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Catalytic properties and polymorphism of serine endopeptidases from the midgut gland of the brown shrimp *Crangon crangon* (Decapoda, Caridea)

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Abstract The brown shrimp *Crangon crangon* is a key species in the coastal areas of the North Sea. It constitutes a significant food source for fishes. Simultaneously, it is an important predator on a wide range of invertebrates. *C. crangon* shows a variety of digestive enzymes that allow to utilizing a wide range of food items. The initial step of alimentary protein digestion, that is the degradation into peptides, is facilitated by set of endopeptidases which are expressed by the midgut gland. In crustaceans, these endopeptidases are often dominated by serine proteinases. *C. crangon*, however, predominantly express cysteine proteinases, while only some specimens show a highly variable pattern of serine proteinases. The composition of these serine endopeptidases was investigated using liquid chromatography, substrate gel electrophoresis and inhibitor assays. Distinctly elevated activities were present only in about 10% of the samples. When activity was detected, two peaks, one with tryptic activity and the other one with chymotryptic activity, could be separated by anionic exchange chromatography. Moreover, specimens with elevated tryptic activities often showed highly polymorphic patterns of endopeptidases after electrophoretic separation. Overall, 30 different bands of endopeptidases were identified. There was no similarity between animals from the same sampling sites, neither between animals of similar size, weight or nutritive state. The polymorphism of proteinase from the midgut gland seems to reflect the high adaptive potential of this species to variable trophic conditions in a continuously changing environment.

Introduction

The brown shrimp *Crangon crangon* is a dominating member of the epibenthic fauna of the North Sea and, particularly, the coastal and Wadden areas (del Norte-Campos and Temming 1994). Its feeding mode is omnivorous and opportunistic (Gibson et al. 1995; Pihl and Rosenberg 1984; Wilcox and Jeffries 1974). Stomach content analysis showed that the composition of ingested organisms widely reflected the composition of potential prey organisms in the habitat (Pihl and Rosenberg 1984; Plagmann 1939). Immediately upon ingestion, the macerated food is hydrolysed by the digestive enzymes.

Crustaceans express a set of highly active digestive enzymes which hydrolyse the major food items. These enzymes include various proteinases, lipases, esterases and glucanases (Dall and Moriarty 1983). Particularly the proteinases appear in many species with high activities (Galgani and Nagayama 1988). Proteolytic activity, in turn, is often provided by enzymes which belong to the class of serine proteinase, i.e. by trypsin-like or chymotrypsin-like enzymes (Dittrich 1992a, b; Sakharov et al. 1994). Decapods investigated so far for trypsin mostly showed high activities of this enzyme. Moreover, trypsin from some crustaceans, like the edible crab *Cancer pagurus*, showed extraordinary high in vitro stability (Saborowski et al. 2004). Trypsin-like enzymes were suggested to be the most important proteolytic enzymes in the digestive tract of

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crustaceans (Dall and Moriarty 1983; García-Carreño et al. 1997; Ceccaldi 1997). However, caridean shrimps of the species *Crangon crangon* and *Crangon allmani* showed extremely variable levels of tryptic activity (Teschke and Saborowski 2005). While most of the shrimps from different coastal areas in the German Bight showed very low or none tryptic activities, about 10% of the animals expressed high or, occasionally, very high activities. Simultaneously, cysteine proteinases such as cathepsin L-like enzymes were present in the midgut glands of both species (Teschke and Saborowski 2005). No obvious relation between the appearance of tryptic enzymes, their activity levels and the diets of shrimps was observed. Therefore, the reason for this unusual pattern of trypsin expression is still unknown. However, this knowledge is important for the understanding of both the digestive physiology of *C. crangon* and for the extraordinary ecological success of this species in the coastal and Wadden areas of the North Sea. Accordingly, the aims of the present work were firstly to verify and to characterize the serine endopeptidases trypsin and chymotrypsin in the brown shrimp *Crangon crangon* and secondly to study the
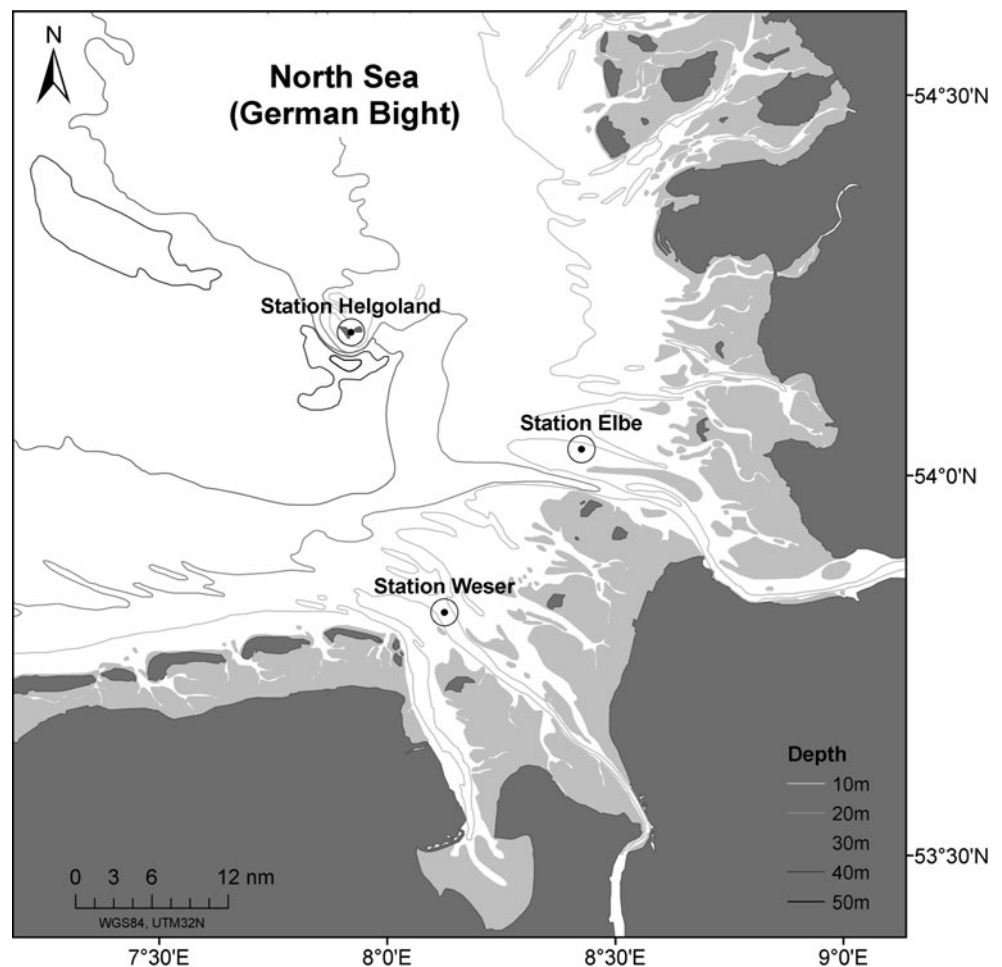
appearance of polymorphic endopeptidases in field samples from the inner German Bight.

Methods

Origin of animals

North Sea shrimps, *Crangon crangon*, were captured in November and December 2003 with R/V Aade by repeated short hols with a dredge at Helgoland Dünenhafen (54°11′ N, 07°54′ E). In March 2004, additional *Crangon* samples were taken with R/V Uthörn from the estuaries of the rivers Weser (54°02′ N, 08°25′ E) and Elbe (53°49′N, 08°07′E), (Fig. 1). Animals were immediately sorted from the net into aquaria and shipped alive to the laboratories of the Marine Station at Helgoland. The next day, individuals were randomly selected from the aquaria. The animals were blotted dry and the fresh weight was recorded. The total length (TL) was measured under a stereo microscope and the sex was determined. The cephalothorax of the shrimps was opened dorsally and the maturation stage of

Fig. 1 The sampling sites in the inner German Bight around the island of Helgoland and in the estuaries of the rivers Weser and Elbe

Table 1 Substrates used for enzyme assays

Enzyme	EC-number	Substrate	Abbreviation	Source
Trypsin (peptidase)	3.4.21.4	Nα-benzoyl–L-arginine–4-nitroanilide–hydrochloride	L-BANA	Fluka, 12915
Trypsin (esterase)	3.4.21.4	Nα-tosyl–L-arginine methyl ester	TAME	Sigma, T4626
Chymotrypsin	3.4.21.1	N-succinyl–ala–ala–pro–phe–p-nitroanilide	SAAPPNA	Sigma, S7388
Collagenase	3.4.24	N-(3-[-2-furyl]acryloyl)–leu–gly–pro–ala	FALGPA	Sigma, F5135
Elastase	3.4.21	N-succinyl–ala–ala–ala–p-nitroanilide	SAAAPNA	Sigma, 4760
Leucyl-aminopeptidase	3.4.11.1	L-leucine–p-nitroanilide–hydrochloride	Leu–pNA	Sigma, L2158
Prolyl-aminopeptidase	3.4.11.5	L-proline–p-nitroanilide trifluoroacetat	Pro–pNA	Sigma, P5276
Alanyl-aminopeptidase	3.4.11.2	L-alanine–p-nitroanilide–hydrochloride	Ala–pNA	Sigma, A9325

the ovaries was recorded. The midgut glands were dissected, weighed and transferred into 1.5-ml reaction cups. One ml of ice-cold demineralized water (a. dem.) was added to each cup.

Preparation of extracts

The midgut glands were homogenized with an ultrasonic cell disrupter (Branson, Sonifier B15 equipped with a microtip). Three bursts of 5 s at 30% of maximum energy were applied while the sample was cooled on ice. Thereafter, the samples were centrifuged for 15 min at 15,000 g and 4 °C. The supernatants were transferred into new reaction cups. The centrifugation step was repeated when the amount of lipids on the surface was high and lipids remained in the aqueous phase. Sub-samples of the extracts (10 μl) were taken, diluted 1:20 with water and used for subsequent enzyme screening. The remaining extracts were stored at −80 °C.

Enzyme and protein assays

Screening for trypsin and chymotrypsin activity was carried out in midgut gland extracts of each animal. On each of two separate microplates 50 μl of diluted extract (1:20 in a. dem.) were applied. The reaction was started subsequently by the addition of 250 μl of 0.1 mol l^{-1} Tris HCl (supplemented with 10 mmol l^{-1} Ca^{2+}) which contained the substrates BAPNA or SAAPPNA (Table 1). The substrates were first dissolved in dimethylsulphoxide (DMSO) and then diluted with buffer (0.1 mol l^{-1} Tris HCl + 10 mmol l^{-1} Ca^{2+}) to a final concentration of 1 mmol l^{-1}. Trypsin activity was measured at pH 8 and chymotrypsin at pH 7. The microplates were incubated at room temperature. The optical density of the plates was read at 405 nm (OD_{405}) after 15, 30 and 45 min. The change in optical density was normalized to fresh weight (ΔOD_{405} min^{-1} g_{fw}^{-1}). Samples that showed activities higher than 10

OD_{405} min^{-1} g_{fw}^{-1} were used for subsequent chromatographic enzyme separation. In these samples, activities of trypsin and chymotrypsin were additionally quantified in a spectrophotometer at 410 nm. Fifty μl of extracts and 930 μl of buffer were mixed in a glass cuvette and incubated for 5 min at 30 °C in a temperature-controlled cuvette holder. Substrate solution (20 μl) was added to start the reaction and the increase in absorbance was continuously monitored for another 3 min. The substrate concentration in the reaction mixture amounted to 1 mmol l^{-1}. The extinction coefficient for substrates that liberate nitroanilide was 10.2 l $mmol^{-1}$ cm^{-1} (Rick 1974a, b). Activity results obtained by each method, microplate screening or cuvette measurements, were closely correlated (trypsin: $r^2 = 0.994$; cymotrypsin: $r^2 = 0.903$; $p < 0.001$, $n = 25$ each). Esterase activity of trypsin using TAME as substrate was measured at 240 nm in quartz cuvettes (Geiger and Fritz 1984). Enzyme activity was normalized for protein or fresh weight. Additionally, the extracts were analysed for a set of endo- and exo-peptidases with the substrates listed in Table 1.

The protein was measured in midgut gland extracts and in separated enzymes after Bradford (1976) with a commercial protein assay (BioRad, 600-0005). Bovine serum albumin (0.1 mg ml^{-1}) was used as protein standard. The assay was carried out in microplates.

Inhibitor assays

Extracts of those midgut glands, which showed elevated trypsin or chymotrypsin activities, were incubated with selected inhibitors (Table 2). These inhibitors either generally inhibited proteases and protease classes, or they inhibited specific enzymes. Enzyme solutions (20 μl) were incubated at 25 °C with 20 μl of inhibitor solutions (1 mmol l^{-1}) and for control with the solvent of the inhibitor solution. After 60 min of incubation, the extracts were subjected to cuvette activity assays for trypsin and

Table 2 List of enzyme inhibitors

Enzyme/enzyme class	EC-number	Inhibitor	Abbreviation	Source
Proteases	3.4.	Phenylmethylsulfonyl fluorid	PMSF	Sigma, P7626
Proteases/Serineproteases	3.4.(21)	Soybean trypsin inhibitor	SBTI	Sigma, T9128
Serineproteases	3.4.21	4-(2-aminoethyl)enzenesulfonyl–fluorid hydrochloride	AEBSF	Merck, 124839
Cysteineproteases	3.4.22	Trans-epoxy-succinyl–L-leucylamido-(4-guanidino)–butane	E64	Sigma, E3132
Trypsin	3.4.21.4	N-p-tosyl–L-lysin chloromethyl ketone	TLCK	Sigma, T7254
Chymotrypsin	3.4.11.1	Nα-p-tosyl–L-phenylalanin chloromethyl ketone	TPCK	Sigma, L4376

chymotrypsin as described previously. The residual activity after inhibition was presented in relation to the uninhibited control which was set to be 100%.

Chromatographic separation of enzymes

Extracts were first desalted and rebuffed over NAP-10 gel-filtration columns (Pharmacia Biotech). Subsequently, the extracts were separated by anion exchange chromatography using an FPLC-system. Extracts (1.5 ml) were applied onto a UNO Q1 R—column (BioRad). Proteins were eluted from the column by increasing the concentration of NaCl from 0 to 1 mol l^{-1} in the buffer (imidazole, 0.01 mol l^{-1}, pH 6.8). The flow rate was 1 ml l^{-1}. The absorbance was detected at 280 nm (protein) with an Uvicord SII monitor. The extracts were separated into 55 fractions of 0.5 ml each and immediately screened for enzyme activities in 96-well microplate assays as described previously. Fractions with maximum enzyme activities were pooled. In order to document the purification progress, the enzyme activities and protein concentrations were measured after each purification step.

Substrate PAGE and IEF

Proteins were separated by SDS-PAGE (12%T, 2.8%C) in a vertical Hoefer SE 250 electrophoresis device. Midgut gland extracts and isolated enzymes were diluted 1:2 with sample buffer which contained 4% SDS. The samples were neither heated nor treated with mercaptoethanol. The gels were loaded with 10–20 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.

Bands of active endopeptidases were visualized according to García-Carreño et al. (1993). After electrophoretic separation, one of the gels was first thoroughly washed in demineralized water (a. dem.). Then, it was incubated in an ice-cooled solution of 3% (w/v) casein (Sigma, C-5890), dissolved in 0.1 mol l^{-1} Tris HCl, pH 8. The casein was allowed to penetrate into the gel for

30 min. Thereafter, the temperature was increased to about 30 °C and the gel was incubated for another 60 min in the casein solution. The casein solution was discarded and the gel was washed several times in a. dem. The gel was coomassie-stained over night and destained with an aqueous solution containing 40% (v/v) methanol and 7% (v/v) glacial acetic acid. Bands with endopeptidase activity appeared pale on an otherwise blue-dyed casein containing gel. The gels were photographed (BioRad, ChemiDoc) and the relative mobility of the bands was analysed using the program QuantityOne (BioRad).

Isoelectric focussing (IEF) was carried out on commercial gels with a pH gradient from 3.5 to 9.5 (Ampholine PAG-plate, Amersham Bioscience 18-1016-67). Ten μl of the samples (crude extracts and isolated enzymes), as well as IEF-standards (BioRad, 161-0310) were applied onto the gel with the help of small pieces of filter papers (5 × 3 mm). IEF was run for 1.5 h at 1,500 V, 50 mA and 30 W in a horizontal system (Pharmacia Biotech, Multiphor II). The protein bands were detected by silver staining (Silver staining kit—protein, Pharmacia Biotech 71-7177-00).

Temperature-activity profile and thermal stability

The thermal profile of enzyme activity was measured at temperatures between 5 and 75 °C. The incubation was performed in a temperature-controlled cuvette holder. Buffer and substrate were incubated for 5 min before the reaction was initiated with enzyme extract.

The thermal stabilities of trypsin and chymotrypsin were determined over a period of 5 h. Samples were incubated at temperatures between 0 and 50 °C. Thereafter, enzyme activities were measured every 30 min under standard conditions (30 °C). In each experiment, the activities of trypsin and chymotrypsin were presented in relation to the maximum average activity.

Statistics

Data sets were presented as mean and standard deviation (SD). Differences among data sets were analysed by

ANOVA followed by a Tukey's multicomparison test. Differences are reported as statistically significant when $p < 0.05$.

The effect of sex, origin (Helgoland vs. Estuaries) and six quantitative independent variables (total length, carapace length, weight, MD index, midgut gland weight and trypsin activity) on the appearance of protein bands were analysed with canonical analysis of principal coordinates (CAP: Anderson and Willis 2003, Anderson 2003). Each term in the analysis was tested using 4,999 permutations of the appropriate units. For these analyses, we used presence/absence data and the Sorensen index. We did not consider the separate effect of Weser and Elbe estuaries due to the relatively low number of individuals collected in each of these sites compared with Helgoland. We tested for differences between Elbe and Weser but we found no significant differences (see "Results").

Patterns obtained with CAP were checked by evaluating the effect of independent variables on the protein bands that most contributed to the formation of the canonical axes. This evaluation was done with a log-linear analysis for the effect of 'Sex' and 'Origin', and Generalized Linear Model (GzLM) with binomial error structure and Logit link function.
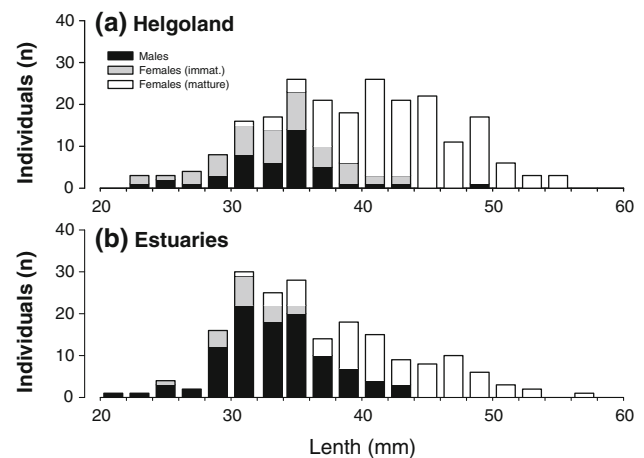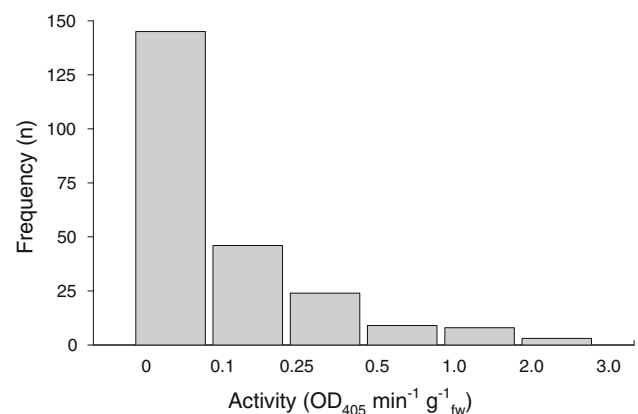
Results

Size and sex distribution

Crangon crangon showed a distinct sexual dimorphism. The largest female was 57 mm long. Males were at maximum 44 mm and on average smaller than the females of the respective sampling site. For a better overview, size classes of 2 mm intervals were classified. The sex distribution within the size classes varied between sampling sites (Fig. 2a, b). The samples from the estuaries contained less immature females (10%) than the samples from Helgoland (20–22%). In general, there were more large animals caught around Helgoland than at the estuaries of Elbe and Weser.

Enzyme activities

The majority of the animals (80%) showed no trypsin activity or very low activity (<0.25 OD_{405} min^{-1} g_{fw}^{-1}). Only few animals had elevated activities of more than 0.25 OD_{405} min^{-1} g_{fw}^{-1} (Fig. 3). Less than 5% of the tested individuals expressed a trypsin activity higher than 1.0 OD_{405} min^{-1} g_{fw}^{-1}. The data follow a Poisson distribution of trypsin activities (occurrences of an event that happens rarely) within the entity of tested animals. The activities of chymotrypsin were much lower than those of trypsin

Fig. 2 Size and sex distribution of *Crangon crangon* from **a** Helgoland and from **b** the estuaries

Fig. 3 Frequencies of trypsin activities in *Crangon crangon* from Helgoland and the estuaries ($n = 235$)

reaching a maximum of only 0.3 OD_{405} min^{-1} g_{fw}^{-1} (data not shown). There was a weak correlation between trypsin and chymotrypsin activities ($r^2 = 0.035$, $p = 0.01$, $n = 376$).
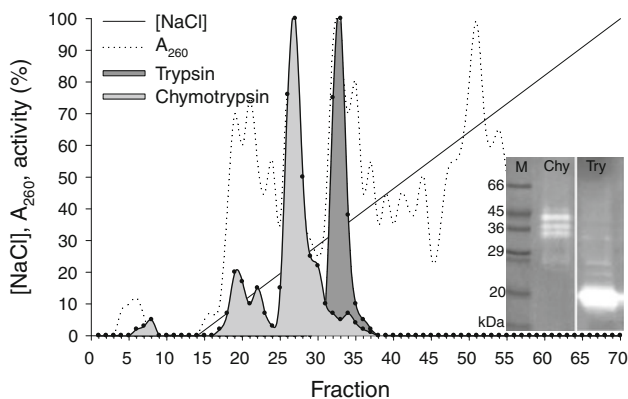
Chromatographic separation

Midgut gland extracts were separated by anionic exchange chromatography. The elution profile showed highest trypsin activity in fractions 31 to fraction 34 (Fig. 4). Chymotrypsin was for the most part separated from trypsin. Highest chymotrypsin activities appeared in fractions 25–29 although some activity was detected in fraction 18–23 and also in fractions 30–35. Trypsin was enriched 13-fold and chymotrypsin 11-fold. Fractions with maximum activity were pooled and used for further characterization. The insert shows the electrophoretic separation and activity staining of chymotrypsin (fraction 28) and trypsin (fraction 33).
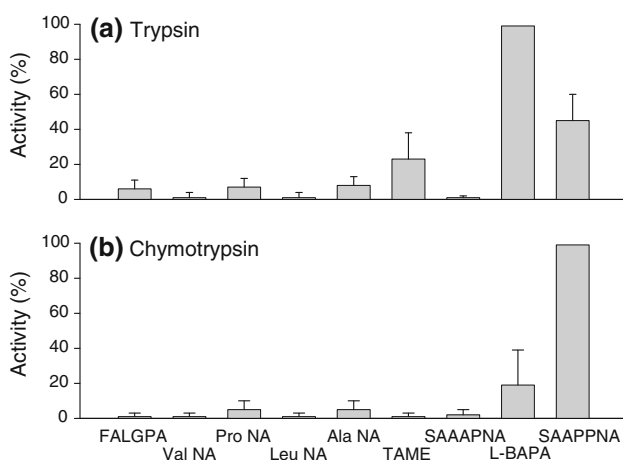
Substrate specificity

Trypsin and chymotrypsin were incubated with different substrates but under otherwise identical conditions. Highest activity for trypsin was obtained with L-BAPA and was set to be 100% (Fig. 5a). SAAPNA and TAME were hydrolysed by *Crangon* trypsin to a lesser extent (45 and 19%) and varied strongly. All other substrates were hydrolysed only marginally.

Chymotrypsin hydrolysed SAAPPNA at highest rates (Fig. 5b). Moreover, L-BAPA was hydrolysed at a lower rate (20%) and showed high variability. A low turnover rate of prolyl- and alanyl-aminopeptidase was recorded (<5%), while no activity was observed with the substrates FALGPA, TAME, SAAAPNA and L-pNA.

Effects of inhibitors

Activities of trypsin and chymotrypsin were measured without inhibitors and set to be 100% (Fig. 6a). Trypsin activity was almost completely inhibited by SBTI, PMSF and AEBSF (1–5% remaining activity), as well as the trypsin-specific inhibitor TLCK (8% remaining activity). TPCK that binds to chymotrypsin also caused a significant decrease in trypsin activity (30% remaining activity).
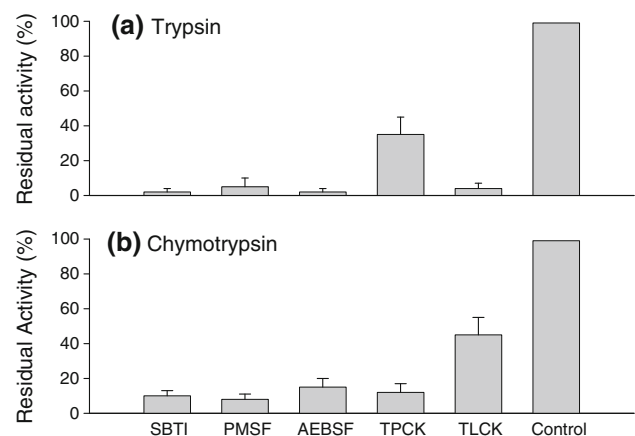
Chymotrypsin activity was strongest reduced by the proteinase inhibitors SBTI, PMSF and AEBSF (10% remaining activity, Fig. 6b). Additionally, TPCK very effectively inhibited chymotrypsin activity to <10%. But also TLCK caused a 50% reduction in chymotrypsin activity.

Operational parameters

The isoelectric points (*pI*) of *C. crangon* trypsin and chymotrypsin are similar amounting to 3.9 for trypsin and 4.0 for chymotrypsin (data not shown).

Thermal profiles: At 5 °C, the tryptic activity amounted to <10% of the maximum. With increasing incubation temperatures, the activity rose exponentially until a maximum activity appeared at 45 °C. Temperatures above 45 °C caused a rapid loss of activity. The thermal profile of chymotrypsin was similar to that of trypsin. However, maximum activity appeared at 55 °C (data not shown).

Thermal stability: When kept at temperatures lower than 40 °C or on ice, the activity of isolated trypsin remained stable for at least 5 h. After 5 h, a slight decrease in activity (80%) was recorded. Incubation of trypsin at 43 °C resulted in reduced enzyme activity after just 1 h. After 5 h, trypsin activity was already reduced to about 50%.

Fig. 4 Example of an elution profile of an extract from the midgut gland of *Crangon crangon* with high trypsin activity

Fig. 5 Hydrolysis of different substrates (for details refer to Table 1) by **a** trypsin and **b** chymotrypsin from midgut gland extracts of *Cangon crangon*. The activities of the respective specific substrates L-BAPA for trypsin and SAAPPNA for chymotrypsin were set to 100% (*n* = 3–5)

Fig. 6 Inhibition of **a** trypsin and **b** chymotrypsin activities of midgut gland extracts from *Crangon crangon* by different proteinase inhibitors (for details refer to Table 2). The 'control' represents untreated samples and was set to 100% (*n* = 3–5)
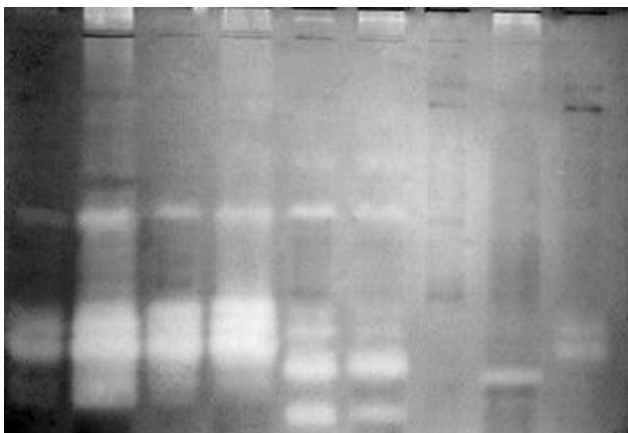
With raising temperatures, the loss of trypsin activity was more rapid, and at an incubation temperature of 50 °C, it was instantly lost. Chymotrypsin also remained stable for at least 5 h when incubated at <40 °C. Higher temperatures caused a rapid decrease in activity (data not shown).

Endopeptidase zymograms

Zymograms of crude midgut gland extracts showed a heterogenous pattern of proteolytic enzymes between different *C. crangon* individuals. An example of 9 individuals is shown in Fig. 7. Single individuals differed from each others in size and number of different endopeptidases as well as in the intensity in which different endopeptidases were expressed. In total, activity bands with 30 different apparent molecular masses were detected. Of 61 analysed shrimps, which expressed trypsin activity only very few shrimps showed the same endopeptidase expression pattern. Only one group of three animals and two other groups of two animals each shared the same enzyme pattern.

Inhibition of endopeptidases activity bands

From the most frequently appearing activity bands, seven bands in the range of 20–85 kDa were selected and their response to different inhibitors was studied. Individual *C. crangon* showed only few of these bands which, moreover, altered between specimens. Therefore, we extracted and combined the results and present them as a sketch (Fig. 8). Inhibition of a midgut gland homogenate with different protease inhibitors resulted in the loss of distinct bands compared with the non-inhibited homogenate which are denoted a 'Control'. The application of specific inhibitors like TLCK (binds trypsin) left more active enzymes in the homogenate than inhibitors that target a broad variety of endopeptidases. AEBSF, for instance, which binds the

Fig. 8 Sketch showing the electrophoretic separation of the 7 most frequent proteolytic activity bands (*light grey*) from midgut gland extracts of *C. crangon*. The inhibition of the activity bands with specific inhibitors (see Table 2) is illustrated. The 'Control'-lane represents full activity of all 7 bands. SBTI, PMSF and AEBSF entirely inhibited all bands. TPCK, for example, inhibited all bands but not the one at 20-kDa

active serine in the catalytic centre of the enzyme, suppressed all proteolytic activity.

In most homogenates, all activity bands were inhibited by the protease inhibitors (SBTI and PMSF) and the serine protease inhibitor (AEBSF). Many bands were inhibited by TPCK as well as TLCK. Inhibitors of metallo and cysteine proteinases affected the proteolytic bands of *C. crangon* to a lesser extent.

A 20-kDa band was inhibited by the general proteinase inhibitors SBTI and PMSF as well as by AEBSF the specific serine proteinase inhibitor and the specific trypsin inhibitor TLCK. TPCK, an inhibitor specific for chymotrypsin, did not affect the 20-kDa activity band. Neither was this band inhibited by metalloproteinase- or cysteine-proteinase inhibitors.

Correlation analysis

Pairwise correlation between tryptic and chymotryptic activities versus morphometric parameters (total length = TL, total weight = TW and midgut gland index = MI) on the other side showed no strong correlation (Table 3). The correlation coefficient was highest for trypsin and chymotrypsin activity (0.188, $p = 0.01$) and second highest for trypsin activity and total length (0.178, $p = 0.014$). Linear regression also showed the best fit for trypsin and chymotrypsin ($r^2 = 0.035$, $p = 0.01$) and for trypsin and total length ($r^2 = 0.032$, $p = 0.014$).

Canonical analysis

Canonical analysis of principal coordinates (CAP) based on 25 protein bands present at least in one individual did not find any significant difference between Elbe and Weser (permutation test: 0.18, $p = 0.85$): data from these

Fig. 7 Example of a native activity gel of midgut gland endopeptidases at pH 8 from different and randomly chosen *C. crangon*

Table 3 Correlation analysis and linear regression between enzyme activities and somatic parameters of all individuals caught at all sampling sites. ($n_{all} = 376$)

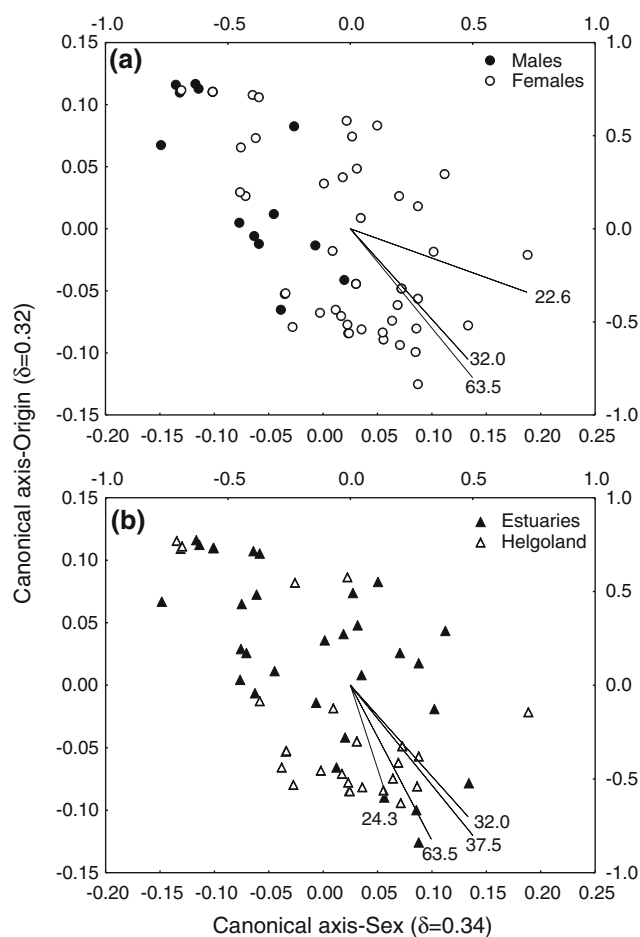| Dependent	Independent	Correlation	Linear regression		
		Coefficient	Coefficient	r^2	p
Chymotrypsin	Total length	0.116	0.316	0.013	0.112
Chymotrypsin	Total fresh mass	0.088	0.341	0.008	0.225
Chymotrypsin	Midgut gland index	0.036	4.406	0.001	0.621
Trypsin	Total length	0.178	2.551	0.032	0.014
Trypsin	Midgut gland index	0.025	15.390	0.001	0.737
Trypsin	Chymotrypsin	0.188	0.967	0.035	0.010

estuaries were pooled and compared with data from Helgoland using all 30 protein bands.

There was a significant association of protein bands with the factors sex, origin and with quantitative variables (CAP: $p < 0.01$ for all tests). For either Sex or Origin, a single canonical axis was obtained by CAP: these axes are plotted together in Fig. 9. The 'leave one out allocation procedure' showed individuals were correctly allocated into groups with 75% success for sex and 73% for origin. The separation of individuals in sexes by the canonical axis can be appreciated in Fig. 9a (horizonal axis); while the separation by origin is shown in Fig. 9b (vertical axis). The bands which most contributed to the formation of the axes were of rather low molecular weight (22.6, 32.0 and 63.5 for sex; 24.3, 32.0, 35.7 and 63.5 for origin). All these bands showed a significant association only with the respective factors (Table 4). For instance, the band 24.3 was significantly associated with origin as suggested by its high canonical correlation with the axis discriminating by origin (Table 4). However, it was not significantly associated with sex, as expected from the low canonical correlation with the axis discriminating by sex. Therefore, results of log-linear and multivariate analyses were consistent with each other.

Protein bands were also significantly associated with six quantitative variables analysed ($p < 0.01$), mostly with total length and carapace length (Fig. 10). The bands that most contributed with this associations were of medium to low molecular weight: 63.5, 43.4 and 26.6 (Table 5). All of them were significantly correlated with carapace length; the band 43.4 was only marginally correlated with total length ($p < 0.10$). Thus, for most cases, results of canonical analysis of principal coordinate and generalized linear model were consistent with each other.

Discussion

North Sea shrimps, *Crangon crangon*, showed extraordinary patterns of the serine proteinases trypsin and

Fig. 9 *Biplot* of first canonical axes, obtained separately from maximization of differences between sexes and origins on the protein polymorphism. These axes are not orthogonal since they belong to separated analyses. Differences between sexes are in **a** along the *horizontal axis*; differences between origins are in the **b**, along the *vertical axis*. Points are individuals, *lines* show the correlation among axes and protein weights (scales at the *upper X* and *right Y* axes). Only the protein weights with highest correlations with the canonical variables are shown
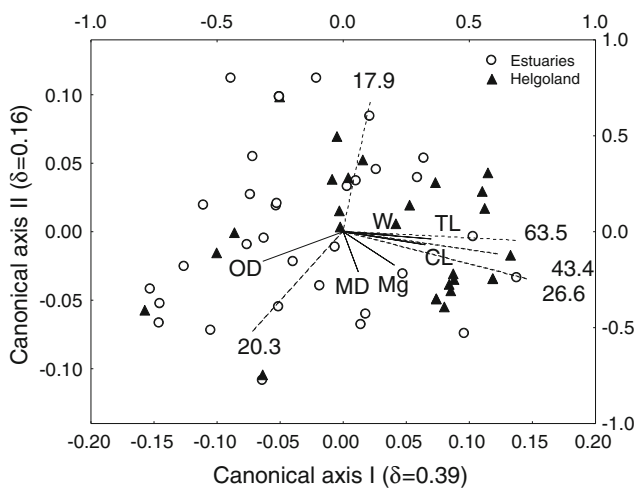
chymotrypsin. Significant activities of these enzymes were present only in 5–10% of the analysed animals. Moreover, *C. crangon* showed a very heterogeneous pattern of

Table 4 The proteinase bands which most contributed to the separation of sexes and origins by canonical analysis of principal coordinates

Molecular weight	22.6	24.3	32.0	35.7	63.5
Sex					
Canonical correlation	0.72	0.15	0.42	0.33	−0.50
% (female/male)	48.9/0.0	35.6/13.3	53.3/20.0	53.3/40.0	57.8/26.7
χ^2	13.75	2.50	4.98	0.76	4.23
p	<0.001	0.11	0.026	0.38	0.039
Origin					
Canonical correlation	−0.34	−0.60	−0.70	−0.82	−0.80
% (female/male)	44.4/25.0	47.2/4.2	61.1/20.8	69.4/20.8	69.4/20.8
χ^2	2.26	13.88	9.47	13.74	13.74
p	0.13	<0.001	<0.005	<0.001	<0.001

Correlation with the canonical axis, frequency of presence of bands, statistics after log-linear analysis to test for dependence of band frequencies on sex or region

Fig. 10 *Biplot* of the first two canonical axes form canonical analysis of principal coordinates to investigate the effect of six quantitative variables (*TL* total length, *CL* carapace length, *W* weight, *MD* index, *Mg* midgut gland weight, *OD* optical density) on the protein polymorphism. Points are individuals, *dashed lines* are protein weights; *continuous lines* are quantitative variables. Only the protein weights with highest correlation with the canonical variables are shown. For each axis, the squared canonical correlation is given as δ^2

endopeptidases. In total, 30 different activity bands were observed under native conditions. Such patterns are quite unusual for crustaceans and, to our knowledge, have not

been reported previously. Of 61 analysed shrimps with high tryptic activities, only very few animals showed similar or identical expression pattern.

Expression of serine proteinases

The LC elution profiles of the specimens with high tryptic activity always showed one distinct trypsin peak and a broad chymotrypsin peak which was flanked by some peaks with lower chymotrypsin activities. These results are in accordance with data previously reported by Teschke and Saborowski (2005). Both enzymes were clearly characterized by the almost exclusive hydrolysis of the specific substrates L-BAPA, TAME and SAAPPNA, while no hydrolysis of collagenase or elastase substrates was observed. The apparent hydrolysis of SAAPPNA by trypsin and L-BAPA by chymotrypsin can be explained by incomplete chromatographic separation. The elution profiles of both enzymes overlapped slightly and, thus, traces of trypsin may have appeared in the chymotrypsin fractions and vice versa.

Trypsin and chymotrypsin from *C. crangon* showed distinct substrate specificities and a clear response to specific inhibitors. Moreover, characteristic features like the apparent molecular mass, the anionic nature of both enzymes showing low pI further support their identity.

Table 5 The proteinase bands which most contributed to the correlation with six quantitative variables in Canonical analysis of principal coordinates

Molecular weight	26.6		43.4		63.5	
CC-1	0.73		0.61		0.68	
CC-2	−0.25		−0.12		−0.05	
	W	p	W	p	W	p
Total length	4.91	0.026	3.17	0.075	4.59	0.032
Carapace length	5.45	0.019	3.90	0.048	4.36	0.037

Correlation with the canonical axis, and statistics of the generalized linear model (Wald test, p values)

In order to exclude the possibility that the lack of tryptic activity in the majority of specimens may have been caused by thermal degradation of the enzyme we studied the stability of trypsin and chymotrypsin at different temperatures. Both enzymes were stable on ice, at room temperature, and at 40 °C for at least 5 h. During the routine analysis, all samples were kept on ice. Therefore, it is unlikely that temperature may have adversely affected enzyme activities during analysis.

Identification of endopeptidases bands

Animals with trypsin activities showed always a proteolytic band of apparently 20-kDa. This band corresponds with the distinct trypsin peak that was separated by liquid chromatography (Teschke and Saborowski 2005). Activity bands with other masses seemed to appear randomly but, as a tendency, their numbers were positively correlated with the trypsin activity of the sample. Most activity bands were inhibited by unspecific protease inhibitors and serine protease inhibitors. Inhibitors of metallo- and cysteine-proteinases had little effect on the proteolytic bands of C. crangon. Although a significant share of proteolytic activity in C. crangon is contributed by cysteine proteinases (Teschke and Saborowski 2005), the low effect of cysteine inhibitors in our study is related to the analytical conditions, particularly the pH. The optimum pH of cysteine proteinases is about pH 5–6. The pH of the gels, however, was 8 and, thus, cysteine proteinases were not active and therefore not detectable on the zymograms.

Crangon crangon showed more than one chymotrypsin isoform by chromatographic as well as electrophoretic separation of midgut gland extracts. Elevated chymotrypsin activity was present as one major and at lest two minor peaks after anionic exchange chromatography. From the midgut gland homogenate, six bands showed chymotrypsin-like activity as demonstrated by their inhibition by TPCK.

Five of the seven proteinase bands were inhibited by TLCK. Six of the seven proteinase bands were inhibited by TPCK. Accordingly, many bands appeared which were inhibited by both TPCK and TLCK. Some of the same enzymes which were inhibited by the serine proteinase inhibitors AEBSF and the proteinase inhibitors SBTI and PMSF were also inhibited by cysteine and metallo proteinase inhibitors. These results indicate multicatalytic properties of these enzymes. A multicatalytic enzyme was described in the lobster Homarus gammarus (Clark et al. 1991). As in Crangon crangon, it was inhibited by both serine and cysteine inhibitors.

Somatic parameters and effects of nutrition

Correlation analysis indicated no significant relation between morphometric parameters and the trypsin activity.

Only the midgut gland index was weakly related with trypsin activity. The variation in enzyme activity was higher in female C. crangon than in male specimens. Highest variation appeared in immature females.

The feeding mode, the choice of prey and differences in food quality may alter enzyme activities. Studies on different Penaeus-species showed that trypsin activities increased when the food was poor in proteins (Le Moullac et al. 1996). In contrast, Fernández et al. (1997) and Córdova-Murueta and García-Carreño (2002) found that the activities of digestive enzymes were correlated with the amount of respective substrates in the diet. Feeding experiments with C. crangon, however, did not show any effect on digestive proteinase activities when algae or mussel tissue was offered as food for 14 days (Teschke and Saborowski 2005). The time of digestion may differ according to the food. In Crangon septemspinosa, some components of a dyed food entered the midgut gland already a few minutes after ingestion and reached the hindgut after 1 h. Little later faecal pellets were released. Other components of the food, however, remained longer in the cardiac stomach, for up to 12 h (Wilcox and Jeffries 1974). Therefore, it cannot be excluded that enzymes which derived from ingested prey organisms may have contributed to the diverse enzyme pattern in C. crangon.

The similarity analysis indicates a relation between endopeptidase pattern and midgut gland index. There was a tendency that individuals with the same endopeptidase pattern also showed similar midgut gland indices. However, no statistically significant relation between band pattern and trypsin- and chymotrypsin activity could be established.

Large shrimp have access to a broader food spectrum. For smaller shrimps, only smaller prey is available. In this respect, the size of prey organisms is related with size of C. crangon as well (Gibson et al. 1995). Therefore, Wahle (1985) suggested to assess the feeding habits of crangonid shrimps always in relation to the life cycle. We showed in this work that this suggestion is not valid for the digestive enzymes of C. crangon. The activities of trypsin and chymotrypsin did not depend on the size of the studied specimens.

The ability to fill the gut is inversely related with the developmental stage of the ovaries (Plagmann 1939). During ovarian growth, feeding activity increases to meet the elevated energy demand and, thus, the gut of females often contains more food. During the time when the ovaries are fully developed the gut and the stomach are less filled because the ovaries occupy a significant amount of space within the cephalothorax (Oh et al. 2001). After spawning, the females carry the eggs ventrally attached to the pleopods until the larvae hatch. During this time, the female cannot moult. Accordingly, growth is retarded and

consequently, the demand of energy is reduced until the larvae have hatched (Oh et al. 2001). The females usually moult after hatching and express their highest feeding activity when the new exo-skeleton is fully hardened (Plagmann 1939; Lloyd and Yonge 1947). As a result, the endopeptidase activity is highest directly after hardening of the new exoskeleton as shown in *Penaeus notialis* by Fernández et al. (1997).

Adaptive polymorphism of endopeptidases

It is widely accepted that heterogeneous and variable environments demand a high degree of physiological flexibility from the organisms living there. And, in turn, organisms living in a variable habitat are expected to show high abilities for adaptation (e.g. Wilcox and Jeffries 1974; Nelson and Hedgecock 1980; Abdullah and Shukor 1993). Christensen et al. (1978) saw in the heterogeneous niche of the oligochaete *Lumbricus lineatus* the reason for the observed protein polymorphism in this species. The unspecific feeding mode of *Crangon septemspinose* was interpreted by Wilcox and Jeffries (1974) as an adaptation to changing feeding conditions in an estuarine environment. *C. crangon* feeds omnivorously as documented from stomach analysis by Plagmann (1939) and Pihl and Rosenberg (1984). The stomach content of different shrimp individuals roughly reflected the composition of smaller benthic individuals at the sampling site. It was dominated by animals while microalgae and parts of macroalgae played a minor role (Plagmann 1939; Pihl and Rosenberg 1984). Accordingly, also the unspecific feeding habits of *C. crangon* can be seen as an adaptation to a variable environment which may be supported by the heterogeneity of digestive serine-endopeptidase due to the variability of food.

Nelson and Hedgecock (1980) studied in 51 species of decapod crustaceans the relation between their ecological niches and the heterozygosity of enzyme coding genes. Species with a broad food spectrum showed a higher degree of heterozygote than feeding specialists. Among the specialists, carnivorous species were more often heterozygote than herbivorous species. The climatic zone had an effect as well. Heterozygosity in crustaceans from the temperate sublittoral zones was double as high as in crustaceans from tropical sublittoral.

Most factors which, according to Nelson and Hedgecock (1980), may trigger heterozygosity do also apply for *C. crangon*. The animals show a broad feeding spectrum acting as predators unselectively feeding omnivorously or carnivorously (del Norte-Campos and Temming 1994; Gibson et al. 1995). The stomach contents of *C. crangon* reflect the relative abundance of potential prey organisms in the respective habitat of the *Crangon* specimens (Plagmann 1939; Pihl and Rosenberg 1984; Ansell et al. 1999; Oh et al. 2001). All individuals studied here came from the sublittoral zone of the southern North Sea. The heterogeneous pattern of endopeptidases in *C. crangon* may suggest heterozygosity of genes coding for serine endopeptidases.

Nelson and Hedgecock (1980) formed for their observations of heterozygosity the term 'adaptive polymorphism' and raised the hypothesis that it represents a suitable tool for studying evolutionary processes in variable habitats. Kimura and Ohta (1971) as well described protein polymorphism as a phase of molecular evolution. It represents the first step in the long lasting establishment of mutations within populations. The present trends in the North Sea support the suggestion that opportunistic species will dominate more in the future (Rachor and Schröder 2003). *C. crangon* is an opportunistic predator. If polymorphic expression of digestive enzymes is an adaptation to variable environmental conditions, then the observed endopeptidase polymorphism of *C. crangon* may reflect the acute process of adaptation of an organism to a changing environment.

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