water, TLCK in ethanol and TPCK in methanol and water. DPCC was dissolved in acetone or propanol while NCDC was used as a suspension in water. PMSF was dissolved in ethanol but did not completely dissolve in propanol. The PMSF-alkanol solution formed a viscous silvery-grey non-particulate precipitate with extract and a suspension in water was employed. The extent of inhibition was expressed as a percentage relative to the specific activity of control digest mixture (DM) containing no inhibitor.

#### **Column Chromatography**

Glass columns (90 cm x 2.5 cm, Wright Scientific Ltd, England), packed with Sephadex G-200 previously equilibrated and degassed in phosphate buffer (0.02 M, pH 6.9), were eluted with the same

buffer at 14-20 ml h<sup>-1</sup> and fractions (6.3 ml) collected in polyethylene tubes using a Biorad model 2110 fraction collector (Biorad Laboratories Ltd., Hemel Hempstead, Herts, England). For enzyme activity assays of fractions, the extract volume was increased and the incubation time also extended in some cases.

## RESULTS

### Pepsin-like Activity

After an incubation period of 60 minutes, the wild *P. indicus* midgut gland/hepatopancreas (H/P) extracts indicated the lack of pepsin activity and thus an absence of this enzyme from the digestive gland of the shrimp (see Fig. 1).

### Trypsin-like activity

An analysis of relative enzyme activity on TAME and BapNA as illustrated in Fig. 2 shows that the optimum pH for *P. indicus* H/P trypsin was around

7.8-8.0, with significant hydrolysis throughout the pH range 7-8. The highest specific activity with TAME was  $103.704 \pm 14.800$  U mg protein<sup>-1</sup> min<sup>-1</sup>. Whereas there was some apparent activity with TAME in strongly acid media (pH 1.5), there was none above pH 10. OTI severely reduced shrimp TAME trypsin activity but was not as effective against Sigma trypsin. The importance of calcium for Sigma and shrimp homogenate trypsins was made obvious by the significant decrease in rates in digest mixtures (DMs) lacking additional CaCl<sub>2</sub> (0.011 M) even when the substrate concentration was doubled. BApNA was a much more convenient and sensitive way of assaying trypsin activity and was stable for several weeks at 30°C. The pH optimum was 7.8-8.0. Supplemental calcium was not as crucial as with TAME and no significant differences were found with shrimp homogenates at pH 7.0. However, at pH >8.5, no activity was observed probably due to unfavourable pH and low calcium amounts. As with carbohydrase (Omondi & Stark, 1995) and total protease activities (Omondi & Stark, 2001), *N. norvegicus* had very low BApNA trypsin activities of  $1.446-1.652 \pm 0.056$

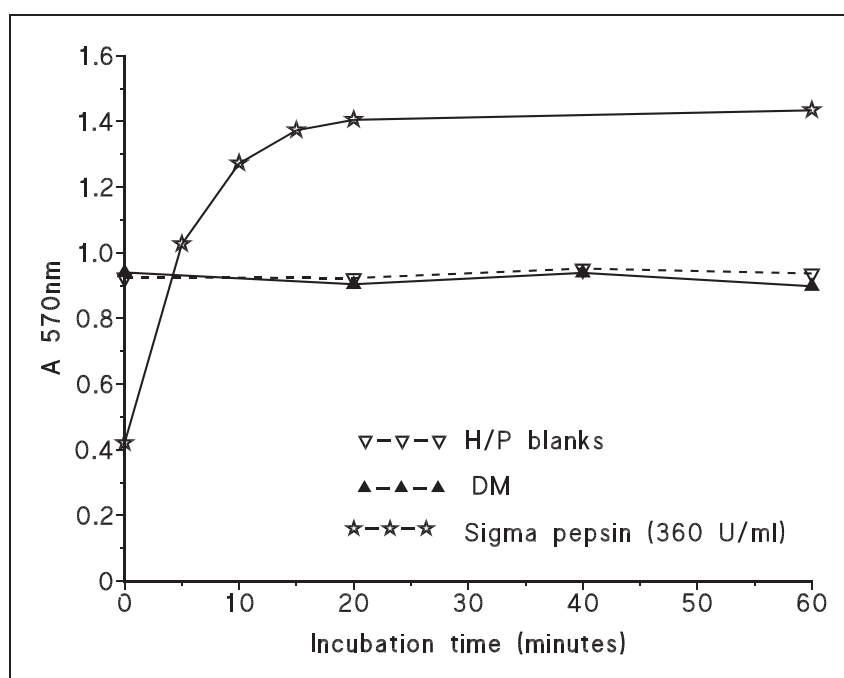


Fig. 1. A time-course activity profile of mammalian (Sigma) pepsin with Ace-Phe-Dit as substrate at 30°C. Values are means  $\pm$  Standard Errors of means

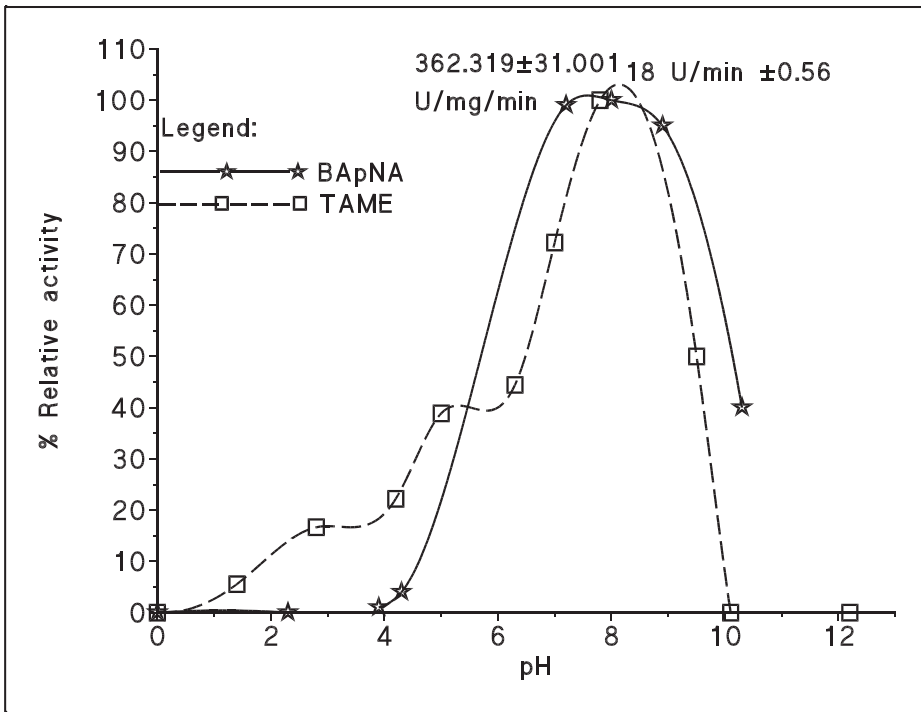


Fig. 2. pH-activity profiles of digestive gland trypsin activity in wild *P. indicus* at 30°C assayed with TAME and BApNA as substrates. Values are means  $\pm$  Standard Errors of the means

compared to  $284.483 \pm 9.069 \text{ U mg}^{-1} \text{ min}^{-1}$  for *P. indicus*.

### Chymotrypsin-like activity

BTEE was problematic due to haziness and precipitation from calcium phosphate and shrimp extract. The pH-activity profile is shown in Fig. 3 where the optimum was pH 7.8-8.2. SApNA was a better substrate with the optimum pH between 7.2-8.0. (see Fig. 3). Use of Triton resulted in higher activities and the molarity of Tris buffer was significant as 0.1 M was better than 0.005 M. It is possible that observations made by some authors that serine protease activities in Tris buffer was less than in citrate phosphate (Schwert & Tanaka, 1955) may depend on Tris concentration. Additional calcium did not significantly affect chymotrypsin activity at all pH values. SApNA chymotrypsin activity was not found in the lobster.

Attempts to distinguish chymotrypsin and trypsin activities using specific substrates and inhibitors were partly successful. NCDC at 1624

$\mu\text{M}$  ( $614.3 \mu\text{g ml}^{-1}$ ) did not significantly inhibit chymotrypsin activity determined with SApNA. This chemical is reportedly a competitive inhibitor/substrate of mammalian chymotrypsin (Erlanger & Edel, 1964) but assays using it as a chromogenic substrate in the present study did not show measurable activity. However, its chloride, DPCC, was a very potent selective inhibitor of chymotrypsin activity as expected (Erlanger & Cohen, 1963, Erlanger *et al*, 1963) inhibiting up to 99 % of shrimp activity within 3 h at optimum acidity. Almost complete inhibition of mammalian Sigma chymotrypsin (Bovine pancreatic chymotrypsin Type IV, 52 BTEE U ( $\mu\text{moles mg}^{-1} \text{ min}^{-1}$ ), occurred within 30 minutes. In contrast, the hydrolysis of BApNA by shrimp homogenate was hardly affected even after long-term pre-incubation with these inhibitors.

The inhibitor TLCK ( $464 \mu\text{M}$ ;  $171.4 \mu\text{g ml}^{-1}$ ) failed to inhibit chymotrypsin activity whereas TPCK inhibited 68 % of that activity. On the other hand, PMSF, did not inhibit either activity significantly. This inhibitor, when dissolved in

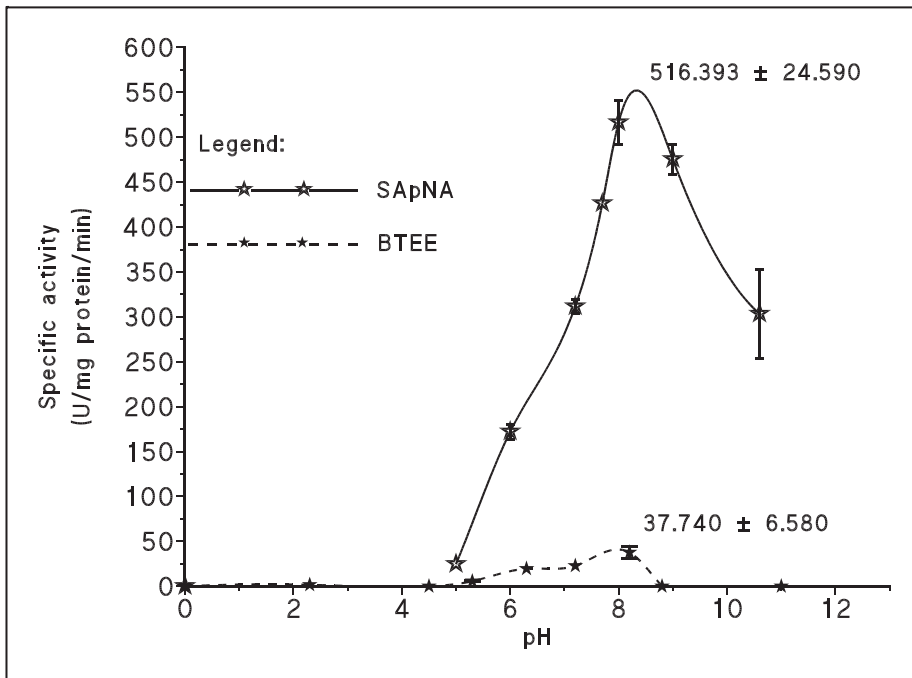


Fig. 3. pH-activity profiles of digestive gland chymotrypsin activity in wild *P. indicus* at 30°C assayed with BTEE and SApNA as substrates. Values are means ( Standard Errors of the means

ethanol or propanol (Fahrney & Gold, 1963, Gold & Fahrney, 1964, Glass, 1989), produced a silvery grey/white viscous precipitate when added at 3278  $\mu\text{M}$  to homogenate and was used suspended in water. In contrast, within 10-20 minutes, BBSTCI caused a 99-100 % inhibition of chymotrypsin and trypsin activities at concentrations as low as 40  $\mu\text{g ml}^{-1}$  but it did not inhibit Sigma trypsin. Finally, AEBSF inhibited 97 % of shrimp trypsin activity but only 27 % of the chymotrypsin, while SBTI inhibited both activities completely.

### Column Chromatography (Sephadex G-200)

Both trypsin and chymotrypsin eluted with the fractions around 30 i.e same as  $\alpha$ -amylase (Omond & Stark, 1995). However, the chymotrypsin activity was spread over more fractions (numbers 15-55) than trypsin (numbers 30-50). The greatest activities were observed with fractions 30-35 for both. This indicated molecular weights between 12.4 and 29 kDa.

### Elastase-like activity

The two synthetic peptides tended to show less alkaline optima. A longer incubation with SAAApNA resulted in greater activity without apparent product inhibition (Fig.4). SAAApNA had a very sharp peak at pH 8.0 while N-CBZ-L-Ala-pNE had a very broad top from pH 5.6-8.2 but with peaks at pH 6.9 and 7.9-8.2 (Fig 5). Both the modified natural substrates had an optimum at pH 8.2-8.5 and there was no activity above pH 9.3. N-CBZ-L-Ala-p-NE was a rather unstable substrate, significantly undergoing rapid hydrolysis even in the absence of extract. The other synthetic substrate was used because, although its insolubility in aqueous media made it inconvenient, initial reaction velocities could be followed in cuvettes in the spectrophotometer. The natural substrates could only be used for fixed time assays to estimate the initial velocities and to complement studies using SAAApNA.

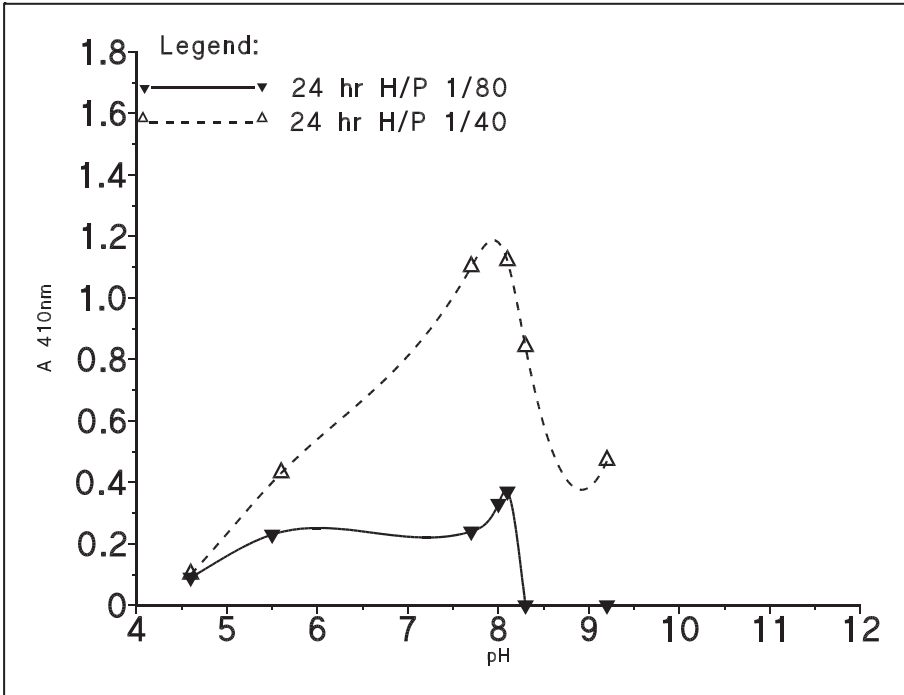


Fig. 4. The pH-activity profile of digestive gland elastase activity in wild *P. indicus* at 30°C assayed with SAAApNA as substrate. Values are means ± Standard Errors of the means

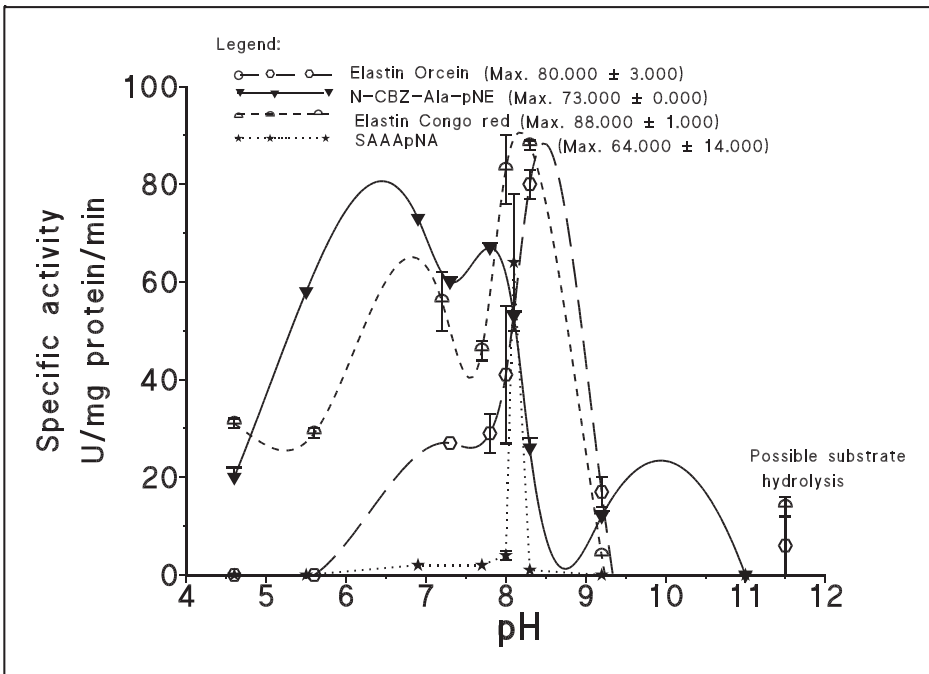


Fig. 5. pH-activity profiles of digestive gland elastase activity in wild *P. indicus* at 30°C assayed with SAAApNA, N-CBZ-Ala-pNE, Elastin Orcein and Elastin Congo red as substrates. Values are means ± Standard Errors of the means

## DISCUSSION

The lack of pepsin in shrimp is consistent with the absence of a true (acidic) stomach in which pepsin activity is found. Many other crustaceans examined have also failed to reveal the presence of pepsin activity. Protein digestion in these animals appears to be undertaken by serine endoproteases aided by exoproteases with the possible involvement of cysteine proteases.

In both UV assays for serine proteases in the present study, substrates were in excess. For trypsin, calcium was present in sufficient concentration (0.011 M) as recommended (Hummel, 1959) and similar quantities were present in the chymotrypsin digest mixtures (DMS) where Tris was used. Supplemental calcium did not significantly affect the BApNA-hydrolysing activity of shrimp homogenate, a finding that agrees with the observations of (Honjo *et al*, 1990). The homogenate may contain adequate calcium unlike purified commercial mammalian enzymes that are salt-free. Since TAME is not hydrolysed by chymotrypsin (Hummel, 1959), it can be concluded that the activity measured is due to trypsin. Under the conditions of reaction for chymotrypsin, BTEE is relatively resistant to trypsin hydrolysis (Hummel, 1959), so it can be concluded that the shrimp homogenate also contains chymotrypsin-like activity. Results from the chromogenic substrate assays confirm this.

Some authors (e.g. Erlanger *et al* 1961) regard BApNA as the most sensitive substrate of trypsin. One of the non-trypsin enzymes able to hydrolyse it is papain, which does not occur in shrimp homogenate. Whereas there may be an appreciable amount of BApNA hydrolysis by non-trypsins, the hydrolysis of SApNA was almost exclusively by shrimp chymotrypsin. This is further evidence for the existence of shrimp chymotrypsin. Serine proteases are now known to be significant in penaeid digestion of proteins (Hernandez-Cortes *et al*, 1997). In the present study, results of experiments using specific inhibitors were confirmatory to a large extent.

DPCC inhibited almost all (99 %) of the chymotrypsin activity but did not alter the hydrolysis of BApNA by shrimp homogenate indicating that the two activities were different,

that both substrates are fairly specific for each activity and that DPCC is an effective, specific inhibitor of chymotrypsin. The rate of DPCC inhibition of human trypsin is reported to be 80 times slower than that of chymotrypsin and reactivation of the latter with nucleophilic reagents such as indole and isonitrosoacetone is also twice as fast as that of trypsin but complete reactivation occurs for both after 18h (Erlanger & Cohen, 1963). In the present study, shrimp trypsin recovered after 20 h but chymotrypsin did not.

Furthermore, as expected, TLCK failed to completely inhibit chymotrypsin activity confirming that the activity involved in the hydrolysis of SApNA was non-trypsin. Inhibitions of upto 55 % of mammalian trypsin and 71 % of lobster H/P protease activity using this inhibitor have been observed (Glass, 1989). At 500  $\mu\text{g ml}^{-1}$ , a 83 % inhibition of *P. indicus* H/P trypsin activity is observed (Honjo *et al*, 1990).

PMSF dissolved in ethanol or propanol precipitated the homogenate, hence was used suspended in water and not surprisingly, did not inhibit either activity significantly contrary to the findings of others where 50-100 % inhibition using concentrations as high as 3000-5000  $\mu\text{M}$  were reported (e.g. Glass, 1989, Honjo *et al*, 1990). PMSF may consequently be a less effective inhibitor of shrimp activity.

Inhibition (97.1 %) of *P. indicus* H/P trypsin activity observed with SBTI (200  $\mu\text{g ml}^{-1}$ ) by Honjo *et al* (1990) is consistent with findings from the present study which also confirm observations that no inhibition of shrimp trypsin activity occurs with TPCK (500  $\mu\text{g ml}^{-1}$ ). Whereas 25 % propanol reportedly elevates *P. indicus* H/P trypsin by 4 times at 30°C and 177 times at 4°C (Honjo *et al*, 1990), in the present study, ethanol, methanol, acetone and propanol concentrations < 5% did not significantly increase enzyme activities.

The estimated molecular weights of shrimp trypsin and chymotrypsin (14.5-29 kD) from the present study are within the range for decapod trypsins (see Vonk & Western, 1984), *P. setiferus* H/P trypsin (Gates & Travis, 1969) and purified mammalian pancreatic trypsin and  $\alpha$ -chymotrypsin (Hess, 1971; Keil, 1971) but are less than one of the two H/P trypsin iso-enzymes found in *P. indicus* of 36 kDa using Mono Q FPLC and SDS-PAGE

(Honjo *et al.*, 1990). However, Sephadex G-100 gel filtration revealed a molecular weight of 18 kDa (see Honjo *et al.*, 1990), similar to that of the present study. In contrast to the present findings, these authors also showed that their enzyme had a broad pH optimum (6.5-11.0) and was most active at 45°C. The stability of the enzyme in alkaline pH has been confirmed by the present study. However, contrary to the findings of Honjo *et al.* (1990), in the present study, there was no shrimp trypsin activity that was almost completely inhibited by PMSF. Such discrepancies may be due to the presence of at least 7 trypsin-like enzymes in *P. indicus* (Honjo *et al.*, 1990). Indications from the present study are that there may also be several variants of chymotrypsin molecular weights.

Decapod and mammalian trypsins have broadly similar molecular weights, substrate specificities and general responses to inhibitors, but differences include greater temperature and pH tolerance and resistance to autolysis by the crustacean enzymes (see Vonk & Western, 1984). The pH optimum in the present study for *P. indicus* H/P trypsin (7.8-8.0) was not very different from that (pH 8.0-8.5) in crabs (Galgani & Nagayama, 1987). The BApNA trypsin pH optimum in the present study indicates that shrimp H/P trypsin has a similar pH optimum to the mammalian enzyme (Hummel, 1959, Erlanger *et al.*, 1961).

The presence of chymotrypsin in *P. indicus* indicates that this endoprotease is of wider occurrence in shrimps than previously thought (Gates & Travis, 1969, 1973). The pH optimum is similar to that of the chymotrypsins of *P. monodon*, *P. japonicus*, *P. penicillatus*, *Metapenaeus monoceros* and *Macrobrachium rosenbergii* (Tsai *et al.*, 1986).

The last serine endo-protease determined in the present study had the most alkaline pH optimum of all assayed enzymes, extending to pH 8.5. This is slightly lower than the pH optimum (8.8) of mammalian pancreatic elastase EC 3.4.21.36 but is still within the optimal pH range of leukocyte elastase EC 3.4.21.37 (Geiger, 1984b) and all the major shrimp H/P proteases. It must therefore contribute significantly to protein digestion as it is an enzyme known to hydrolyse a wide range of proteins (Clark *et al.*, 1985b). In agreement with Glass (1989) and (Clark *et al.*, 1985a), the pH

optima varied with the different substrates. In *P. kerathurus*, elastase could not be demonstrated with unmodified Elastin but collagenase was present (Galgani *et al.*, 1984). The shrimp elastase partly behaves like that of the Dover sole with respect to some substrates (Clark *et al.*, 1985b). The pH optima with the two artificial substrates were almost identical between the shrimp and fish (pH 8.1-8.2) but with natural substrates, including undyed Elastin, fish pH optima were more alkaline (8.3-9.8) than shrimp ones <pH 8.5. Fish elastase was inhibited by PMSF (35-92 %), TPCK (16-23 %) and TLCK (12-41 %) and had a molecular weight of 19.5 kDa on Sephadex G-200. Unlike in fish where there exists a wide discrepancy in pH optima between the synthetic and natural substrates (Clark *et al.*, 1985b) possibly indicating different enzymes, the present study found a lesser difference and may be measuring the activity of one enzyme. The minor discrepancies may be due to the difficulties associated with working with these insoluble substrates (Clark *et al.*, 1985a, 1985b). More studies of crustacean, especially shrimp, lobster and crab elastase activity are necessary.

In the present study, the residual activity observed in shrimp in the presence of selective inhibition of the three serine proteases indicates the presence of other types of proteases, probably cysteine ones. It would appear that the latter may be more important in the lobster (low total and trypsin activities and no chymotrypsin) while the former dominates shrimp dietary protein digestion. This differential importance of serine and cysteine proteases between crustacean groups has been observed by several authors (e.g. Ceccaldi, 1998; Garcia-Carreno *et al.*, 1997; Hernandez-Cortes *et al.*, 1997; Le Moullac *et al.*, 1997).

## CONCLUSIONS

In view of the recognition of the need for capacity building in aquaculture feed technology and nutrition in sub-Saharan Africa (FAO, 2001), the present study has demonstrated considerable proteolytic activity in *P. indicus* within the physiological pH (7-8) range and active at natural ambient water temperatures of the shrimp. Until locally formulated feeds are available, high-protein

diets developed for other cultured shrimp species elsewhere may be tried as long as they are economically affordable and justifiable.

It may not be necessary to supplement shrimp enzyme levels by using live or freshly killed prey organisms. Organisms with much lower enzyme levels such as some bivalve molluscs and asteroidea may be tried as protein and mineral/vitamin sources.

High and varied protease activity may preclude the use of supplemental amino acids in artificial diets (Lee *et al*, 1980) which reduces the costs of manufacturing these diets. However, such feed prices may still be unaffordable to prospective African shrimp farmers and efforts must be made to develop alternative aquafeeds using local ingredients. Examples might include aquatic floral resources like the currently problematic, but abundant, Lake Victoria water hyacinth (*Eichhornia crassipes*) and marine seaweeds of the East African coast.

The high enzymatic activity in *P. indicus* may be a source of commercial enzymes, which could be extracted from shrimp waste. By this further processing of shrimp after removal of conventional eatable parts, environmental pollution may be reduced.

*Nephrops*, and probably other lobsters, when cultured, may require considerable enzyme input from prey organisms with high enzyme activities such as other crustacea, echinoidea, polychaetes and cephalopods. Its diet may also need simpler food components such as dipeptides, amino acids and disaccharides. This means that artificial diets made from such components, and rich in protein precursors or protein, may be that much more expensive, limiting lobster culture unless final market prices can offset the high cost of production due principally to the high feed costs.

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