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# Studies on Digestive Proteases from Midgut Glands of a Shrimp, *Penaeus indicus*, and a Lobster, *Nephrops norvegicus*

*Part 1. Proteolytic Activity*

**James G. Omondi*,[1] and James R. Stark[2]**

*[1]Department of Zoology, C.B.P.S., University of Nairobi, Box 14576, Nairobi, Kenya, E-mail: jgo@ics.uonbi.ac.ke; and [2]Department of Biological Sciences, Heriot-Watt University, Edinburgh, Scotland, UK*

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## Abstract

Digestive gland protease pH optima and specific activities determined in *Penaeus indicus* with casein, azocasein, Azocoll, and Congo red fibrin as substrates were pH 7.7–9.2, 210–371 μmol of tyrosine/mg of homogenate protein/min; pH 7.8, 36; pH 6.0–7.0, 7; and pH 8.9–9.2, 7A Δ0.001 U/mg of homogenate protein/min, respectively. Activity in the shrimp was stable during frozen storage but relatively labile and very low (1.043 azocasein units) in the Norwegian lobster, *Nephrops norvegicus*. The high activity in shrimp is significant in aquaculture and may be a source of proteolytic enzymes for industrial use. The rapid deterioration after landing may be a consequence of the high and stable activity. The low activity in the lobster may present a problem in culture and requires a more critical choice of feed as well as further investigation. 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride was a very convenient, fast-acting, and effective inhibitor of shrimp trypsin and chymotrypsin but did not completely inhibit general protease activity in shrimp and had a negligible effect on the lobster. A significant component of that activity may be from nonserine proteases (such as the exoproteases carboxypeptidase A and B and the leucine aminopeptidases), whose proportion relative to the serine proteases may be greater in the lobster.

*Author to whom all correspondence and reprint requests should be addressed.

**Index Entries:** Shrimp midgut gland digestive proteases; enzyme pH-activity profiles; casein; sulfanilamide azocasein; Azocoll; Congo red fibrin; enzyme inhibitors; proteolytic enzymes; *Penaeus indicus*; *Nephrops norvegicus*.

## Introduction

Over the last 20 yr, declining shrimp catches and increasing demand have stimulated the phenomenal expansion of shrimp farming *(1)*, but unlike Southeast Asia and South America, Africa has not exploited this resource much. A well-managed shrimp industry controls the resource better, boosts local protein reserves, provides greater income, and can diversify developing economies. Most African countries rely on single items for export (cash crops, minerals, and tourism, or combinations of these), but the benefits of shrimp farming may not be realized soon because the necessary indigenous technical expertise and research base are underdeveloped. Consequently, Africa's penaeids such as *Penaeus indicus*, *P. monodon*, *P. notialis*, *P. semisulcatus*, *Metapenaeus monoceros*, and *Parapenaeopsis atlantica (2)* have been little studied. *P. indicus* dominates shrimp fisheries along the eastern coast and appears suited for culture, but at the present it has no local diet formulations. Without biochemical/physiological data for comparison, feeding regimes used in shrimp culture elsewhere cannot be adopted with certainty. The present study was undertaken to provide information on the digestive capacity of *P. indicus* with a view to developing appropriate feeds. The Norway lobster, *Nephrops norvegicus*, was used for comparison because it was more readily available.

## Materials and Methods

### Sample Collection

Wild adult *P. indicus* came from Kenya *(3)* and adult *N. norvegicus* from the east coast of Scotland. The latter were transported live to Edinburgh, where some were immediately frozen in liquid nitrogen and stored at –22.4°C and some were kept for a few months in recirculating seawater (35%) aquaria held at 15°C and fed a shrimp diet *(3)*.

### Chemicals

Buffers and pH measurements were as described in ref. *3*. Reagents (analytical grade), inhibitors, and commercial enzymes were from Sigma-Aldrich-Fluka, UK, Calbiochem-Novabiochem, UK, or British Drug Houses, UK.

### Preparation of Enzyme Extracts

Homogenate preparation was done as described in ref. *3* but *N. norvegicus* samples were diluted 1/5 to 1/10 w/v. After centrifugation, fat mats were removed by glass wool filtration. Most lobster samples were used within 1 wk of preparation. Small quantities were thawed and used without refreezing.

### *Estimation of Enzyme Activities*

Initial total proteolytic activity was estimated using casein *(4–9)*. Calibration graphs were prepared using L-tyrosine (Tyr), and specific activity was expressed as U (μmol) of Tyr released/(mg of homogenate protein·min) under the experimental conditions. Assays were in triplicate. Amino acids from casein were also reacted with ninhydrin or Folin reagent, and absorbances were read at 570 and 578 nm, respectively, against appropriate blanks. Azocasein *(10–13)* was also used with assays in duplicate, and results were reported as specific activity Units (U[mg·min]) or as activity Units (U[mL·min]), 1 U being the quantity of enzyme causing an increase in absorbance of 0.001/min ($\Delta A_{440nm}$ 0.001/min). An Azocoll substrate suspension *(13,14)* in distilled water (0.3%, 1.6 mL) was incubated with buffer (1.0 mL) and extract (0.8 mL, 1/50 w/v) at 30°C and continuously agitated in capped Sterilin centrifuge tubes (BDH) for 1 h on a Denley Spiramix 5 (Denley, UK) or an STR 6 Platform shaker (Stuart Scientific, UK). The reaction was stopped by the addition of NaOH (1 *M*, 2.0 mL), the assay mixture was centrifuged for 10 min at 750*g* (Denley BS400 or BR401, Denley), the supernatant was decanted, and the absorbance was read at 520 nm against substrate blanks at each pH. Assays were in duplicate and results were reported as specific activity units or activity units ($\Delta A_{520nm}$ 0.001/min). Congo red (0.04 g) was suspended in centrifuge tubes with buffer (4.0 mL) and extract (0.8 mL, 1/50 w/v) for 1 h with constant agitation *(14)*. The assay mixture was then centrifuged, the supernatant was filtered, and the absorbance was read at 495 nm against substrate blanks. Assays were in duplicate and the results were reported as specific activity or activity units ($\Delta A_{495nm}$ 0.001/min).

To distinguish between different endo- and exoprotease activities *(9,14–31)*, inhibitors (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride [AEBSF], phenylmethyl sulfonylfluoride [PMSF], soybean trypsin inhibitor, Bowman-Birk chymotrypsin-trypsin inhibitor, *N*-α-isoyl-L-lysine chloromethyl ketone [TLCK], *N*-isoyl-L-phenylalanine chloromethyl ketone [TPCK], diphenylcarbamyl chloride, 2-nitro-4-carboxyphenyl-diphenyl carbanate [NCDC], and Ovomucoid) were preincubated with extracts before assays, and inhibition was expressed as a mean percentage relative to the control assay mixtures without inhibitor *(32)*.

Protein assays, absorbance measurements, and statistical analyses (using Minitab™, significant level $p \leq 0.05$) were conducted as described in ref. *3*.

## Results

Caseinolytic activity in *P. indicus* had an alkaline optimum (pH 8.0–9.0) with a minor peak around pH 3.5–4.3 (Fig. 1) and appeared depressed at pH 4.7–5.4. Supernatants had the same pH optimum but reduced activity, with specific activity at 5500 rpm being significantly greater than at 12,000 and 18,000 rpm. The latter two were not statistically different from each other. At pH 6.0, specific activity at 3615*g* was significantly greater

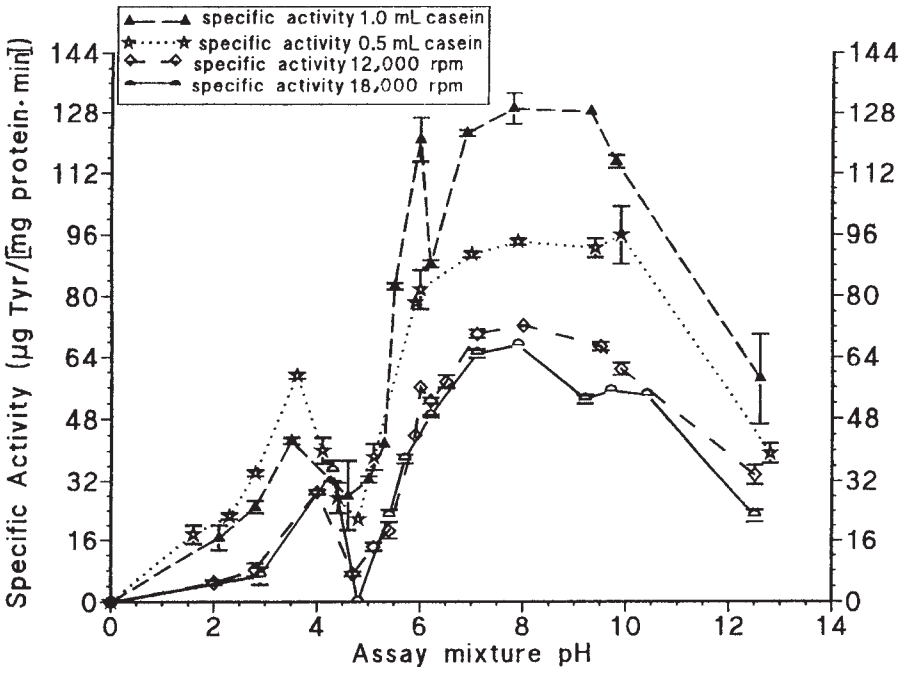


Fig. 1. pH profile of digestive gland protease activity in wild *P. indicus* at 30°C assayed with "soluble" casein ($A_{280\,nm}$) as substrate (0.5 mL, 1%; 5500 [+1.0 mL casein], 18,674*g* and 39,000*g* supernatants). Values are means ± SEM represented by the error bars. Statistics by Minitab.

than at 0, 18,674*g* and 39,000*g*, but the latter two were significantly different from each other. Most hydrolysis occurred in the first 0.5 h (Fig. 2), and activity in the shrimp was much greater than that in the lobster (Fig. 3).

In the shrimp, activity in Tris buffer was significantly higher than in phosphate or citrate-phosphate (Table 1). In lobster 1, there was a significant decrease in activity during storage. Values in Tris were slightly (5%) but statistically less than those in the first phosphate buffer, but were similar to those in the other buffers. All values for the three "fresh" lobsters (*Nephrops* 1 a, 2, and 3) were significantly greater than those for *Nephrops* 1 b at all pH values.

Table 2 shows the effects of buffer type, pH, and centrifugation speed on protease activity in the lobster. Activity of the uncentrifuged sample in citrate-phosphate buffer was significantly greater than in Tris but was not different from values in phosphate buffers. Supernatant activities at pH 6.8 were significantly greater than at pH 7.8–8.0. At pH 6.8, specific activities in the 0–18,674*g* fractions were statistically similar but lower than those at 50,277*g*. At pH 7.8, specific activity in uncentrifuged homogenate was significantly higher than at 746*g* but was similar to the 3615*g* fraction. Values in fractions above 3615*g* were significantly greater than those at lower rpms. At pH 8.0, in phosphate buffer 1, the uncentrifuged fraction had significantly higher specific activity than the 746–18,674*g* fractions but not the

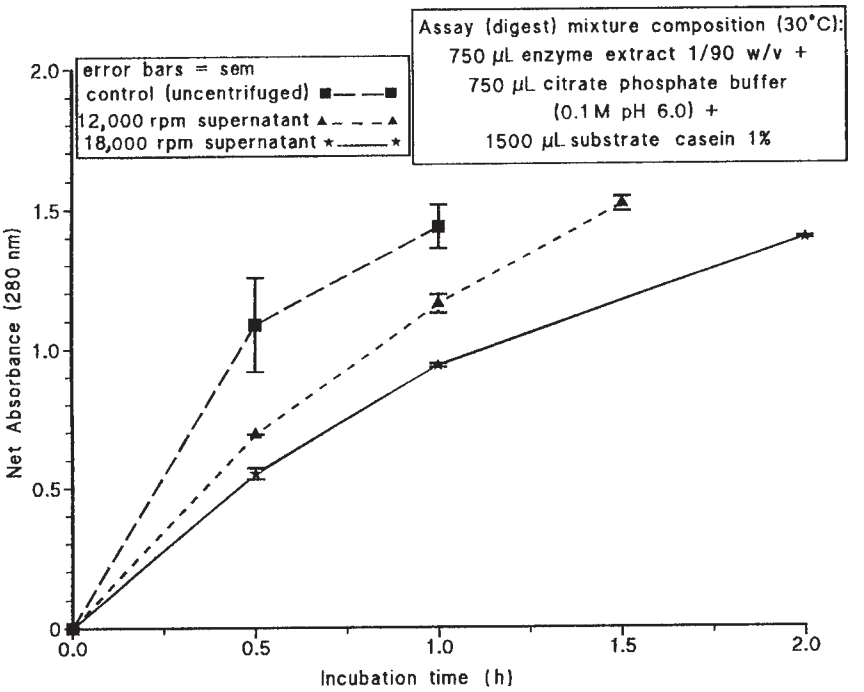


Fig. 2. Rate of increase in absorbance at 280 nm during the hydrolysis of "soluble" casein as a measure of protease activity in the digestive gland of wild *P. indicus.* Values are means ± SEM, represented by the error bars. Statistics by Minitab.

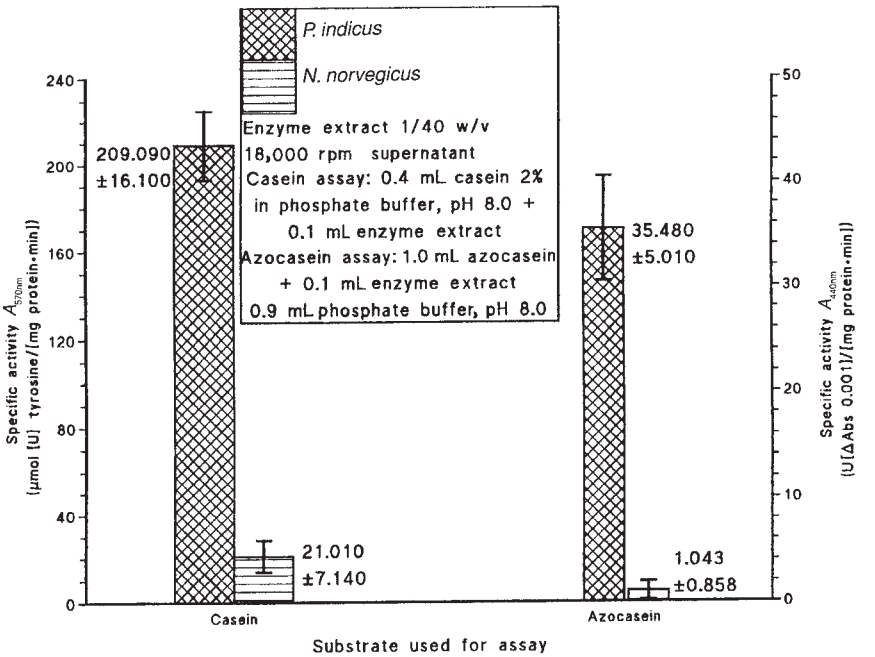


Fig. 3. Differences in protease-specific activities of homogenate supernatants from the digestive glands of wild *P. indicus* and *N. norvegicus* assayed at 30°C with casein and azocasein (1.25%) substrates at pH 8.0. Values are means ± SEM, represented by the error bars. Statistics by Minitab.

Table 1
Effect of Buffer Type and pH on Protease Activities in Midgut Gland Extracts from Shrimp (1/80 w/v, 39,000*g*) and Lobster (1/5 w/v, 50,277*g*) Using Azocasein (1.25% w/v) as Substrate at 30°C[a]

| Buffer | Citrate phosphate, pH 6.8 (0.1 *M*), substrate dissolved in buffer | Tris-HCl, pH 7.8 (0.05 *M*), substrate dissolved in buffer | Phosphate 1, pH 8.0 (0.1 *M*), substrate dissolved in water | Phosphate 2, pH 8.0 (0.1. *M*), substrate dissolved in buffer |
|---|---|---|---|---|
| *P. indicus* | 0.516 ± 0.005[b] | 0.645 ± 0.003[b] | 0.567 ± 0.002[b] | 0.549 ± 0.013[b] |
| | 29.656 ± 0.288[c] | 37.041 ± 0.143[c] | 32.586 ± 0.115[c] | 31.552 ± 0.747[c] |
| *N. norvegicus* 1 a | nd[b] | 0.597 ± 0.003[b] | nd[b] | nd[b] |
| | nd[c] | 1.428 ± 0.006[c] | nd[c] | nd[c] |
| *N. norvegicus* 1 b | 0.279 ± 0.017[b] | 0.320 ± 0.003[b] | 0.336 ± 0.000[b] | 0.353 ± 0.032[b] |
| (6.5 wk, –22.4°C) | 0.465 ± 0.028[c] | 0.533 ± 0.005[c] | 0.560 ± 0.000[c] | 0.589 ± 0.054[c] |
| *N. norvegicus* 2 | nd[b] | 0.583 ± 0.000[b] | nd[b] | nd[b] |
| | nd[c] | 1.653 ± 0.000[c] | nd[c] | nd[c] |
| *N. norvegicus* 3 | nd[b] | 0.570 ± 0.000[b] | nd[b] | nd[b] |
| | nd[c] | 1.856 ± 0.004[c] | nd[c] | nd[c] |

[a]*N. norvegicus* 1 were kept in aquarium for weeks and frozen (–22.4°C) 1.5 yr before assay. 1 b is homogenate 1 a after 6.5 wk. *N. norvegicus* 2 and 3 were kept in aquarium for weeks, removed when normal (2) or stressed (3), and stored (–22.4°C) 1–1.5 wk or assayed immediately. Shrimp was frozen for 1.5 yr before assay. Shrimp assay was performed 9 wk after that in Table 4.

[b]Mean net absorbance ± SEM.

[c]Specific activity (U[mg·min]).

nd = not determined.

Table 2
Effect of Buffer Type and Centrifugation Speed on Protease Activity in Midgut Gland Extracts from Wild, Unacclimatized Norwegian Lobster (1/5 w/v) Using Azocasein (1.25% w/v) as Substrate at 30°C[a]

| | Buffer | | | |
|---|---|---|---|---|
| rpm | Citrate phosphate, pH 6.8 (0.1 *M*), substrate dissolved in buffer | Tris-HCl, pH 7.8 (0.05 *M*), substrate dissolved in buffer | Phosphate 1, pH 8.0 (0.1 *M*), substrate dissolved in water | Phosphate 2, pH 8.0 (0.1 *M*), substrate dissolved in buffer |
| 0 | 0.254 ± 0.003[b] | 0.199 ± 0.003[b] | 0.243 ± 0.004[b] | 0.250 ± 0.006[b] |
| | 0.783 ± 0.008[c] | 0.614 ± 0.009[c] | 0.749 ± 0.011[c] | 0.770 ± 0.017[c] |
| 2500 | 0.332 ± 0.004[b] | 0.210 ± 0.004[b] | 0.288 ± 0.001[b] | 0.280 ± 0.007[b] |
| | 0.769 ± 0.010[c] | 0.493 ± 0.009[c] | 0.667 ± 0.003[c] | 0.647 ± 0.015[c] |
| 5500 | 0.338 ± 0.003[b] | 0.256 ± 0.002[b] | 0.301 ± 0.000[b] | 0.299 ± 0.005[b] |
| | 0.772 ± 0.007[c] | 0.585 ± 0.002[c] | 0.687 ± 0.007[c] | 0.683 ± 0.012[c] |
| 12,500 | 0.309 ± 0.001[b] | 0.231 ± 0.002[b] | 0.280 ± 0.001[b] | 0.288 ± 0.003[b] |
| | 0.769 ± 0.003[c] | 0.557 ± 0.003[c] | 0.696 ± 0.002[c] | 0.717 ± 0.008[c] |
| 20,500 | 0.360 ± 0.003[b] | 0.243 ± 0.008[b] | 0.305 ± 0.002[b] | 0.302 ± 0.003[b] |
| | 1.000 ± 0.008[c] | 0.554 ± 0.017[c] | 0.846 ± 0.004[c] | 0.838 ± 0.007[c] |

[a]After homogenization, samples were kept at –22.4°C for 5.5 wk before assay.
[b]Mean net absorbances (440 nm) ± SEM.
[c]Specific activity.

20,500 rpm one. Specific activities at 2500–5500 and 5500–12,500 were similar, whereas those between 2500 and 12,500–20,500, 5500–20,500, and 12,500–20,500 rpm were different, with greater values observed at higher speeds. In phosphate buffer 2, the apparent decrease in specific activity with increase in centrifugation speed from 0 to 12,500 was only significant between 0 and 2500–5500. Activities at 2500–12,500 rpm were similar, but that at 20,500 rpm was significantly greater.

Table 3 shows the stability of shrimp activity during long-term storage. The lobster sample was prepared from freshly killed laboratory-acclimatized animals and used within 24 h. The decline in lobster activity between 1 and 4–22 h was highly significant, but the difference between 4 (1.5 U/mg) and 22 h (1.4 U/mg) was not. Reducing the substrate concentration in the assay mixture significantly reduced activities (Table 4).

All inhibition by AEBSF in the shrimp (Table 5) was significant. The lowest concentration preincubated for 11.5 d (–22.4°C) inhibited to the same extent as at 4 h but twice as high as at 1 h. At 63 µm, most inhibition (43–49%) occurred within 10 min, significantly increasing to 55–60% at 0.5 h with no further increase at 1–2 h (43–54%). However, at 250 µm, there was a significant increase in inhibition with time from 1–2 h. The greatest inhibition occurred with 500 µM within 0.5 h, but this did not increase after 1 h. The highest concentration used (1000 µM) did not increase the inhibition at 0.5 h, even with a longer preincubation (20 h). Thus, for short incubation periods, there was an increase in inhibition with increased inhibitor concentration. However, inhibition by 1000 µM (63%) was less than that by 500 µM (88%), and there was no significant difference between inhibitions by 63 and 250 µM at 1 h. Inhibition by 8 µm (37%) at 4 h increased to 50% with 63 µM and 58% with 250 µm (2 h). With longer periods (>20 h), the effect of inhibitor concentration appeared to decline and the difference in the inhibition by 250 and 1000 µm was only 5%. An 11-d preincubation with 4 µm caused an inhibition that was only 12% less than that owing to 250 µm after 20 h.

AEBSF had a lesser impact on lobster. At 4 µm, it did not have any effect after 1 h, which was even greater than the 4- and 22-h controls. However, after 4 and 22 h, the activity significantly declined relative to the 1- to 22-h controls. Whereas the 1-h inhibited activity was significantly greater than the 4 and 22 h, the difference between the latter was not significant. The inhibition at 23.25 h was calculated using the 22-h control. At 8 µM, a 1-h preincubation resulted in activities that were statistically greater than the 1- to 22-h controls. A 2-h preincubation decreased the activities by 35% to be significantly lower than the 1-h controls, but they were greater than the 4- to 22-h controls. Inhibition was significantly greater with 4 (1–23.25 h) than 8 µM at 1–2 h. A 24-wk-old AEBSF solution (4–2000 µM) produced no inhibition.

Maximum activity in the shrimp with Congo red and azocoll was pH 9.0 and 5.7–6.9, respectively (Fig. 4). However, there was considerable apparent activity at both pH extremes.

Table 3
Protease Activity Assays over a 3-d Period on Same Digestive Gland Homogenates from *P. indicus* and *N. norvegicus* with Azocasein (1.25% w/v stock solution) as Substrate and Tris Buffer (0.05 *M*, pH 7.8) at 30°C[a]

| | Assay | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Shrimp | 0.623 ±0.002[b] | 0.628 ±0.007[b] | 0.636 ±0.002[b] | 0.630 ±0.019[b] | 0.616 ±0.002[b] | 0.638 ±0.002[b] | 0.737 ±0.000[b] | 0.760 ±0.001[b] | 0.614 ±0.011[b] | 0.618 ±0.001[b] | 0.716 ±0.105[b] | 0.821 ±0.020[b] |
| | 18.879 ±0.060[c] | 19.015 ±0.197[c] | 19.273 ±0.061[c] | 19.076 ±0.567[c] | 18.674 ±0.055[c] | 19.334 ±0.060[c] | 22.333 ±0.000[c] | 23.015 ±0.015[c] | 19.664 ±0.337[c] | 19.792 ±0.016[c] | 22.949 ±3.370[c] | 26.298 ±0.625[c] |
| Nephrops | 0.361 ±0.027[b] | 0.216 ±0.006[b] | 0.191 ±0.008[b] | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| | 2.550 ±0.191[c] | 1.527 ±0.040[c] | 1.349 ±0.057[c] | nd | nd | nd | nd | nd | nd | nd | nd | nd |

[a]All three assays for Nephrops sample (1/10 w/v, 50,277*g*) were performed over a 22-h period with storage in ice (12 h) in a freezer (–22.4°C, 10 h). Shrimp samples (1/40 w/v, 39,000*g*) were kept at 0°C during the day for assays and at –22.4°C for overnight storage.
[b]Mean net absorbances (440 nm) ± SEM.
[c]Specific activity.

Table 4
Effect of Substrate Concentration on Protease Activities of Midgut Gland Homogenates from Wild Shrimp (1/80 w/v, 39,000*g*) and Laboratory-Acclimatized Wild Lobster (1/5 w/v, 50,277*g*) Assayed with Azocasein and Tris Buffer (0.05 *M*, pH 7.8) at 30°C[a]

| | Azocasein stock concentration (%) | | |
|---|---|---|---|
| | 1.250 | 0.625 | 0.313 |
| | Azocasein assay concentration (%) | | |
| | 0.625 | 0.313 | 0.157 |
| *P. indicus* | 0.611 ± 0.011[b] | 0.483 ± 0.001[b] | 0.316 ± 0.009[b] |
| | 35.087 ± 0.603[c] | 27.730 ± 0.029[c] | 18.161 ± 0.517[c] |
| *N. norvegicus* 1 | 0.597 ± 0.003[b] | 0.322 ± 0.007[b] | 0.176 ± 0.005[b] |
| | 1.428 ± 0.006[c] | 0.771 ± 0.017[c] | 0.422 ± 0.012[c] |
| *N. norvegicus* 2 | 0.583 ± 0.000[b] | 0.268 ± 0.002[b] | 0.143 ± 0.001[b] |
| | 1.653 ± 0.000[c] | 0.760 ± 0.006[c] | 0.404 ± 0.001[c] |
| *N. norvegicus* 3 | 0.570 ± 0.001[b] | 0.260 ± 0.003[b] | 0.132 ± 0.001[b] |
| | 1.856 ± 0.004[c] | 0.845 ± 0.008[c] | 0.430 ± 0.004[c] |

[a]*N. norvegicus* 1–3 are as described in Table 1 in which assay 1 b was performed 6.5 wk after these. The difference between the two shrimp assays (Table 4 and then Table 1) was 9 wk.
[b]Mean net absorbances (440 nm) ± SEM.
[c]Specific activity.

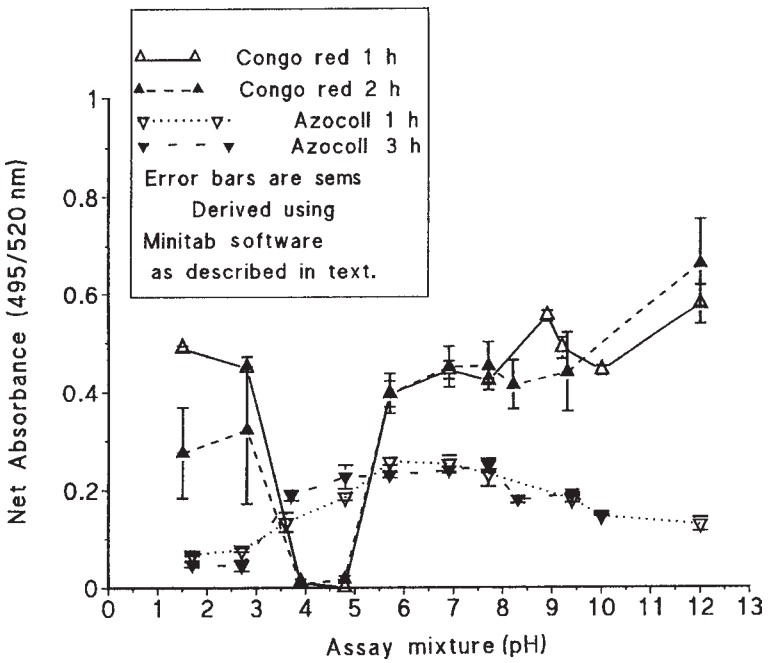


Fig. 4. Protease pH activity profiles of homogenate supernatant (39,000*g*, 1/50 w/v) from the digestive gland of wild *P. indicus* assayed at 30°C with Congo red fibrin and azocoll as substrates, respectively. Values are means ± SEM, represented by the error bars. Statistics by Minitab.

Table 5
Inhibition of Protease Activities in Digestive Gland Extracts from Shrimp and Lobster by AEBSF at pH 7.8 Using Azocasein (1.25%) as Substrate and Tris-HCl Buffer (0.05 *M*) at 30°C[a]

| | Duration of incubation (h) | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3.91 | | | | | | 7.81 | 62.5 | | | | 250 | | | 500 | | 1000 | | |
| AEBSF (μ*M*) | 1 | 4 | 22 | 23.25 | 264 | 268 | 4 | 0.2 | 0.5 | 1 | 2 | 1 | 1.5 | 20 | 0.5 | 1 | 0.5 | 1 | 20 |
| *P. indicus* (1/40) | 17.4 *** | 33.5 *** | nd | nd | 35.8 | 43.4 | 34.06 *** | 45.1 | 56.8 | 45.1 | 50.4 | 53.5 | 57.9 | 54.9 | 83.6 | 87.9 | 62.8 | 62.6 | 59.5 |
| *N. norvegicus* (1/10) | 3.3 | 24.1 | 23.0 | 49.2 | nd | nd | 34.6* 0*** | 0** | nd | 0** | nd | 0** | nd | 0** | nd | 0** | nd | 0** | 0** |

[a]Percentage inhibition was derived by expressing the mean net absorbance at 440 nm of the test assay mixture containing AEBSF as a percentage of the mean net absorbance of the corresponding control assay mixture without inhibitor. There was a decline in protease activity in lobster homogenates at room temperature (18–20°C) in the absence of inhibitor (34.7%, first 4 h) and in inhibitor samples (36.4–46.0%) in the same period. Differences were not statistically significant. *, An increase in inhibition at 2 h with respect to the inhibition at 1 h; **, tests were done with a different freshly prepared homogenate (from 20 lobsters frozen for 14 wk) using 24-wk-old inhibitor and 7-wk-old substrate solutions (stored at 0–4°C); ***, *P. indicus* homogenate had a dilution factor of 1/160 w/v. Assays were done at the same time as Nephrops**. Decline in protease activity in lobster homogenates at room temperature (18–20°C) in absence of inhibitor (34.7%, first 4 h) and in inhibitor samples (36.4–46.0%) in the same period.

## Discussion

The pH-activity profile of proteolysis in wild *P. indicus* determined using casein (Fig. 1) was much broader (three peaks: pH 3.5, 6.0, and a major one at 8.0–10.0) than the simpler peak at pH 7.8–8.0 in *P. monodon (14,33,34)*. Activities in *P. indicus* in the present study were much higher than those in *P. monodon*, which had a much higher (180 mg/g of tissue) hepatopancreatic protein concentration *(34)* compared to 59–64 mg/g in our study. Alkaline pH maxima (8.5–9.5) are also found in *P. japonicus* and *P. kerathurus*, but in the latter there is no activity below pH 6.0 *(35)*. Whereas the acidic activity may have been an artifact *(36)*, it resembles the European lobster, *Homarus gammarus*, whose digestive extracts show two peaks, with the second and major peak at pH 5.5 *(14)*. Three-peaked (pH 2–3, 5.5, 9.0) profiles have also been demonstrated in turbot, *Scophthalmus maximus (37)*, but the present study did not find pepsin activity with *N*-acetyl-phenylalanyl-3,5-L-diiodotyrosine *(32)*. Activity around pH 7.8–8.0 was likely owing to the serine endoproteases trypsin and chymotrypsin and the exoproteases LAP 1, LAP 2, carboxypeptidase A (CPa), and CPb. The higher pH activity may have been the combined effects of these six and another serine endoprotease, elastase *(32)*. In Dover sole (*Solea solea*), almost all proteolytic activity around pH 7.0–8.0 is owing to trypsin, chymotrypsin, and collagenase, whereas activity at pH 9.0–10 is entirely owing to elastase, the dominant protease in juvenile fish *(38–41)*. In Atlantic halibut (*Hippoglossus hippoglossus*) and turbot, pH 4.5–5.3 caseinolytic activity may be cathepsin-like, but high activity above pH 10.5 with either casein or hemoglobin may reflect substrate disintegration in highly alkaline environments in which the enzyme may be denatured *(33,42)*. Several crustacea possess cathepsin-like activity *(7)*. This activity is absent from *P. monodon*, Dover sole, and European lobster, but digestive fluid at the foregut of *P. monodon* has a pH of 6.0–7.0 *(33)*, and the present peak at pH 6.0 may be a true physiological activity.

In 50 marine invertebrate species, taxonomic position and other unnamed factors determine the amount and distribution of proteases *(7)*. Great variation exists even within species, and although crustacea and asteroidea have very high activity, some bivalves and echinoidea have negligible amounts. The casein-ninhydrin specific activity in shrimp from the present study (209 U) was similar to that at 37°C in tunicates (120–168 U) and some molluscs (69–540 U) but much less than that (402–3400 U) in polychaetes, gastropods, cephalopods, echinoderms and the crustaceans, *Pachycheles stevens*, *Hemigrapsus sanguineus*, *Hapalogaster dentata*, *Cancer pygmaeus*, and *Pagurus ochotensis (7)*. This may be relevant in formulating diets for shrimp and lobster culture because it may be advantageous to include organisms with high protease activity and protein content in the feeding regime during growth. Varied activities in five *Penaeus* spp. led to the conclusion that age and diet affect levels but not species nor sex *(43)*.

Other factors may contribute to variations in measured protease activities. Because of the variable nature of casein, its various components with different solubilities may be differentially hydrolyzed by different proteases *(8)*. There may also be highly selective proteases that do not significantly hydrolyze specific natural macromolecular substrates such as casein, hemoglobin, Congo red, or azocoll. Therefore, assays may indicate low activity, underestimating enzymatic proteolysis since specific proteases are not detected. Homogenates may also contain unknown inhibitors. In digestive gland extracts of the Japanese Spiny lobster (*Panulirus japonicus*), CPa and LAP are only detectable after removal of inhibitors present in the crude extract by gel filtration *(13)*. In *N. norvegicus* in the present study, such inhibitors, if present, may be antagonized by AEBSF, apparently increasing activity that may not have been very noticeable because it was matched by a decrease owing to enzyme lability.

The profile in *N. norvegicus* with azocasein indicated a maximum around pH 8.0 for laboratory-acclimatized specimens but pH <7.0 for others. Three peaks of protease activity (pH 2.4, 6.4, and 8.2, constituted by three different enzymes, two of which are lost during purification) have been found in *N. norvegicus* head extracts using casein *(36)*, with greater activity at pH 6.4 than at pH 8.2 *(44,45)*. A caseinolytic optimum at pH 5.7–6.2 occurs in *H. gammarus (14,33)* with activities similar to our *N. norvegicus*, but with no alkaline activity.

Tris elevated activity in shrimp but not in lobster (Tables 1 and 2). Thus, agents and sources of proteolysis in the two may differ.

In diet evaluation, it is important to consider units in which results are expressed as moulting cycle, nutritional status, season, sexual condition, and ontogenetic stage, all of which affect the size, histological condition, water content, and protein concentration of the hepatopancreas *(46)*. Units expressed as micromoles/minute differ from units derived as ΔA0.001/minute (Fig. 3). Furthermore, the number of micromoles at 570 nm was much lower than calculated at 280 nm.

Activity in lobster rapidly deteriorated in marked contrast to the more durable activity in shrimp frozen for more than 1.5 yr in which subsequent assays over a 3-mo period (total time since shrimp collection: 1 yr 11 mo) on the same supernatant did not reveal any significant decrease in activity. In *P. setiferus*, a 6-mo storage (–70°C) does not reduce most protease activities except CPa *(46)*. Hepatopancreatic proteases in Carp are stable for 1 mo at 0°C, but trypsin, elastase, and some chymotrypsin activities decline *(47)*. A situation similar to that in the lobster occurs in starfish in which protease activity declines considerably as soon as the animals are removed from the sea *(7)*. It is possible that, in addition to the unstable nature of lobster protease activity, stress-related protease inhibitors may be responsible for the decline in activity that AEBSF seemed to arrest (*see* Table 5). Since AEBSF is an irreversible serine protease inhibitor that completely inhibited shrimp trypsin/chymotrypsin activities *(32)*, trypsin may be responsible for deactivating/digesting other proteases in lobster, and its activity may

be enhanced by stress. Postdeath trypsin activity has been reported to increase in Spiny lobster (*Panulirus homarus* L.) and penaeids (*P. aztecus* and *P. setiferus*) during the activation of phenol oxidase in melanosis *(48)*. This may explain why penaeids and their reptantian relatives such as *Macrobrachium rosenbergii (49)* rapidly deteriorate after landing, which has been attributed to autolytic proteases diffusing from the digestive gland *(50)*. Even in refrigeration, deterioration occurs, lowering food crustacean quality *(51)*. *P. indicus* or its proteases may be more resistant to physiological stress.

The present results indicate that purification by centrifugation alone does not increase protease activity much in the lobster even though the highest activities were at 50,227*g*. Routine extractions are done at lower speeds *(5,11,47,52,53)*. Most of the digestive protease activity in the lobster and shrimp does not, therefore, appear to be membrane bound, but in the shrimp, the bound component may be greater. In *P. monodon*, 85% of protease activity in gut homogenates is water soluble; centrifugation at 100,000*g* loses only 15% of activity *(34)*. The membrane-bound protease activity may be contributed by peptidases such as LAP 2 (microsomal LAP). In *P. setiferus*, no LAP 1 or 2 activities are found with L-leucine hydrochloride or L-leucine β-naphthylamide, but substantial nongut/nonhepatopancreatic soluble activity is demonstrable with L-leucinamide-*p*-nitroanilide hydrochloride (LpNA) *(46)*. Very low LAP activity is found in the hepatopancreas of the Japanese Spiny lobster with LpNA *(13)*. Centrifugation in *P. monodon* and *H. gammarus* significantly reduces the soluble protein content of hepatopancreatic and gut extracts, but there is no substantial enzyme loss with the pellet fraction *(14)*. The membrane-bound protease activity can be released by Triton X-100, but the resulting increase in activity is not significant. Repeated liquid nitrogen freezing and thawing results in the highest proteolytic specific activity *(14)*. In the present study, liquid nitrogen was not used repeatedly, but some of the higher specific activities for shrimp in Table 4 may be explained by this freezing-thawing-freezing hypothesis.

Activity in shrimp was much greater than that in lobster at all pH values, and *N. norvegicus* may rely on exogenous enzymes for digestion since low activity was also observed in assays for total amylase, maltase, and specific α-amylase *(32)*. The European lobster requires a high protein diet and artificial lobster feeds contain expensive protein *(14)*. In light of very low protease activities in *N. norvegicus* in the present study and in the European lobster *(14)*, it may be interesting to establish whether that expensive protein is efficiently digested and, if so, whether by endogenous or exogenous enzymes (bacteria and prey organisms). In intensive culture under closed systems, live prey organisms may not be available. A preliminary survey of the gut and digestive gland of *N. norvegicus* found very low numbers of a single *Vibrio* type (A. Monteiro Rocha, Heriot-Watt University, personal communication) that may not account for significant protease activity. Larval stages of lobster may be especially enzyme starved,

and feeding a high inert protein diet may be wasteful. It may be better to use amino acids, peptides, and simple polypeptides instead, if leaching loss can be minimized. By contrast, the high and varied protease activities in the shrimp justify the inclusion of high-protein feeds in its diet. A substantial bacterial input can be discounted since the numbers found in the hepatopancreas were not high. A positive result in an enzyme assay in homogenates may not necessarily represent physiologically expressible activity. Protease, amylase, chitinase, chitobiase, and nonspecific esterase activities are found in crustacean tissues outside the digestive system and in homogenized whole-animal extracts *(46)*. Some of the enzymes, especially lysosomal ones, are only involved in intracellular assimilation. An assay may reveal an activity that has not been secreted and is not available for extracellular digestion in the gut lumen. However, in *Palaemon serratus*, intracellular concentrations of digestive enzymes directly reflect concentrations in the gut lumen *(54)*, so activities in our study may be good indicators of relative activities in the digestive gland and gut lumen.

Since 40–50% of proteolytic activity in adult penaeids is trypsin *(35)*, AEBSF inhibition represents most of the serine protease activity (trypsin, chymotrypsin, and elastase). In the lobster, there was no chymotrypsin *(32)*, and serine activity may be mostly trypsin but it was not the major part (<24%) of the total protease activity and was not affected much by the inhibitor. In the shrimp, the significant activity (12–64%) left after AEBSF inhibition implies that trypsin contribution to proteolysis may be <50% and that its contribution to serine protease activity is substantially less. This high residual protease activity may be contributed jointly by CPa, CPb, and LAP *(32)*. We did not find the low molecular weight protease (<11 kDa) described in several crustacea as being significant in proteolysis *(35,55–57)*. If present, it and collagenase *(35)* may constitute the remainder of the residual proteolytic activity. The effectiveness of AEBSF at very low concentrations may preclude a review of its toxicity to humans and its effect on human proteases at these low concentrations, with a view to using it as a spoilage retardant in landed shrimp and human treatment. However, AEBSF may not be an effective melanosis arrester in lobster. It has been considered for use in the prevention of thrombosis at concentrations as high as 130–1000 µm *(58,59)*. In comparison, PMSF, used at 1000 µm to inhibit rapidly human erythrocyte cholinesterase, pancreatic chymotrypsin, and pancreatic trypsin, and to penetrate the blood-brain barrier, is described as being of low toxicity and has been tested as a possible prophylactic agent against human pancreatitis *(60)*. At 2000 µm, PMSF inhibits 53–100% of serine protease activity in *C. sapidus* in 1 h *(52)*. In the present study, it precipitated shrimp homogenate.

## Conclusion

This study demonstrates high proteolytic activity in *P. indicus* at physiological pH and temperature. High-protein diets of other cultured shrimp may therefore be used as long as they are economically affordable and

justifiable. It may be unnecessary to supplement shrimp proteases with live or freshly killed prey. Organisms with lower levels may be used as supplemental protein and mineral/vitamin sources. *P. indicus* may be a source of commercial enzymes that could be extracted from shrimp waste, reducing environmental pollution. Our study shows that cultured lobster requires considerable enzyme input. Its proteolytic activity seems to be expressed by nonserine soluble proteases not inhibited by AEBSF whereas the shrimp appears to have significant susceptible serine endoproteases contributing about 43–60% of the total. The unusual activity in *Nephrops* requires further investigation.

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