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Cysteine proteinases substitute for serine proteinases in the midgut glands of *Crangon crangon* and *Crangon allmani* (Decapoda: Caridea)

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Abstract

The utilization of dietary proteins in crustaceans is facilitated by a set of peptide hydrolases which are often dominated by "trypsin-like" serine proteinases. As expected, the North Sea shrimps *Crangon crangon* and *Crangon allmani* showed in their midgut glands high proteolytic activities. However, the majority of animals lacked trypsin and chymotrypsin. Conversely, a minority of about 10% of the animals had elevated trypsin activities. The appearance of trypsin was neither related to the mode of feeding nor to the nutritive state of the animals. When present, trypsin was expressed in both species as a single isoform of apparently 20 kDa. The lack of serine proteinases was also confirmed by inhibitor assays. AEBSF, a serine proteinase inhibitor, slightly reduced total proteinase activity by less than 10%. In contrast E 64, a cysteine proteinase inhibitor, caused a reduction of more than 70% of total proteinase activity, indicating that a substantial share of proteolytic activity is caused by cysteine proteinases. Cathepsin L-like proteinases were identified as major cysteine proteinases.

A comparison with the eucarid crustaceans *Pandalus montagui*, *Pagurus bernhardus*, *Cancer pagurus* and *Euphausia superba* showed a similar high level of total proteinase activity in all species. Trypsin, however, varied significantly between species showing lowest activities in Caridea and the highest activity in *E. superba*. E 64 suppressed total proteinase activity by more than 70% in *Crangon* species but not in *C. pagurus* and *E. superba*. In contrast, the serine proteinase inhibitor AEBSF had only little effect in Caridea but was most effective in *P. bernhardus*, *C. pagurus* and *E. superba*. The results may indicate different traits of food utilization strategies in some eucarid crustaceans. Caridea may express predominantly cysteine proteinase, while in Anomura, Brachyura and Euphausiacea, serine proteinases may prevail.

Keywords: *Crangon crangon*; *Crangon allmani*; Enzymatic food utilization; Trypsin; Cathepsin

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1. Introduction

The common shrimp *Crangon crangon* is up to 70 mm long and inhabits sandy and muddy substrates of shallow coastal waters. It is distributed from the Black Sea over the Mediterranean to the Baltic Sea. However, it is most abundant in the North Sea where it holds a key position in the Wadden Sea ecosystem (Tiews, 1970; Redant, 1984). Due to its benthic life style and feeding habits, *C. crangon* significantly influences the structure and function of the in- and epifauna (Reise, 1978; Beukema, 1992). Simultaneously, *C. crangon* is an important food source for many fish species and also represents an important target in coastal fisheries with average annual landings of approximately 20,000 tons.

Crangon allmani is smaller, less abundant, and thus economically less important than *C. crangon*. It shows a more reddish pigmentation and can be easily distinguished by a deep longitudinal groove and two parallel carinae on the dorsal side of the sixth abdominal segment. *C. allmani* prefers colder conditions than *C. crangon*. In Northumberland waters, higher numbers of *C. allmani* were recorded only between October and April, when the water temperatures were low (Allen, 1960). In the German Bight, the probably only reproducing population of *C. allmani* appears in the Helgoland Trench, a 60 m deep depression south of the island of Helgoland (Blahudka and Türkay, 2002).

Both *Crangon* species are predominantly carnivorous but may also be considered omnivorous while feeding on benthic microalgae (Plagmann, 1939; Pihl and Rosenberg, 1984; van der Veer et al., 1998; Oh et al., 2001). Due to temporary as well as seasonal changes in benthic communities, they must be able to cope with different prey and thus utilize a broad and variable spectrum of diets. According to the most frequent food items, crustaceans predominantly express proteases, glucanases and lipases to hydrolyze the major biotic macromolecules. Proteinases, in particular, are always represented in crustaceans by a wide range of different and highly active enzymes (e.g., Galgani and Nagayama, 1987; Gibson and Barker, 1979; Garcia-Carreño, 1992). Especially, the serine proteinase trypsin, also referred to as "trypsin-like" proteinase (Honjo et al., 1990; Dittrich, 1992a,b) is considered to be the major proteolytic enzyme in many crustaceans. It is synthesised in large quantities and may represent up to one-third of the soluble proteins in the digestive gland (Ceccaldi, 1998). Many investigations confirmed high trypsin activities in different crustacean species (e.g., Murhty and Saxena, 1979; Garcia-Carreño et al., 1994, 1997; Johnston et al., 1995; Hernández-Cortés et al., 1997; Le Moullac et al., 1997). However, preliminary but repeated enzyme analysis of the midgut glands of *C. crangon* as well as *C. allmani* showed unusually low or even no trypsin activities, while in the same samples, total proteolytic activities were high (Saborowski, unpublished). The reasons for low trypsin activities in *Crangon* species are unknown yet.

The aim of the present study, therefore, was to investigate the presence of trypsin and to evaluate the relevance of serine proteinase as digestive enzymes in *C. crangon* and *C. allmani*. Midgut gland extracts of both species were analysed for total proteinase and trypsin. Specific characteristics of crude extracts and isolated proteins were examined by activity measurements and inhibitor assays. The results were compared with those of other eucarid crustaceans. Additionally, feeding experiments on *C. crangon* were performed to study the effects of starvation and different diets on enzyme activities.

2. Materials and methods

2.1. Animals

C. crangon and *C. allmani* (Caridea) were caught with a bottom trawl between February and October 2002 near the island of Helgoland in the German Bight (North Sea, 54°11′N, 7°55′E). The animals were immediately transferred to the laboratories of the Marine Station Helgoland. Only healthy adult specimens in the intermoult stage were selected for further investigations.

Additionally, the aesop shrimp *Pandalus montagui* (Caridea), the hermit crab *Pagurus bernhardus* (Anomura) and the edible crab *Cancer pagurus* (Brachyura) were obtained from bottom trawls south off Helgoland. Antarctic krill, *Euphausia superba* (Euphausiacea) were captured with a midwater trawl in February 2001 off the Antarctic Peninsula during the expedition ANT XVIII/4 of R/V Polarstern. Krill

were shock frozen after capture and shipped to the laboratory at −80 °C.

2.2. Preparation of the midgut gland extracts

The *Crangon* catches were dominated by females. Therefore, only these were selected for further procession. Freshly caught specimens were weighed and decapitated. The midgut gland was carefully dissected, weighed, and stored at −80 °C until use. In the case of *P. bernhardus* and *C. pagurus*, not the entire midgut gland but subsamples of the organ were dissected and stored. Frozen *E. superba* were dissected on a precooled cooling element. Care was taken to avoid thawing of the midgut gland during dissection.

Extracts of tissues from individual midgut glands (50–100 mg) were prepared in 1 ml cold deionised water. Homogenization was performed on ice with an ultrasonic cell disrupter (Branson, Sonifier B 15, microtip 101-148-063) with three bursts of 5 s and a break of 10 s in between. The homogenates were subsequently centrifuged for 15 min at 15,000×*g* and 4 °C. The supernatants were transferred into new reaction cups and stored at −80 °C until analysis.

2.3. Enzyme assays

Total proteinase activity was determined by the hydrolysis of azocasein as described by Garcia-Carreño (1992) and modified by Saborowski and Buchholz (1999). The reaction mixture contained 200 μl Tris–HCl (0.1 $mol \cdot l^{-1}$, pH 6.0, supplemented with 0.01 $mol \cdot l^{-1}$ $CaCl_2$) and 20 μl of the midgut gland extract. After preincubation for 5 min at 30 °C in a thermomixer (Eppendorf, 5437), the reaction was initiated by the addition of 50 μl of azocasein solution (Fluka, 11615, 1% w/v in buffer). After another 30 min of incubation, the reaction was terminated by the addition of 500 μl of trichloroacetic acid (TCA, 8% w/v) and subsequent cooling on ice. The mixture was centrifuged for 15 min at 15,000×*g* and 4 °C. The absorbance of the supernatant was measured photometrically at 366 nm. Samples were assayed in triplicate with two blanks for which TCA was added prior to the enzyme extract. Total proteinase activity was expressed as the change in absorbance per time and weight ($\Delta E_{366} \cdot min^{-1} \cdot g_{fw}^{-1}$).

Trypsin activity was assayed with Nα-benzoyl-DL-arginine-*p*-nitroanilide (L-BAPNA, Merck 1.10754) as substrate (Erlanger et al., 1961). The extracts (50 μl) were mixed thoroughly in a cuvette with 930 μl of 0.1 $mol \cdot l^{-1}$ Tris–HCl, pH 8.0, containing 0.01 $mol \cdot l^{-1}$ $CaCl_2$ and incubated for 5 min. The reaction was started by the addition of 20 μl of substrate solution (BAPNA, 0.05 $mol \cdot l^{-1}$ in DMSO). The substrate concentration in the cuvette was 1 $mmol \cdot l^{-1}$. The change of absorbance at 405 nm was recorded for another 3 min at constantly 30 °C. The activity was expressed as $U \cdot g_{fw}^{-1}$ (=$\mu mol \cdot min^{-1} \cdot g_{fw}^{-1}$) using the extinction coefficient ε_{405}= 10.2 $l \cdot min^{-1} \cdot cm^{-1}$.

2.4. pH profiles

The effects of pH on total proteinase activity of *C. crangon* and *C. allmani* were measured between pH 5 and pH 8 using 0.1 $mol \cdot l^{-1}$ Tris–HCl (+10 $mmol \cdot l^{-1}$ $CaCl_2$). The activities were expressed in relation to the average of all measurements (100%) of either species, respectively.

2.5. Inhibition studies

Midgut gland extracts (20 μl) were incubated at 25 °C with 20 μl of aqueous solutions (1 $mmol \cdot l^{-1}$) of the serine proteinase inhibitor AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride, Sigma A 8456) and the cysteine proteinase inhibitor E 64 (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane, Sigma E 3132). After 60 min, 180 μl of Tris–HCl buffer (0.1 $mol \cdot l^{-1}$, pH 6.0, 10 $mmol \cdot l^{-1}$ $CaCl_2$) were added and extracts were assayed for proteinase activity as described above. The activity was calculated in relation to a control which received no inhibitors but water only.

2.6. Chromatography

Proteins in the midgut gland extracts of *C. crangon* and *C. allmani* were separated by anion exchange chromatography using a FPLC-system (AP Biotech). First, extracts were desalted and rebuffered into the appropriate elution buffer. One milliliter of extract was loaded onto a NAP™-10 Sephadex G25-column (AP Biotech) and eluted with 1.5 ml of 10 $mmol \cdot l^{-1}$

imidazole buffer, pH 6.8 (buffer A). Subsequently, the processed extracts were loaded onto a UNO™ Q1-R anion exchange column (1 ml, BioRad, Hercules, USA). In order to remove unbound proteins, the column was washed with 5 ml of buffer A. Then, the bound proteins were continuously eluted with a linearly increasing concentration of NaCl from 0 to 1 $mol \cdot l^{-1}$ using 10 $mmol \cdot l^{-1}$ imidazole supplemented with 1 $mol \cdot l^{-1}$ NaCl, pH 6.8 (buffer B). Simultaneously, absorbance at 280 nm and conductivity were monitored and recorded. The flow rate was 1 $ml \cdot min^{-1}$ and the final elution volume was 35 ml. Fractions of 500 μl each were collected.

The fractions were assayed for total proteinase activity by azocasein hydrolysis. Fifty microliters of each fraction were incubated at 30 °C with 170 μl of buffer A. The assay was processed as described above.

Trypsin activity in the fractions was measured with a microassay using L-BAPNA as substrate. The substrate solution (50 $mmol \cdot l^{-1}$ in DMSO) was diluted 1:50 with buffer A. Fifty microliters of each fraction were transferred onto a 96-well microplate. The reaction was started by the addition of 250 μl of the substrate containing buffer. After 5–10 min of incubation at 25 °C, the optical density was read at 405 nm.

Chymotrypsin was determined as described for trypsin. However, SAAPPNA (*N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine 4-nitroanilide, Sigma S 7388) was used as substrate.

Cathepsin L activity was measured fluorometrically using *Z*-phenylalanine-arginine-7-amido-4-methylcoumarin (1 $mmol \cdot l^{-1}$ in DMSO). Twenty microliters of each fraction were transferred into reaction tubes to which 180 μl assay/activator buffer (340 $mmol \cdot l^{-1}$ sodium acetate, 60 $mmol \cdot l^{-1}$ acetic acid, 4 $mmol \cdot l^{-1}$ EDTA, pH 5.5) were added. After short incubation for 1 min at 30 °C, the reaction was initiated by the addition of 200 μl of substrate solution (diluted 1:50 with distilled water prior to use). After 10 min of incubation at 30 °C and permanent shaking, the reaction was terminated by the addition of 200 μl stop solution (100 $mmol \cdot l^{-1}$ sodium monochloroacetate, 70 $mmol \cdot l^{-1}$ acetic acid, 30 $mmol \cdot l^{-1}$ sodium acetate, pH 4.3). The fluorescent product 7-amido-4-methylcoumarin (AMC) was measured with a spectrofluorometer (Kontron SFM 25) at an excitation of 370 nm and an emission of 460 nm.

Fractions with maximum activities of total protease, trypsin and chymotrypsin, respectively, were pooled for electrophoretical protein and zymogram analysis. The pools were desalted and rebuffered into 1 ml of deionised water over a NAP™-10 Sephadex G-25 column (0.5 ml, AP Biotech). These isolated enzymes were freeze-dried and redissolved in distilled water before use.

2.7. SDS-PAGE and Substrate-SDS-PAGE

Proteins were separated by SDS-PAGE after Laemmli (1970) using 12%T gels in a vertical Hoefer SE 250 electrophoresis device (80×100×1.5 mm).

Midgut gland extracts and isolated enzymes were diluted 1:2 with sample buffer which contained 4% SDS. In order to maintain enzyme activity, the samples were neither heated nor treated with mercaptoethanol. SDS in the final sample concentration of 2% did not affect proteolytic activity. Two identical gels were loaded with 10 to 20 μl of samples per lane and 5 μl of molecular weight markers (Low Range Markers, Sigma M 3913). Electrophoresis was performed at constant current of 15 mA per gel and at 2–4 °C.

One of the gels was immediately fixed and coomassie-stained for protein detection. The other gel was used for zymogram analysis according to Garcia-Carreño et al. (1993). The gel was first immersed in a casein solution (3% w/v in 50 $mmol \cdot l^{-1}$ Tris–HCl, pH 8) for 30 min at 5 °C. At low temperature, the casein penetrated into the gel while the enzyme activity remained low. In a second incubation step, the temperature was raised to 25 °C. The enzyme activity increased and casein was hydrolyzed for another 60 min. The gel was washed thoroughly with distilled water and was immediately fixed and coomassie-stained. Opaque zones on a blue background indicated endopeptidase activity. Molecular weight standards appeared darker than the background.

Samples showing activity bands were further assayed for group specificity by inhibitor tests. E 64 was used as specific inhibitor for cysteine proteases. TLCK (Nα-*p*-tosyl-lysine chloro-methyl ketone) was used as a specific inhibitor of trypsin. Solutions of TLCK (10 $mmol \cdot l^{-1}$ in 1 $mmol \cdot l^{-1}$ HCl, pH 3.0) and E 64 (1 $mmol \cdot l^{-1}$ in a. dest.) were

separately incubated with the sample in a ratio of 1:2 and for 1 h at 25 °C. Controls were run with pure distilled water. Finally, the mixture was treated with sample buffer (1:2) and 10 to 20 µl were loaded onto the gel.

2.8. Feeding experiments

Freshly caught nonovigorous females in the intermolt stage were transferred into filtered seawater of 8 °C and acclimated for 24 h to the experimental conditions. A control group of 10 animals was killed prior to the feeding experiments. The midgut glands were dissected, weighed and stored at −80 °C until analysis. Thirty specimens were incubated individually for 14 days in 500-ml beakers at 8 °C and dimmed light. Ten animals were fed with mussel tissue of *Mytilus edulis*; another 10 animals received commercial *Spirulina platensis* tabs, and the remaining 10 individuals were starved until the end of the experiment. Feeding was carried out daily ad libitum. Seawater was also exchanged daily. The experiments were terminated after 14 days. Dissection and preparation of the midgut gland extracts as well as enzyme analysis were carried out as described above.

2.9. Statistical analysis

Statistical analysis was performed with the computer programme SigmaStat 2.03 (SPSS, Illinois, USA). Differences among means were analysed by ANOVA followed by a Student–Newmann–Keuls test. Data were expressed as mean±standard deviation. The significance level was set at $P \leq 0.05$. The feeding experiments were analysed with a Chi-square test.

3. Results

3.1. Activities of total proteinase and trypsin

The specific activity of total proteinase (Fig. 1a) was lowest in *P. montagui* (0.21 ± 0.18 $\Delta E \cdot min^{-1} \cdot g_{fw}^{-1}$) and highest in *E. superba* (0.38 ± 0.15 $\Delta E \cdot min^{-1} \cdot g_{fw}^{-1}$). *C. crangon* and *C. allmani* showed intermediate activities of 0.34 ± 0.05 and 0.33 ± 0.08 $\Delta E \cdot min^{-1} \cdot g_{fw}^{-1}$. The activities of total proteinase did not differ significantly between species ($P > 0.05$).

In contrast, significant differences were evident between the specific trypsin activities ($P < 0.05$, Fig. 2b). Lowest trypsin activities appeared in *C. crangon*

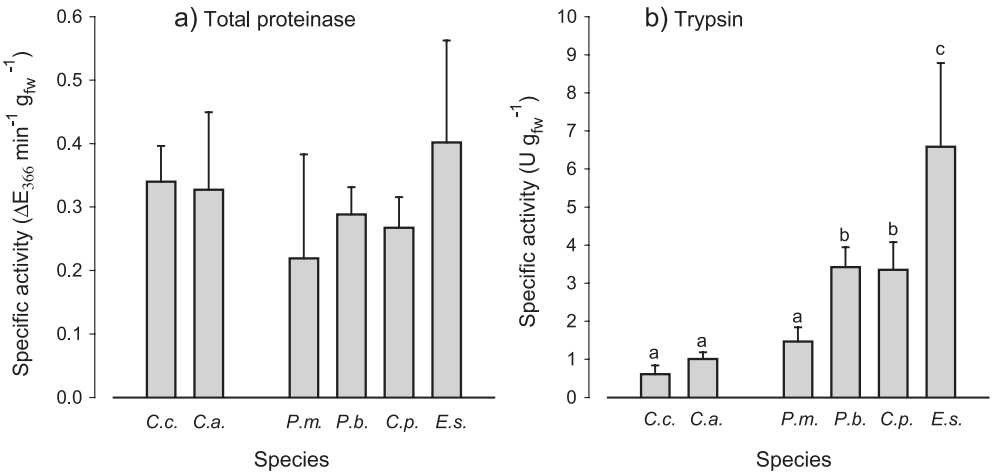

Fig. 1. Weight-specific activities of (a) total proteinase and (b) trypsin of *Crangon crangon* (*C. c.*), *Crangon allmani* (*C. a.*), *Pandalus montagui* (*P. m.*), *Pagurus bernhardus* (*P. b.*), *Cancer pagurus* (*C. p.*) and *Euphausia superba* (*E. s.*). Significant differences of trypsin activity between species were indicated by different letters (ANOVA, $P < 0.05$). The bars show means+S.D, (n=3).

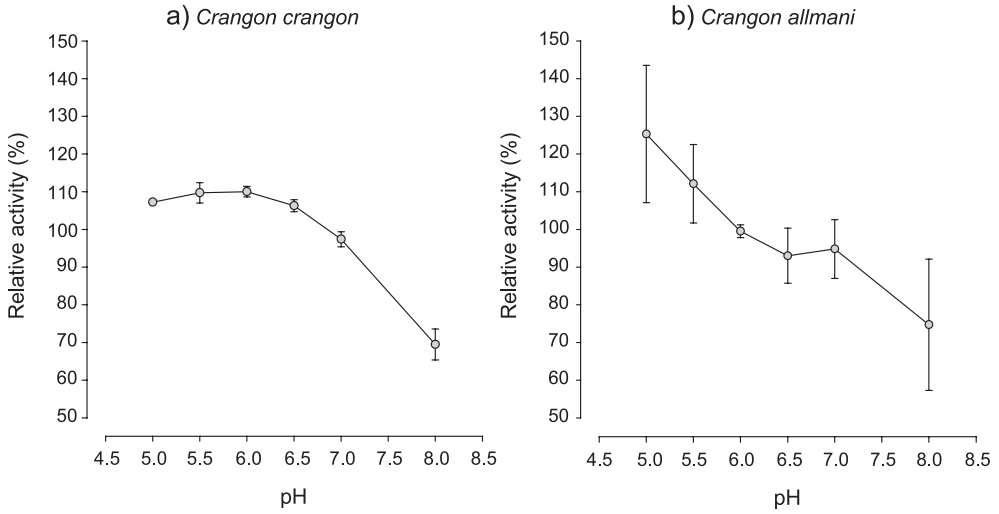

Fig. 2. Effect of pH on total proteinase activity of (a) *C. crangon* and (b) *C. allmani*. The activities at different pH were calculated in relation to the mean of all measurements in either species which was set to be 100% (means±S.D., n=3).

(0.6±0.1 $U \cdot g_{fw}^{-1}$) and *C. allmani* (1.0±0.1 $U \cdot g_{fw}^{-1}$) as well as *P. montagui* (1.3±0.4 $U \cdot g_{fw}^{-1}$). Trypsin activities of *C. pagurus* and *P. bernhardus* were significantly higher, amounting to 3.3±0.6 and 3.4±0.7 $U \cdot g_{fw}^{-1}$, respectively. Trypsin activity of krill *E. superba* (6.6±2.0 $U \cdot g_{fw}^{-1}$) significantly exceeded the activities off all other species.

3.2. pH profiles

Midgut gland extracts of *C. crangon* showed highest activities of total proteinases between pH 5 and 7. A maximum of activity appeared at pH 5.5 to 6.0 (Fig. 2a). Activities rapidly decreased towards pH 8.

A totally different course of total protease activity was evident for *C. allmani* extracts. The activity decreased continuously from pH 5 to 8 (Fig. 2b). An apparently constant level of activity was present between pH 6.5 and 7.

3.3. Inhibitory studies

Total proteinase activities in midgut gland extracts of *C. crangon* and *C. allmani* were inhibited more than 70% by E 64 but less than 10% by AEBSF (Fig. 3). Similarly, total proteinase from *P. montagui* was inhibited on average 60% by E 64 and 10% by AEBSF. In contrast, E 64 inhibited proteinase activity of *P. berhardus*, *C. pagurus* and *E. superba* only slightly while AEBSF caused a reduction of 30–50%.

3.4. Frequency of total proteinase and trypsin

The midgut glands of 30 randomly selected *C. crangon* and *C. allmani* were analysed for total protease activity and trypsin. Total proteinase showed distinct activity in all samples. Trypsin, in contrast, was low in most samples but also showed high activities in some samples. The values were classified into three groups: low activity (<2.0 $U \cdot g_{fw}^{-1}$), moderate activity (2.0–6.0 $U \cdot g_{fw}^{-1}$) and high activity (>6.0 $U \cdot g_{fw}^{-1}$).

In *C. crangon*, 20 extracts (67%) had low trypsin activities. Six extracts (20%) showed moderate activities and 4 extracts (13%) showed high activities (Table 1).

In *C. allmani*, 26 extracts (87%) had low trypsin activities. Four extracts (13%) exhibited moderate activities and only one extract (3%) showed high trypsin activity (Table 1).

3.5. Chromatography and gel electrophoresis of extracts with low trypsin activity

Midgut gland extracts of *C. crangon* and *C. allmani* with a low weight-specific trypsin activity

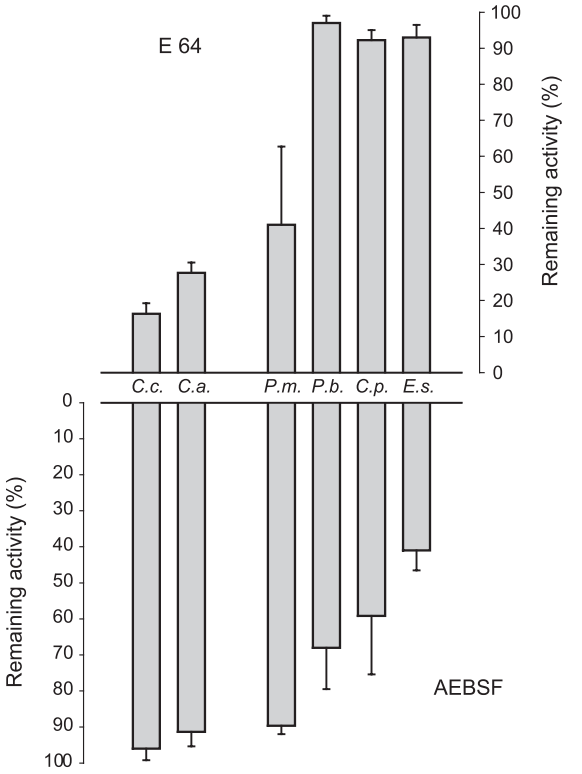

Fig. 3. Effect of the cysteine proteinase inhibitor E 64 and the serine proteinase inhibitor AEBSF on the total proteinase activity of *Crangon crangon* (*C. c.*), *Crangon allmani* (*C. a.*), *Pandalus montagui* (*P. m.*), *Pagurus bernhardus* (*P. b.*), *Cancer pagurus* (*C. p.*) and *Euphausia superba* (*E. s.*). The remaining activities were calculated and expressed in relation to the activity of the untreated controls which were set to be 100% (means±S.D., n=3).

(<2 $U \cdot g_{fw}^{-1}$) were analysed by anion exchange chromatography. The collected fractions were assayed for total proteinase, trypsin and chymotrypsin.

Both species showed a broad elution profile of total proteinase activity between fractions 30 and 50 which corresponded with a NaCl concentration of 0.2 to 0.6 $mol \cdot l^{-1}$. In extracts of *C. crangon*, two major peaks of total proteinase appeared around fraction 34 (0.28 $mol \cdot l^{-1}$ NaCl) and fraction 39 (0.36 $mol \cdot l^{-1}$ NaCl), respectively (Fig. 4a). A similar pattern was evident in extracts of *C. allmani* which showed two peaks of total proteinase activity around fraction 36 (0.32 $mol \cdot l^{-1}$ NaCl) and fraction 43 (0.45 $mol \cdot l^{-1}$ NaCl; Fig. 5a). Despite the distinct appearance of total proteinase activity, neither *C. crangon* nor *C. allmani* showed any detectable activities of trypsin or chymotrypsin.

Fractions with maximum total proteinase activity (P1) were analysed by SDS-PAGE and substrate-SDS-PAGE (Figs. 4 and 5). One protein fraction of 21 kDa appeared in P1 of *C. crangon* as well as in P1 of *C. allmani* after SDS-PAGE (lane 2) (Figs. 4b and 5b). In both species, this band corresponded to an activity zone with a molecular weight between 21 and 23 kDa after substrate-SDS-PAGE (lane 4; Figs. 4c and 5c). A slight reduction of activity was evident of both bands when samples were treated with the cysteine inhibitor E 64 (lane 5).

3.6. Chromatography and gel electrophoresis of extracts with high trypsin activity

Extracts of *C. crangon* and *C. allmani* with a trypsin activity higher than 2.0 $U \cdot g_{fw}^{-1}$ also showed distinct peaks of total proteinase activity between 0.2 and 0.6 $mol \cdot l^{-1}$ NaCl with maximum activities around fraction 34 (*C. crangon*) and fraction 36 (*C. allmani*; Figs. 6a and 7a).

Distinct activity peaks of trypsin as well as chymotrypsin were detected in both species. Trypsin eluted with a sharp peak in fraction 35 to 37 (0.32 $mol \cdot l^{-1}$ NaCl) in extracts of both species. In *C. crangon*, trypsin activity and the maximum of total proteinase activity did not appear in the same fractions (Fig. 6a). In *C. allmani*, however, total proteinase and trypsin matched exactly in fraction 36 (Fig. 7a).

Chymotrypsin of *C. crangon* appeared with a single distinct activity peak around fraction 28 which corresponded to a NaCl concentration of 0.15 $mol \cdot l^{-1}$ (Fig. 6a). In contrast, *C. allmani* showed two distinct chymotrypsin peaks, a minor one at fractions 24/25 (0.10 $mol \cdot l^{-1}$) and a major one around fraction 34 (0.28 $mol \cdot l^{-1}$; Fig. 7a).

Fractions with maximum activity of total proteinases, trypsin and chymotrypsin were subjected to

Table 1
Frequencies of low, moderate and high trypsin activities in the shrimps *C. crangon* and *C. allmani* from the field

Trypsin activity level	*Crangon crangon*	*Crangon allmani*
Low (<2 $U \cdot g_{fw}^{-1}$)	20	26
Moderate (2–6 $U \cdot g_{fw}^{-1}$)	6	4
High (>6 $U \cdot g_{fw}^{-1}$)	4	1

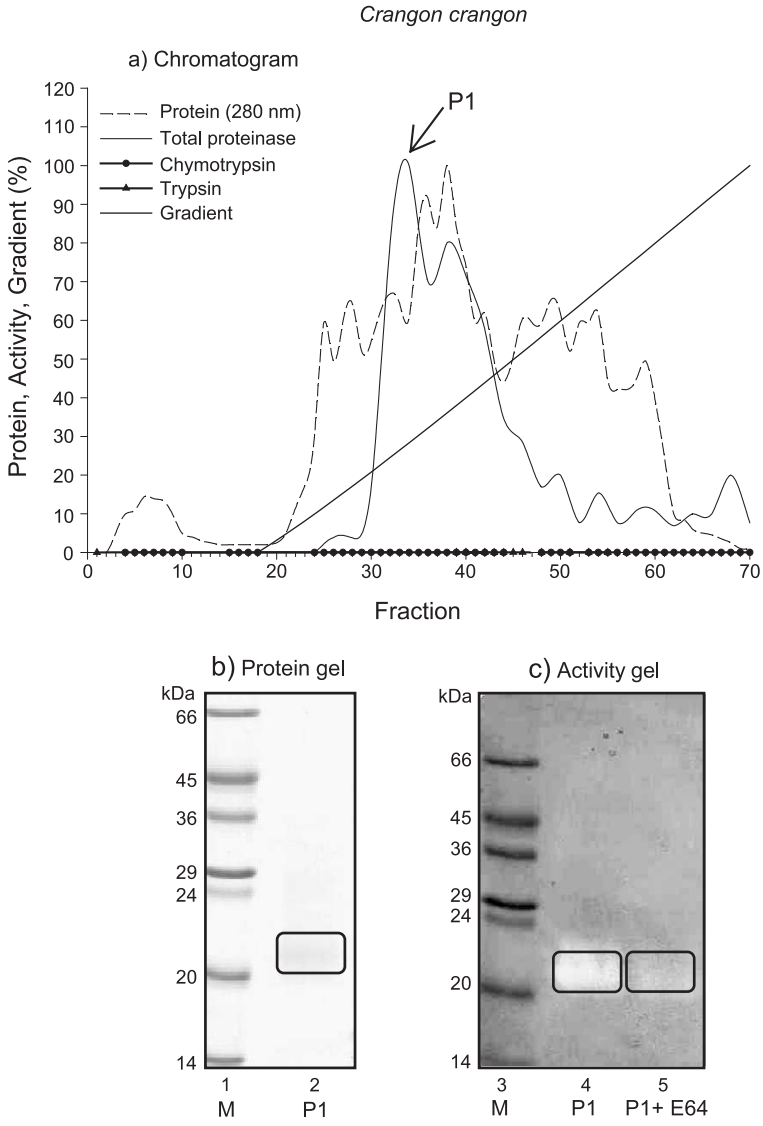

Fig. 4. *C. crangon*: (a) Chromatographic separation of a midgut gland extract with a low specific trypsin activity (<2.0 U · g_{fw}^{-1}). The maximum of the total proteinase activity is indicated (P1). Neither trypsin nor chymotrypsin were detected. Protein-gel (b) after SDS-PAGE and activity gel, (c) after substrate-SDS-PAGE of fractions with maximum proteinase activity (P1). Bands of proteolytic activity and corresponding protein bands are marked with boxes (M=markers).

SDS-PAGE and substrate-SDS-PAGE (Figs. 6 and 7). In *C. crangon* extracts, the fractions with highest chymotrypsin activities (CT) showed three distinct activity bands of 35, 38 and 43 kDa (Fig. 6c, lane 6). In the fractions of maximum protease activity (P), two activity bands were present (Fig. 6c, lane 7). One activity band of 21 kDa corresponded with a distinct protein band (Fig. 6b, lane 3). Furthermore, an activity zone with two separate bands of 23.5 and 24 kDa appeared on the activity gel. However, no corresponding protein bands were detected. Incubation with the cysteine protease inhibitor E 64 caused a distinct reduction of activity in these bands (Fig. 6c, lane 8). The

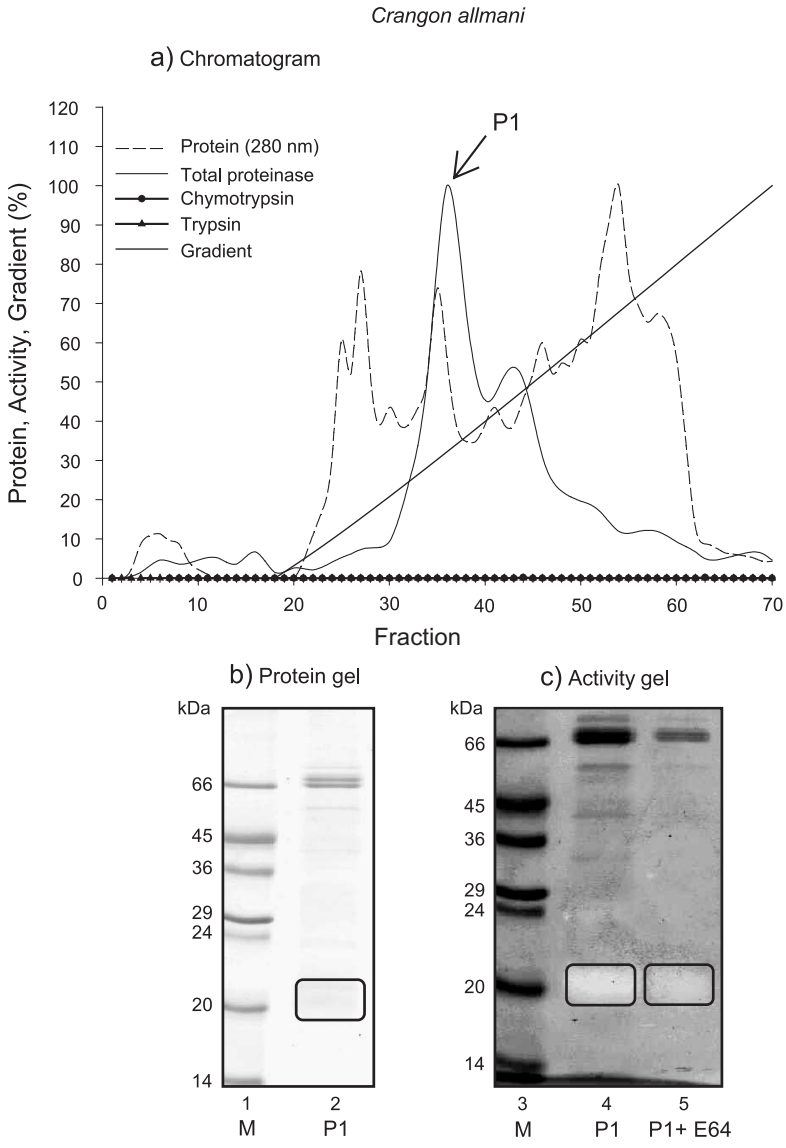

Fig. 5. *C. allmani*: (a) Chromatographic separation of a midgut gland extract with a low specific trypsin activity (<2.0 U · g_{fw}^{-1}). The maximum of the total proteinase activity is indicated (P1). Neither trypsin nor chymotrypsin were detected. Protein-gel (b) after SDS-PAGE and activity gel, (c) after substrate-SDS-PAGE of fractions with maximum proteinase activity (P1). Bands of proteolytic activity and corresponding protein bands are marked with boxes (M=markers).

fractions with maximum trypsin activity (T) showed a very strong activity zone of about 20 kDa (Fig. 6c, lane 9) which, although weaker, also appeared in lanes 7 and 8. The protein gel showed that this zone was caused by at least two bands of 20.5 and 20 kDa (Fig. 6b, lane 4). The activity was distinctly reduced after incubation with the trypsin inhibitor TLCK (Fig. 6c, lane 10).

In *C. allmani*, the fractions with the first maximum of chymotrypsin activity (CT1) did not show any recognizable activity zones on the substrate-SDS-PAGE (Fig. 7c, lane 6) nor protein

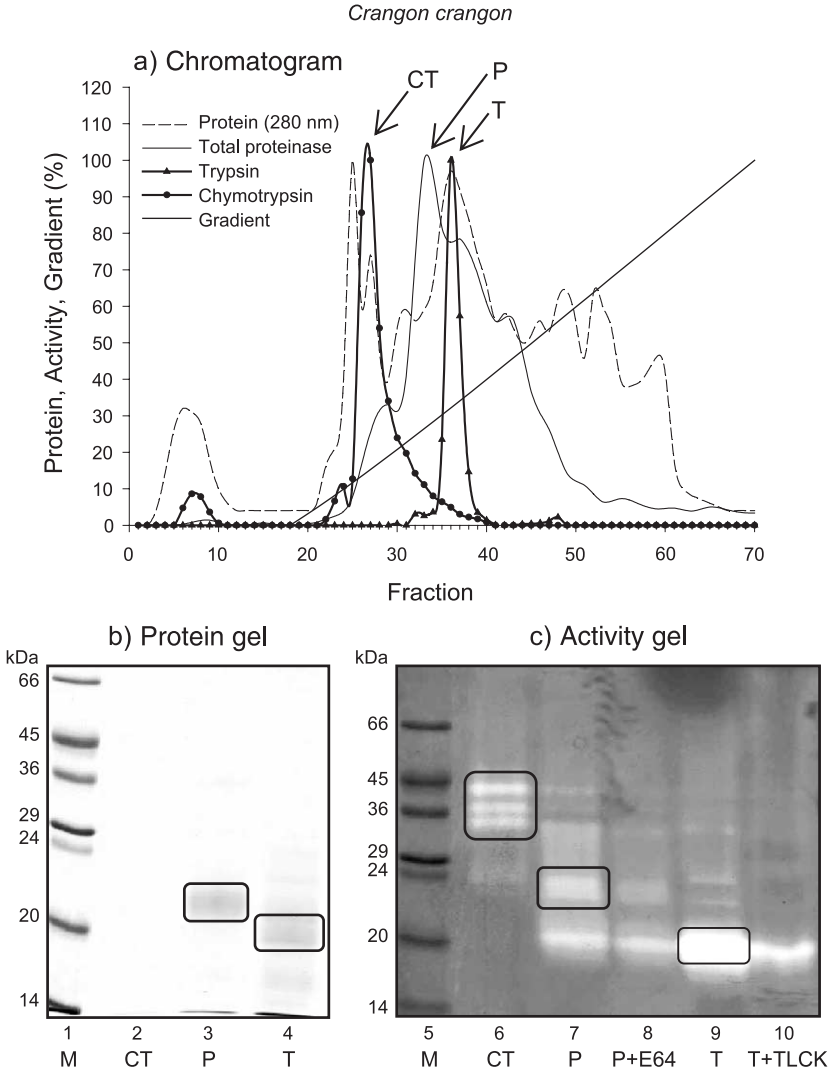

Fig. 6. *C. crangon*: (a) Chromatographic separation of midgut gland extract with a high specific trypsin activity (8.2 $U \cdot g_{fw}^{-1}$). The maximum of the total proteinase activity (P1), trypsin (T) and chymotrypsin (CT) are indicated. Protein-gel (b) after SDS-PAGE and activity gel, (c) after substrate-SDS-PAGE of fractions with maximum proteinase activity (P1), trypsin activity (T) and chymotrypsin activity (CT) as well as inhibitors E 64 and TLCK. Bands of proteolytic activity and corresponding protein bands are marked with boxes (M=markers).

bands on SDS-PAGE (Fig. 7b, lane 2). In the fractions of maximum chymotrypsin activity (CT2), however, three distinct activity bands appeared with a molar weight of 20, 22 and 23 kDa (Fig. 7c, lane 7). However, no distinct protein bands were evident (Fig. 7b, lane 3). The fractions with maximum total proteinase as well as maximum trypsin activity (P+T) showed a strong activity zone at 20 kDa in lane 8 (Fig. 7c). Corresponding protein bands were present on the SDS-PAGE (Fig. 7b, lane 4). The trypsin inhibitor TLCK caused a disitinct reduction in activity (Fig. 7c, lane 9). In contrast, activity was not influenced by E 64 (Fig. 7c, lane 10).

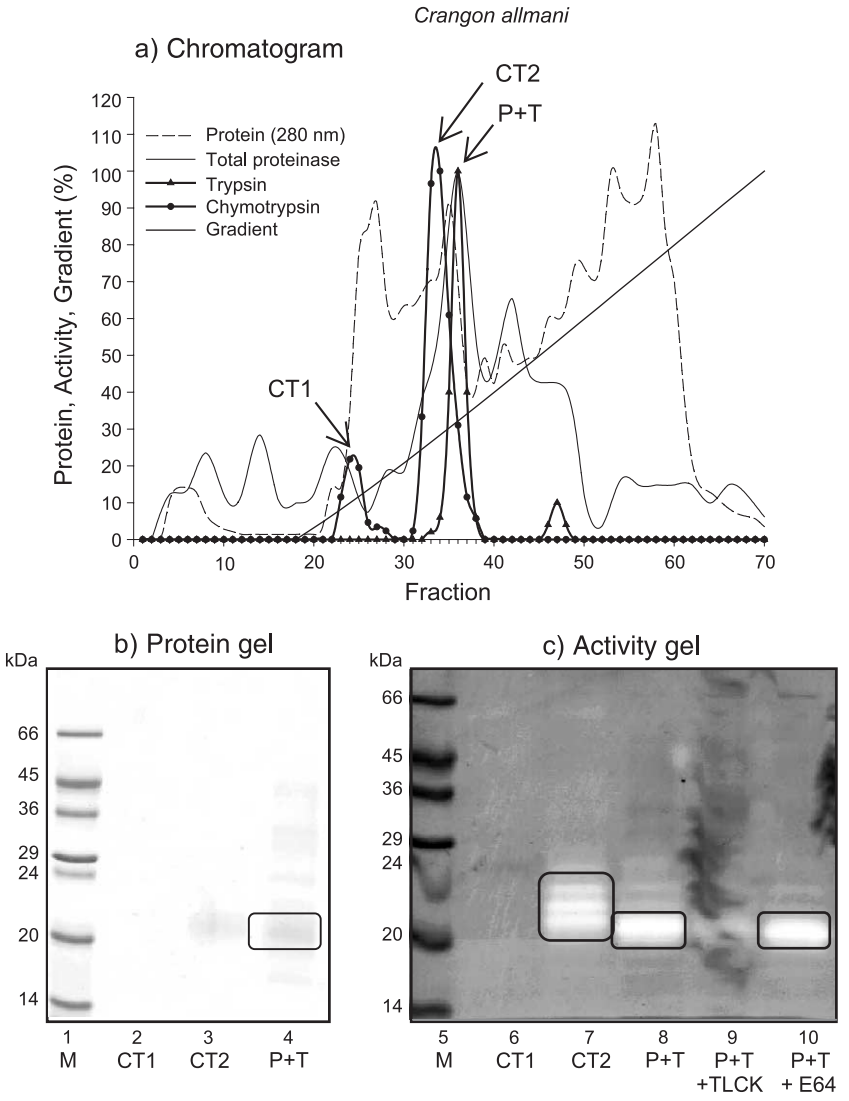

Fig. 7. *C. allmani*: (a) Chromatographic separation of midgut gland extract with a specific trypsin activity of 3.4 $U \cdot g_{fw}^{-1}$. The maximum of the total proteinase activity and trypsin (P+T) and chymotrypsin (CT1 and CT2) are indicated. Protein-gel (b) after SDS-PAGE and activity gel, (c) after substrate-SDS-PAGE of fractions with maximum proteinase and trypsin activity (P1), and chymotrypsin activity (CT1 and CT2) as well as inhibitors E 64 and TLCK. Bands of proteolytic activity and corresponding protein bands are emphasized (M=markers).

3.7. Cathepsin L-like activity in chromatographically separated midgut gland extracts

Extracts of *C. crangon* and *C. allmani* with low trypsin activities were chromatographically separated and assayed for Cathepsin L (Fig. 8a,b). Both extracts showed three wide peaks of Cathepsin L activity at NaCl concentrations of 0.1, 0.15 and 0.28 $mol \cdot l^{-1}$. However, the intensity of peaks differed between species. In the extract of *C. crangon*, the maximum of Cathepsin L eluted at a concentration of 0.15 $mol \cdot l^{-1}$ NaCl (Fig. 8a). In

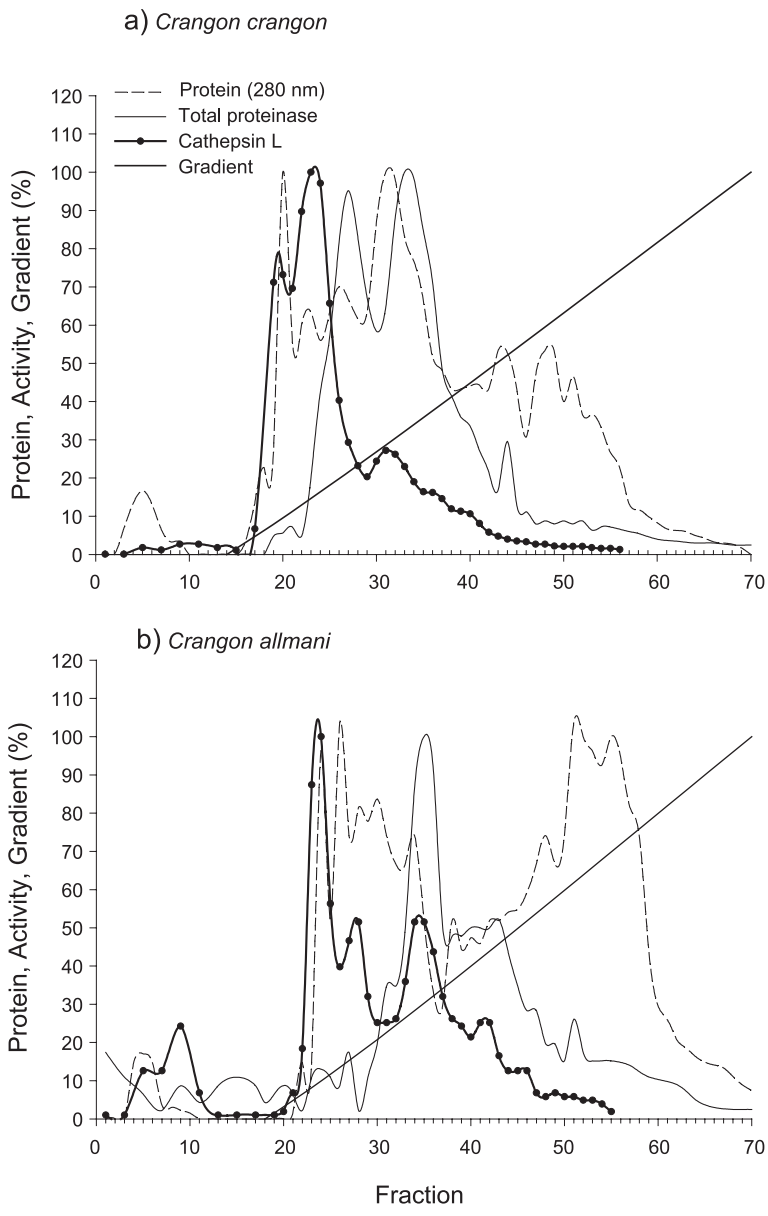

Fig. 8. Profiles of cathepsin-L-like activities in midgut gland extract of (a) *C. crangon* and (b) *C. allmani* with a low specific trypsin activity (<2.0 $U \cdot g_{fw}^{-1}$). The legend applies to both figures.

extracts of *C. allmani*, highest Cathepsin L activity appeared at a concentration of 0.1 $mol \cdot l^{-1}$ NaCl (Fig. 8b). In neither species cathepsin L profiles matched exactly with the elution profiles of total proteinase activity.

3.8. Feeding experiments

The specific activity of total proteinase did not differ significantly between the treatments ($P>0.05$, ANOVA; results not presented as graph). It ranged

Table 2
Frequencies of activity levels in shrimps *C. crangon* which were exposed to different feeding conditions

Trypsin activity level	Feeding conditions			
	Control	*Spirulina*	*Mytilus*	Hunger
Low (<2 $U \cdot g_{fw}^{-1}$)	7	4	10	7
Medium (2–6 $U \cdot g_{fw}^{-1}$)	2	1	0	0
High (>6 $U \cdot g_{fw}^{-1}$)	1	1	0	1
$\hat{\chi}^2$		0.3095	4.2857	2.000
Difference from control		n.s.	n.s.	n.s.

The appearance of activity levels in the treatments did not differ significantly (n.s.) from those of the control group [$\hat{\chi}^2$<5.99= $\hat{\chi}^2$(2,0.05); P>0.05].

between 0.3 and 0.5 $\Delta E \cdot min^{-1} \cdot g_{fw}^{-1}$. The activity in the control group amounted to 0.35 $\Delta E \cdot min^{-1} \cdot g_{fw}^{-1}$. Shrimps fed with mussels and starved shrimp had highest activities of 0.47 and 0.49 $\Delta E \cdot min^{-1} \cdot g_{fw}^{-1}$. Lowest activities of total proteinase were present in shrimps fed with *Spirulina*-tabs (0.3 $\Delta E \cdot min^{-1} \cdot g_{fw}^{-1}$).

The appearance of low, moderate and high trypsin activities in the feeding experiments did not differ significantly from the control group [$\hat{\chi}^2$<5.99= χ^2(2,0.05); P>0.05: Table 2]. Most animals showed low trypsin activities while only few animals appeared with moderate and high trypsin activities.

4. Discussion

The North Sea shrimps *C. crangon* as well as *C. allmani* always had high activities of total proteinase in their midgut glands. Our work, however, indicated two further characteristics. Firstly, only few shrimps had high trypsin levels, while in most animals, the trypsin activities were extremely low or even lacking. Secondly, the serine proteinases trypsin and chymotrypsin were substituted by cysteine proteinases such as cathepsin L.

4.1. Variation of trypsin

Trypsin and chymotrypsin are important endopeptidases in crustaceans. Upon excretion into the extracellular space of the midgut gland tubules and passage into the stomach, they facilitate the first steps of protein hydrolysis. Variation of enzyme activity, as observed here for trypsin, may be due to the quality or quantity of food (Saborowski and Buchholz, 1999), the effect of inhibitors (Garcia-Carreño et al., 1999), the moult cycle (Dall et al., 1990) or the contribution of exogenous enzymes (Donachie et al., 1995).

The available information about alimentary effects on the variation of digestive proteinases is contradictory. According to Guillaume and Ceccaldi (2001), carnivorous species are expected to have higher proteinase activities while omnivorous species would have lower proteinase activities but, instead, increased activities of carbohydrases, such as amylase. Indeed, Le Moullac and Van Wormhoudt (1994) found in larvae of the shrimp *Penaeus vannamei* elevated chymotrypsin activities along with increasing dietary casein levels. In the same experiment, however, the authors found a negative relation between dietary protein levels and trypsin. Moreover, Fernández Gimenez et al. (2001) reported an opposite relation between feeding habits and enzyme activities: e.g., the omnivorous *P. vannamei* and *P. monodon* had higher proteinase activities than the carnivorous shrimp *P. californiensis* and *Pleoticus muelleri*.

In our experiments, feeding different diets did not affect enzyme activities in *C. crangon*. After 10 days of feeding mussels, algae or starvation, neither total proteinase nor trypsin activities varied from controls and field samples. Moreover, the pattern of frequency of specimens with predominantly low activities and few with high activities remained similar. Accordingly, alimentary effects cannot explain the distinct variation in trypsin activities of *C. crangon* and *C. allmani*.

The strong intraspecific variation of trypsin activity seems not to be caused by low molecular weight effectors or by competitive inhibitors in the crude extract. Potential effectors were most likely separated from the target enzymes during the chromatographic processes. When high trypsin activity was measured in crude extracts, an active trypsin protein could always be isolated by gel filtration and anion-exchange chromatography. In contrast, extracts with lacking trypsin activity never developed significant activity after the purification process. Even the lack of Ca^{2+}, which is an important activator in vertebrate trypsins, caused no distinct loss of activity. However, it cannot be excluded that noncompetitive inhibitors might have bound irreversibly to the enzymes and

inactivated them; for example, Garcia-Carreño et al. (1999) found in midgut glands of the shrimp *Litopenaeus vannamei* a soluble molecule which inhibited trypsin. In order to clear this uncertainty, we conducted control assays of extract mixtures with low and high trypsin activity at different ratios. We did not find any indication that low activity extracts, possibly containing inhibitors, had adverse effects on trypsin activity above the expected decrease of activity due to dilution (results not shown).

According to Dall et al. (1990), activities of digestive enzymes are least affected by moulting processes during the intermoult stage. In *P. muelleri*, enzyme activities were lowest during the intermoult stage while they were highest in the postmoult stage (Fernández Gimenez et al., 2001). In *P. vannamei*, trypsin activity was significantly influenced by starvation as well as by moulting (Muhlia-Almazán and García-Carreño, 2002). In order to minimize moult-related physiological effects, we analysed exclusively shrimps in the intermoult stage. We also excluded a possible effect of gender on trypsin variability by using only adult females in this study.

The contribution of endosymbionts to significant amounts of digestive enzymes in crustaceans is ambiguous. The occurrence of bacteria in the digestive tract was studied in few crustaceans, such as the Antarctic krill (Donachie and Zdanowski, 1998) and isopods (Zimmer et al., 2001). While bacteria were considered important in the breakdown of plant and detrital material in a thalassinid decapod (Pinn et al., 1997), their role was denied in Northern krill (Donachie et al., 1995). If high trypsin activities in *Crangon* species were produced by symbiotic bacteria, it seems unlikely, that only a minority of the population, which otherwise is exposed to the same environmental conditions, hosts significant amounts of enzyme-producing symbionts. Furthermore, the supply with different feeds and starvation during the feeding experiments should alter the microbial conditions within the gut as well as the residence time of the gut content. Both factors can be expected to entail variations in the population of endosymbionts and, consequently, changes in enzyme activities as well as the expression of different trypsin isoforms. However, the trypsin isoforms of *C. crangon* had always the same chromatographic characteristics and the same molecular weight, when present. Therefore, we believe that the contribution of bacteria to the variation in trypsin activity of *C. crangon* is not significant.

4.2. The presence of cysteine proteinases

Cysteine proteinases were thoroughly studied in vertebrates where they appear in different tissues and are involved in the lysosomal hydrolysis of endogeneous and exogeneous proteins (see Barrett and Kirschke, 1981; Kirschke et al., 1998). In crustaceans, however, knowledge about cysteine proteinases is still limited. Sequences and gene structures were analysed for some cysteine proteinases (Laycock et al., 1991; Le Boulay et al., 1995, 1996, 1998); however, their role in the process of digestion is still poorly understood.

The activities of total proteinase in *C. crangon* and *C. allmani* were highest between pH 5.0 and 6.0. Thus, our results comply with those of Degkwitz (1957) who found in *C. crangon* maximum proteinase activity at pH 5.5 to 6.0. Generally, maximum activities of most crustacean proteinases range between pH 5.0 and 9.0 (Garcia-Carreño, 1992; Garcia-Carreño et al., 1994; Ceccaldi, 1997). Trypsin-like serine proteinases typically show pH-optima at neutral or slightly alkaline conditions between pH 7.5 and 9.0; for example, total proteinase of the shrimp *P. muelleri* which has a high amount of trypsin-like serine proteinases has a pH-optimum between pH 7.5 and 8.0 (Fernández Gimenez et al., 2001). In *P. vannamei*, where the major proteinases were the serine proteinases trypsin and chymotrypsin, total proteolytic activity was highest at slightly alkaline conditions (Le Moullac et al., 1997). In contrast, cathepsin-like cysteine proteinases show highest activities between pH 4 and 7. The maximum activity of cysteine proteinase from *P. vannamei* was at pH 5.1 (Le Boulay et al., 1996). Because of the low pH-optimum in both *Crangon* species, we agree with Degkwitz (1957) that the majority of proteolytic enzymes in *C. crangon* and *C. allmani* may consist of cathepsin-like proteinases rather than trypsin-like enzymes.

Total proteinases in midgut gland extracts of *C. crangon* and *C. allmani* were inhibited more than 70% by E 64 but less than 9% by AEBSF. In contrast to *Crangon* species, E 64 caused no distinct loss of

total proteinase activity in *E. bernhardus*, *C. pagurus* and *E. superba*. However, activity was reduced at least 50% by AEBSF. E 64 is a highly selective and irreversible inhibitor for cysteine proteinases (Barrett et al., 1982; Katunuma and Kominami, 1995) while AEBSF specifically inhibits serine proteinases (Markwardt et al., 1974). Accordingly, the inhibitor assays confirm the predominance of cysteine proteinases in the midgut gland extracts of both *Crangon* species while serine proteinases represented the dominant endopeptidases in *E. superba*, *E. bernhardus* and *C. pagurus*, respectively (Osnes and Mohr, 1985; Tsai et al., 1986).

Chromatographic separation of *Crangon* extracts confirmed the previous results from crude extracts. In both species, extracts with low trypsin activity showed a distinct pattern of total proteinase activity but neither trypsin nor chymotrypsin were detected. On the contrary, *Crangon* extracts with high trypsin activities did show distinct peaks of trypsin and chymotrypsin. In many crustaceans, the number of trypsin isoforms varies between species but mostly at least two or three isoforms are present (e.g., Honjo et al., 1990, Fernández Gimenez et al., 2001, Muhlia-Almazán and García-Carreño, 2002). Both *Crangon* species, however, expressed only a single trypsin isoform. The isolated trypsin of either species had the same apparent molecular weight of 20 kDa and each enzyme showed a distinct activity band which was clearly inhibited by TLCK, a specific trypsin inhibitor (Garcia-Carreño, 1992). The estimated molecular weight of 20 kDa, however, does not reflect the true molecular mass of the protein. Most digestive enzymes have a compact molecular structure which makes them resistant against hydrolysis. Due to the native conditions of the electrophoresis, the protein was not unfolded and, therefore, the size of the molecule was underestimated. In contrast to trypsin, the chymotrypsin isoforms differed between species. The zymograms showed three activity bands in either species which ranged between 35 and 43 kDa in *C. crangon* and between 20 and 23 kDa in *C. allmani*.

We detected cysteine proteinases in FPLC fractions of *C. crangon* by activity measurements and inhibitor assays. Cathepsin L was identified with the specific substrate *Z*-phe-arg-7-amido-4-methylcoumarin hydrochloride. It appeared in three broad peaks which partly eluted separate from the maximum of total proteinase activity. Maximum inhibition appeared within the peaks of highest total proteinase activity. The appearance of cathepsin isoforms in crustaceans is in agreement with Laycock et al. (1989, 1991) and Le Boulay et al. (1996) who isolated cathepsin-L-like cysteine proteinases from lobsters and shrimps. A detailed characterization of North Sea shrimp cathepsins, however, was beyond the scope of this work but will be the subject of future studies.

5. Conclusions

In conclusion, our work has shown that *Crangon* species exhibit an unusual pattern of trypsin activity which, to our knowledge, has not been described previously in any crustacean species. The lack or the presence of trypsin was neither related to the mode of feeding nor to the nutritive state of the animals. The share of the serine proteinase trypsin on the total proteolytic activity varied distinctly between species. On one hand, *C. crangon*, *C. allmani* as well as *P. montagui* had lowest trypsin activities and lowest shares of serine proteinases, while on the other hand, both were highest in *E. superba*. Conversely, the share of cysteine proteinases was highest in *Crangon* species but lowest in *E. superba*. Although the number of analysed species is limited, the results may indicate different traits of food utilization strategies in eucarid crustaceans.

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