ORIGINAL PAPER

Proteasomal activities in the claw muscle tissue of European lobster, *Homarus gammarus*, during larval development

Sandra Götze · Reinhard Saborowski

Abstract Decapod crustaceans grow discontinuously and gain size through complex molt processes. The molt comprises the loss of the old cuticle and, moreover, substantial reduction and re-organization of muscles and connective tissues. In adult lobsters, the muscle tissue of the massive claws undergoes significant atrophy of 40–75% before ecdysis. The degradation of this tissue is facilitated by calcium-dependent proteases and by the proteasome, an intra-cellular proteolytic multi-enzyme complex. In contrast to the adults, the involvement of the proteasome during the larval development is yet not validated. Therefore, we developed micro-methods to measure the 20S and the 26S proteasomal activities within mg- and sub-mg-quantities of the larval claw tissue of the European lobster, *Homarus gammarus*. Within the three larval stages (Z1–3) we distinguished between sub-stages of freshly molted/hatched (post-molt), inter-molt, and ready to molt (pre-molt) larvae. Juveniles were analyzed in the post-molt and in the inter-molt stage. The trypsin-like, the chymotrypsin-like, and the peptidyl-glutamyl peptide hydrolase activity (PGPH) of the 20S proteasome increased distinctly from freshly hatched larvae to pre-molt Z1. During the Z2 stage, the activities were highest in the post-molt animals, decreased in the inter-molt animals and increased again in the pre-molt animals. A similar but less distinct trend was evident in the Z3 stages. In the juveniles, the proteasomal activities decreased toward the lowest values. A similar pattern was present for the chymotrypsin-like activity of the 26S proteasome. The results show that the proteasome plays a significant role during the larval development of lobsters. This is not only reflected by the elevated activities, but also by the continuous change of the trypsin/chymotrypsin-ratio which may indicate a shift in the subunit composition of the proteasome and, thus, a biochemical adjustment to better cope with elevated protein turnover rates during larval development.

Keywords Lobster larvae · Molt · Claws · Muscle atrophy · 20S and 26S proteasome

Communicated by G. Heldmaier.

S. Götze · R. Saborowski (✉)
Alfred Wegener Institute for Polar and Marine Research,
Functional Ecology, PO Box 120161,
27515 Bremerhaven, Germany
e-mail: Reinhard.Saborowski@awi.de

S. Götze
Phillipps-Universität, FB Biologie, Karl-von-Frisch-Straße 8,
35043 Marburg, Germany

Introduction

Crustaceans have to shed their exoskeleton when they grow. To be able to slide out of their old shell, they have to reduce muscle tissue. This mold-induced muscle atrophy is most distinct in the large claws of the thoracopods (Mykles 1999a) where the muscle tissue may be degraded by 40–75% (Skinner 1966; Shean and Mykles 1995). Although the muscle is weakened, its contractile function remains largely intact. This is due to the fact, that the muscle fibers and myofibrils are less degraded but, instead, the filament packing is compressed. So, the ratios of thin to thick myofilaments decrease from 9:1 to 6:1 (Mykles and Skinner 1981). This reduction and re-organization is accompanied by a drastic increase in both protein degradation and protein synthesis (e.g.,El Haj et al. 1996). The high protein turnover rate seems to be necessary to perform the complex remodeling process. Two different but

complementary proteolytic systems are mainly responsible for the muscle degradation in lobsters: calcium-dependent calpain-like proteases and proteasome (Mykles 1990, 1999b; Mykles and Haire 1991; Koenders et al. 2002).

The proteasome is a highly conserved multi-catalytic protease complex, which is present in prokaryotes as well as in all tissues of eukaryotes (Baumeister et al. 1998; De Mot et al. 1999). Since the majority of redundant proteins and peptides are degraded via the ATP/ubiquitin-dependent proteasomal pathway, the proteasome seems to hold a regulatory key position within cells (Hochstrasser 1995). It is involved in, e.g., cell-cycle control, cell differentiation, apoptosis, antigen processing and signal transduction (Voges et al. 1999). The proteasome of eukaryotes consists of a core complex, denoted as 20S proteasome (~ 700 kDa), which forms together with two regulatory subunits, the 26S proteasome (~ 2.5 MDa). The catalytic centers are located within the barrel-shaped 20S core unit (Baumeister et al. 1998).

The 20S core complex is made up of 28 subunits, which are organized in four-stacked rings. The two outer rings consist of seven different α-subunits and form a pore on each side, which is accessible only by unfolded proteins or short polypeptides. The two inner rings consist of seven different β-subunits and only they buid up the hydrolytic chamber. However, only three subunits of each β-ring are proteolytically active. The sites are named after their preferred cleaving mechanism: the trypsin-like site ($\beta 2$) cleaves preferentially after basic residues, the chymotrypsin-like site ($\beta 5$) cleaves after large hydrophobic residues, and the peptidyl-glutamyl-peptidase hydrolase-like (PGPH) site, recently more correctly referred to as caspase-like site ($\beta 1$), cleaves after acidic residues. The subunit composition of the proteasome can vary depending on, e.g., post-translational modification, on the target tissues, or the age of the organism (Frisan et al. 1998; Dahlmann et al. 2000; Husom et al. 2003). Depending on the subunit composition, the proteasome may display different catalytic cleavage preferences.

The early developmental stages of crustaceans are subjected to immense morphological and anatomical changes through a series of rapidly succeeding molts. Within about 14 days, the larvae pass three zoea stages and molt into the first juvenile stage (Charmantier et al. 1991; Schmalenbach and Fanke 2010). Similar to the adults, the larvae have to reduce and re-organize their muscles as well as the connective tissue. So far, only limited information is available about the mechanisms, which control these vital physiological processes in crustacean larvae.

We raise the hypothesis that the proteasome is already involved in the molts between the larval stages. Therefore, the aim of the present work was to study the contribution of the 20S and the 26S proteasome during larval development of European lobsters (*Homarus gammarus*). As target organ, we chose the claws of the larvae because the muscle tissue in these claws most probably undergoes similar modification as in adults. Moreover, the claws consist mainly of muscle and connective tissue and, thus, have the advantage of providing solely the proteasomal enzymes of interest. Using entire larvae would be detrimental because the comparatively huge midgut gland would contribute highly active digestive enzymes, including trypsin and chymotrypsin. Since these enzymes are able to degrade the substrates used for proteasomal measurements, the digestive enzymes would interfere with the proteasomal enzymes or mask their activities. However, since the claws are extremely small, weighing mostly less than 2 mg, we first had to establish sensitive micro-assays to guarantee reliable and reproducible enzyme measurements. We focused our studies on the ontogenetic changes and molt cycles of individual larvae. Crude extracts were prepared from claws and the proteasomal activities were verified by the highly specific effectors epoxomicin and PA28 (Mykles 1996; Meng et al. 1999; Kisselev and Goldberg 2001). The results will help to better understand larval development and those processes that influence molt success in early developmental stages of decapod crustacea.

Materials and methods

Maintenance and rearing of lobster larvae

Lobster larvae of all three zoea stages (Z1–Z3) as well as the first juvenile stage (J1) were obtained from the lobster rearing facility of the Marine Station Helgoland and shipped to the laboratories in Bremerhaven. Immediately after arrival, the larvae were separated and maintained individually in 100-ml beakers filled with filtered natural seawater. The water temperature ranged between 17 and 19°C. The seawater was exchanged daily and the larvae were fed with freshly hatched nauplii of the brine shrimp *Artemia salina*. The overall mortality of the lobster larvae in the rearing experiments was less than 10%.

One group of larvae ($n = 15$) was allowed to grow up until they reached the juvenile stage. This approach was used to determine the duration of the larval stages at the given experimental conditions (Table 1). Additional data about the duration of larval development were provided by Schmalenbach and Fanke (2010).

The samples were taken after molting (post-molt), during the inter-molt phase (inter-molt), and before the next molt (pre-molt) of each stage. Z1 larvae were sampled after hatching (Z1/1) to obtain the post-molt individuals. When the developmental times were unknown, the larvae were first raised until they molted into the subsequent stage.

Table 1 Developmental time of the larval stages (mean ± SD, $n = 15$) and sampling schedule after hatch or molt, respectively, of the larval and juvenile stages

Stage	Duration (days)	Time of sampling (days)		
		Hatched/post-molt	Inter-molt	Pre-molt
Zoea 1	4.3 ± 0.8	Max. 12 h after hatch	3.0 ± 0.5	±1 day before molt
Zoea 2	8.1 ± 2.4	Max. 12 h after molt	3.0 ± 0.5	±1 day before molt
Zoea 3	12.8 ± 1.1	Max. 12 h after molt	5.5 ± 0.5	±1 day before molt
Juvenile 1	20.3 ± 3.2	Max. 12 h after molt	10.0 ± 0.5	(not sampled)

Thereafter, controlled sampling could be performed after defined periods.

The post-molt individuals were sampled at the latest 12 h after hatching or molting, but preferably as early after ecdysis as possible. The inter-molt individuals were sampled after they passed half of the predicted duration of the respective stage. To sample the pre-molt animals in due time, the larvae of the respective batch were controlled for the first animal to molt into the next stage. When this happened, it was assumed that the larvae of this batch were also at a stage shortly before the molt and, consequently, were sampled. A detailed schedule illustrating the molt intervals and the sampling strategy is shown in Fig. 1.

Extract preparation

From single larvae of each molting stage, both claws were completely excised, briefly blotted dry on filter paper and transferred into a reaction tube. The claws were weighed and homogenized in 45 µl of homogenizing buffer (20 mmol l^{-1} Tris/HCl, 1 mmol l^{-1} EDTA, 5 mmol l^{-1} MgCl$_2$, pH 7.5) using a micro-pestle. Subsequently, the extracts were centrifuged for 10 min (9000g, 4°C) and split into two samples of 20 µl each. The aliquots were stored at −80°C until further analysis.

Proteasomal activities

The proteasomal activities were investigated in claw muscle extracts of lobster larvae from each molt stage ($n = 6$–10 individuals).

The trypsin-like, chymotrypsin-like and peptidylglutamyl peptide hydrolase activities (PGPH) of the 20S proteasome were assayed by using the fluorogenic substrates Boc-Leu-Arg-Arg-AMC (PeptaNova, 3140), Suc-Leu-Leu-Val-Tyr-AMC (Enzo Life Sciences, P-802) and Z-Leu-Leu-Glu-AMC (Enzo Life Sciences, 9345). Additionally, the trypsin-like and the chymotrypsin-like activities were verified by inhibition of the enzymes by the highly specific inhibitor epoxomicin (PeptaNova, 4381-0.02). The PGPH activity was not inhibited but, instead, activated by the endogenous regulatory complex PA28 (11S regulatory subunit, Enzo Life Sciences, 9420-0025). The chymotrypsin activity of the 26S proteasome was determined with the substrate for the chymotrypsin-like activity and with the inhibitor. The only difference was the composition of the assay buffer. The buffer for measuring the 20S proteasome contained 0.1 M Tris at a pH of 8, while the buffer for measuring the 26S proteasome contained 5 mmol l^{-1} Tris, 4 mmol l^{-1} HCL, 5 mmol l^{-1} MgCl$_2$ and 2 mmol l^{-1} ATP at the same pH.
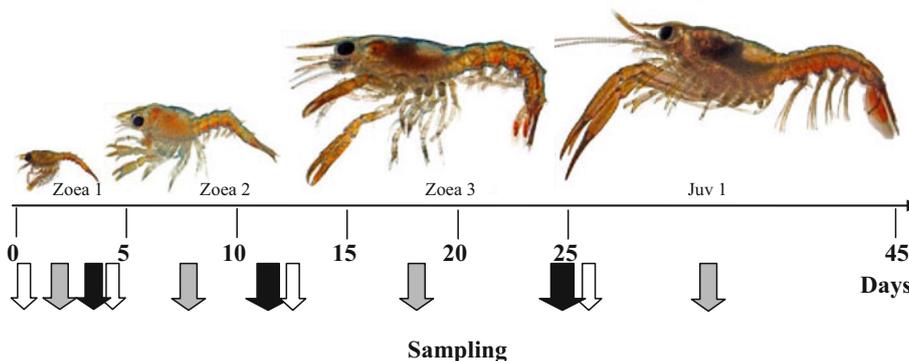
Fig. 1 Stage duration of the larvae and the sampling intervals (in days). The *hollow white arrows* indicate when the post-molt individuals were sampled, the *gray arrows* indicate the sampling of the inter-molt individuals and the *black arrows* the sampling of the pre-molt individuals. The sizes of the larvae are not shown in scale. The lengths (from the rostrum to the tip of the telson) ranged from 9 mm in Zoea 1 to 13 mm in Zoea 3. The first juvenile stage was on average 16 mm long

The sensitivity of the 20S proteasome against epoxomicin as well as its specificity was first examined with claw muscle tissue of adult European lobster samples. The applied concentrations of epoxomicin ranged for the chymotrypsin-like activity from 2.5 to 50 μmol l^{-1} and for the trypsin-like activity from 10 to 100 μmol l^{-1}. Blanks were run to determine the auto-fluorescence of the substrate and of the inhibitor, respectively. Additionally, one approach served as control for the level of uninhibited activity.

The specificities of the effectors, epoxomicin and PA28, were determined by comparing the grade of inhibition or the grade of activation in samples containing 20S proteasome or gastric fluid, respectively. The gastric fluid is rich in digestive enzymes such as trypsin, chymotrypsin and other proteases, but does not contain proteasome. Digestive trypsin and chymotrypsin are capable of degrading the same fluorogenic substrates as the proteasome. However, in contrast to the proteasome, the gastric enzymes should neither be affected by epoxomicin nor by PA28. The gastric fluids of three adult European lobsters were obtained directly from the stomach with a syringe connected to a plastic tube. The samples were centrifuged for 15 min at 1,3000g and 4°C to remove food residues and were diluted 1:20 with demineralized water (a. dem.) before they were applied into the reaction tubes. First, the trypsin and the chymotrypsin activities of the gastric fluids were determined to obtain the unaffected level of activity. Concurrently, assays were run with epoxomicin with concentrations of 100 μmol l^{-1} for the trypsin-like activity and 5 μmol l^{-1} for the chymotrypsin-like activity. Additionally, it was investigated whether the gastric enzymes were able to degrade the PGPH substrate and whether the activity can be enhanced by PA28. The PA28 concentration amounted to 4 μg ml^{-1}.

Enzyme micro-assays with the NanoDrop device

Fluorometric enzyme micro-assays were carried out with a NanoDrop device as described by Götze and Saborowski (2011). The instrument requires only 2 μl of reaction mixture. Accordingly, the total volume of the reaction mixture was reduced to 25 μl. It contained 17.5 μl buffer, 5 μl enzyme solution (sample), 1.25 μl of substrate solution (final concentration for the trypsin-like activity was 0.75 mmol l^{-1}, for the chymotrypsin-like activity 0.5 mmol l^{-1} and for the PGPH-like activity 1.0 mmol l^{-1}), and, in case, 1.25 μl of the inhibitor epoxomicin or the activator PA28 (final concentration 100 and 5 μmol l^{-1}, respectively). All substrates, except PA28 which was delivered already in solution, were dissolved in dimethyl sulfoxide (DMSO) and prepared as 10- or 20-fold stock solutions freshly before use. The reaction mixtures were incubated in micro-tubes at 37°C. The released fluorescence was measured exactly after 1 h, while 2 μl of the reaction

mixture was taken from the reaction tubes and pipetted onto the optical pathway of the NanoDrop 3300 device (PeqLab). The fluorescence was measured at 365 nm (ex) and 437 nm (em). Blanks containing only buffer and the substrate but no enzyme were run in parallel to control substrate autolysis.

Protein assay

Dissolved protein was quantified after Bradford (1976) with a commercial dye reagent (BioRad, 500-0006). The reagent was diluted 1:5 with a. dem. before use. Bovine serum albumin (BSA, BioRad, 76290A) was used as standard. Increasing amounts of the standards from 0 to 5 μg were applied in duplicate onto a 96-well plate and filled up with a. dem. to 25 μl. Samples (5 μl) were applied in duplicate and filled up to 25 μl. The reactions were started with the addition of 125 μl of the diluted dye reagent (1:5 with a. dem.) to each standard and each sample. After 5 min of incubation, the optical density was read at 600 nm with a microplate reader (Thermo Scientific, Multiscan SF).

Quantification and data analysis

Trypsin and chymotrypsin activities were measured with and without the inhibitor epoxomicin. Only the shares of the enzyme activities which were inhibited were regarded as true proteasomal activities. The relative fluorescence (RF) obtained from the enzymatic measurements was quantified in relation to the protein content of the sample and expressed as Units g_{Prt}^{-1} (1 U = 1 μmol min^{-1}). A calibration curve was established with 7-amino-4-methylcoumarin (AMC, Fluka, 08440). The AMC was dissolved in DMSO and prepared as tenfold stock solutions for concentrations from 0.025 to 10 μmol l^{-1}. Then, 2.5 μl of the particular concentration was added to 22.5 μl of assay buffer and the fluorescence was measured with the NanoDrop device. Each concentration was measured in five replicates, which were used to calculate a calibration curve.

The data were expressed as mean specific activities (U g_{Prt}^{-1}) and the standard errors of the means (SEM). Data sets were subjected to a one-way ANOVA followed by a Tukey HSD test (Statistica 7.1, Systat Software, Inc.). Significant differences between groups were indicated by different letters when p values ≤ 0.05. Graphs were created with the program SigmaPlot 10.0 (Systat Software, Inc.).

Results

Weight and protein content

The size, fresh weight and protein content of the claws increased significantly during the development of the

lobster larvae. The fresh weight and protein content displayed a statistically significant correlation (Fig. 2, $r^2 = 0.6$, $n = 110$, $p < 0.001$).

The weight of a pair of claws increased significantly from stage to stage (Fig. 3). The claws of the Z1 larvae weighed on average 0.63 mg. The weight increased until the first J1 stage to about 2.9 mg. The protein content rose continuously from 15.9 µg in Z1 to 23.7 µg in Z3. Between Z3 and J1, the protein content increased about 2.3-fold to an average of 55.3 µg.

The weight and the protein content did not change significantly within the three molt stages of Z1 (Fig. 3). Both values, however, increased in Z2 larvae significantly. This trend continued for the fresh weight within the Z3 larvae. The claws of pre-molt Z3 individuals were significantly heavier than those of post-molt Z3. The protein content rose from the post-molt to the inter-molt individuals significantly, but the pre-molt animals possessed less protein. The two juvenile molt stages showed significantly higher weights than the larvae. The juvenile stages did not differ in their protein content.

Inhibition and activation of proteasomal activities

The trypsin-like and the chymotrypsin-like 20S proteasomal activities of the claw muscle extracts of the adult European lobsters were significantly inhibited by epoxomicin (Fig. 4).

Over 93% of the chymotrypsin-like activity was inhibited when epoxomicin was present in the lowest concentrations of 2.5 µmol l^{-1}. Around 50% of the trypsin-like activity was inhibited by 10 µmol l^{-1} epoxomicin. Increasing concentrations of epoxomicin did not considerably enhance the inhibition of the chymotrypsin-like
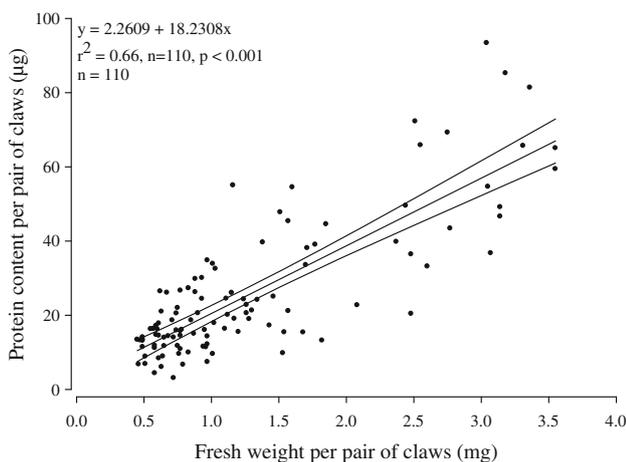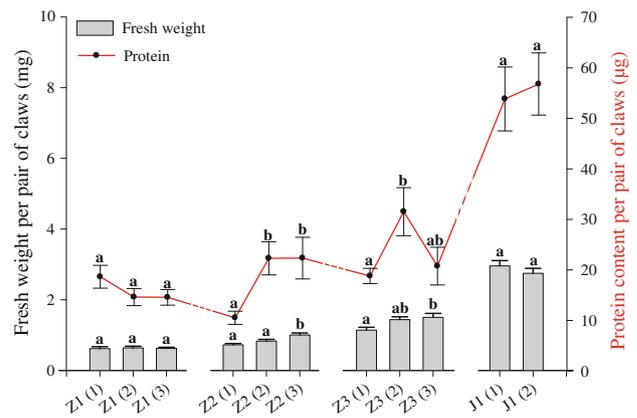
Fig. 3 Fresh weight and the protein content of one pair of claws of the developmental stages. The *bars* represent the average fresh weight of a pair of claws and the *line* represents the average protein content of a pair of claws. The values are expressed as mean ± SEM ($n = 10$). *Different letters* indicate significant differences within the respective stages ($p < 0.05$). Z1–Z3, Zoea 1–Zoea 3; J1, Juvenile 1. The *numbers 1–3 in brackets* indicate the molt stage: (1), freshly hatched or post-molt stage; (2), inter-molt stage; (3), pre-molt stage

activity of the 20S proteasome. At the highest concentration of 50 µmol l^{-1}, only 3% of the initial activity remained. The trypsin-like activity was inhibited by 75% at the highest epoxomicin concentration of 100 µmol l^{-1}.

In the gastric fluids of the adult European lobsters, the trypsin-like, chymotrypsin-like and PGPH-like activities were not significantly affected by epoxomicin (Fig. 5a). The chymotrypsin-like activity was only reduced by 3%. The trypsin-like activity (103%) and the PGPH activity (104%) were slightly higher than in the uninhibited assays.

In contrast to the gastric fluid, the activities of the larval and juvenile lobster samples were highly affected by the inhibitor as well as by the activator (Fig. 5b).

The chymotrypsin-like activities of all investigated stages were inhibited by more than 90%. The highest inhibition occurred in the Z3 stage where only 5.6% of the initial activity remained. The average inhibition amounted to 90.7%. No significant differences appeared between the stages. The trypsin-like activity was inhibited on average by about 75%. As in the chymotrypsin-like activity, no differences between the stages were detected. The endogenous activator complex PA28 enhanced PGPH activity on average by 45%.

Enzymatic activities of the 20S- and 26S proteasome

The catalytic activities of the three active sites of the 20S proteasome changed significantly within the larval development. The average trypsin-like, chymotrypsin-like and PGPH-like activities increased within the Z1 stages and remained at a high level until Z3. All three activities decreased significantly when the larvae developed into the

Fig. 2 Correlation between the fresh weight and the protein content per pair of claws ($n = 110$). Indicated is the regression line with the confidence intervals (95%)

In Fig. 2: $y = 2.2609 + 18.2308x$; $r^2 = 0.66$, $n = 110$, $p < 0.001$; $n = 110$

Fig. 4 Inhibition of the 20S proteasomal activity by different concentrations of epoxomicin. The residual activities (%) are presented for the chymotrypsin-like activity (**a**) and for the trypsin-like activities (**b**)
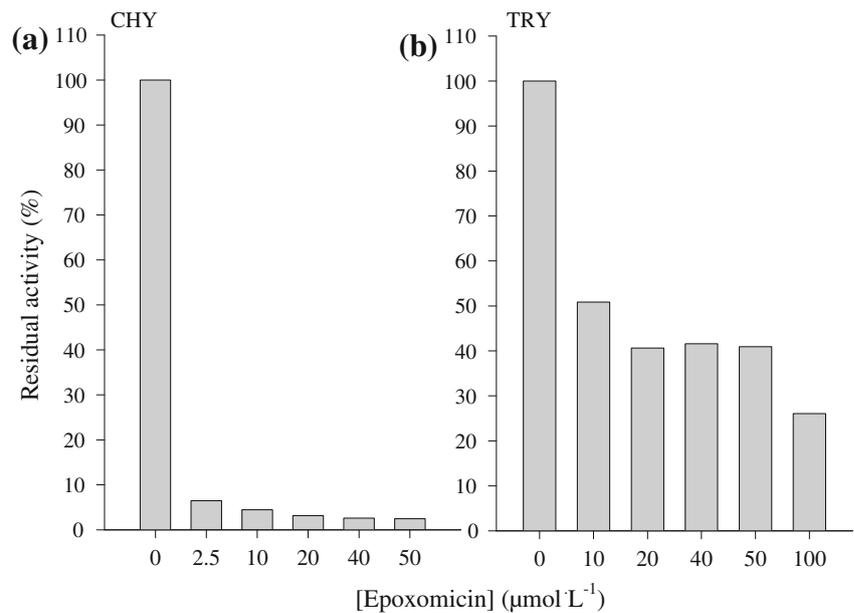
Fig. 5 Effects of epoxomicin and PA28 on the 20S proteasomal activities of larvae and adult lobsters. **a** Impact on the digestive enzymes trypsin-like, chymotrypsin-like and PGPH activity in the gastric fluid (GF) of adult European lobsters ($n = 3$). **b** The effect of epoxomicin and PA28 onto the three proteasomal activities of the larval (Z1–Z3) and the juvenile stage (J1). Values are expressed as mean of remaining activity (%) ±SEM ($n = 30$ per stage). The activities of the untreated samples were set as 100%, and the remaining activities were calculated accordingly
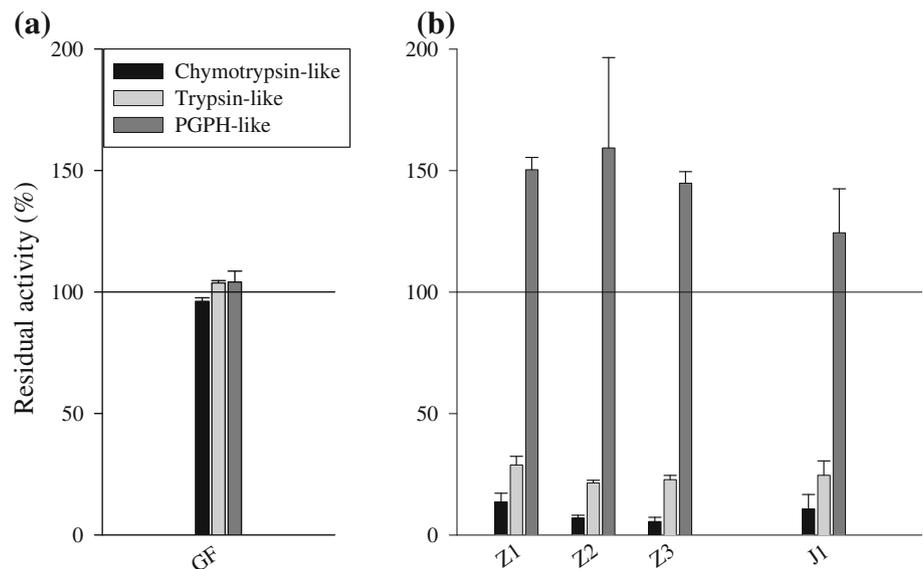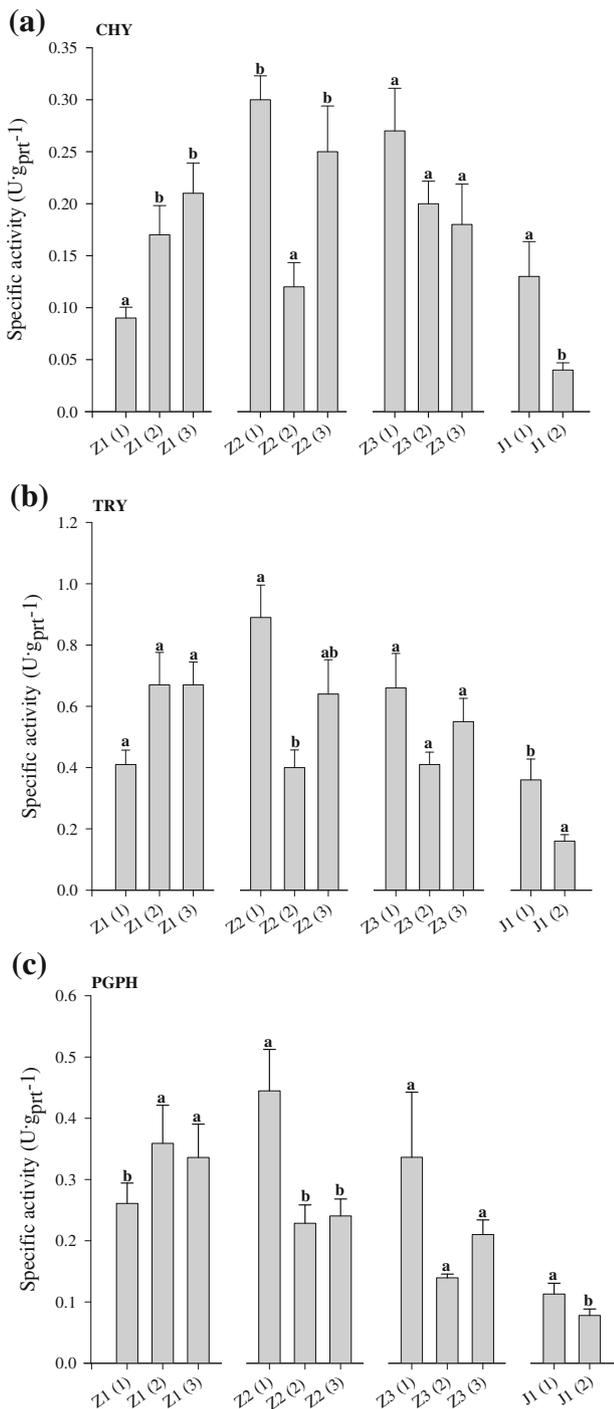

juvenile stage. A similar pattern was present in the chymotrypsin-like activities of the 26S proteasome. Again, the highest activities appeared in the Z2 and Z3 stages.

Changes in activities were detected between the different molt stages (Fig. 6a–c). The chymotrypsin-like activity of the 20S proteasome increased within Z1 individuals from initially 0.09 U g_{prt}^{-1} and reached the highest in post-molt Z2 individuals with 0.30 U g_{prt}^{-1} (Fig. 6a). Then, the activity decreased drastically in inter-molt individuals (0.12 U g_{prt}^{-1}), but reached the highest again in the subsequent pre-molt individuals. Although the activity did not change significantly within Z3 molt stages, a decreasing trend was evident. This trend distinctly continued in the juvenile stage where the activities decreased from

0.13 U g_{prt}^{-1} in post-molt individuals to 0.04 U g_{prt}^{-1} in inter-molt juvenile lobsters.

Similar trends were also found for the trypsin-like (Fig. 6b) and the PGPH-like activities (Fig. 6c). In both cases, the activities showed an increasing, though not significant, trend from freshly hatched Z1 larvae to the post-molt individuals of this stage. The inter-molt Z2 possessed lowest trypsin-like (0.40 U g_{prt}^{-1}) and PGPH-like (0.23 U g_{prt}^{-1}) activities and both activities increased again in post-molt Z3 individuals. Furthermore, both activities decreased in inter-molt individuals of Z3. This was followed by an increasing trend in pre-molt individuals again, although these differences were not statistically significant. In the juvenile lobsters, both activities decreased again.

(a)

(b)

(c)

Fig. 6 Chymotrypsin-like (**a**), trypsin-like (**b**) and PGPH-like (**c**) activities of the 20S proteasome of the investigated larval and juvenile stages ($n = 10$, mean ± SEM). *Different letters* indicate significant differences within the respective stages ($p < 0.05$)
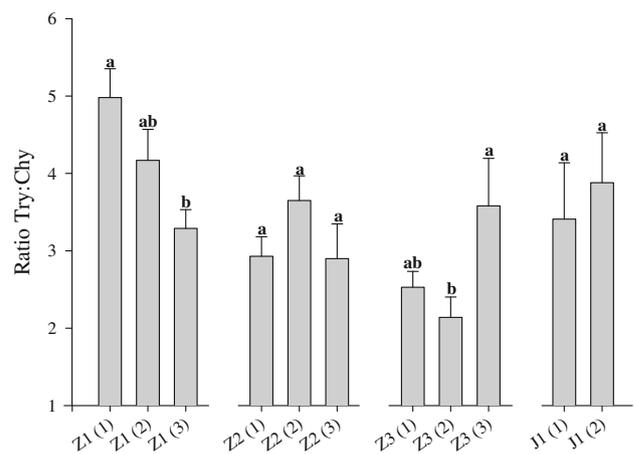
The chymotrypsin-like activities of the 26S proteasome followed a similar pattern as the 20S activities. Within the Z1-stage, the activity almost doubled from initially 0.12 to 0.19 U g_{prt}^{-1} in the inter-molt stage (Fig. 7). Highest activities were present in the pre-molt phases of Z2 and Z3 (0.23

and 0.24 U g_{prt}^{-1}, respectively) whereas lowest activities appeared in the inter-molt stages (0.12–0.13 U g_{prt}^{-1}).

Each stage possessed an individual ratio between the trypsin-like and the chymotrypsin-like activities (Fig. 8). In all stages, the trypsin-like activity dominated. In freshly hatched Z1 individuals, the trypsin-like activity was almost five times higher than the chymotrypsin-like activity. In the subsequent stages, until post-molt Z2, the ratio of trypsin to chymotrypsin decreased to about 3. Inter-molt Z2 animals showed again slightly higher trypsin-like activities. The ratio changed in the following stages in favor of the chymotrypsin-like activity until the larvae developed into the inter-molt Z3 (2.1). The ratio increased significantly to 3.6 in pre-molt Z3. In the juvenile lobsters, the trypsin-like activity was fourfold higher than the chymotrypsin-like activity.
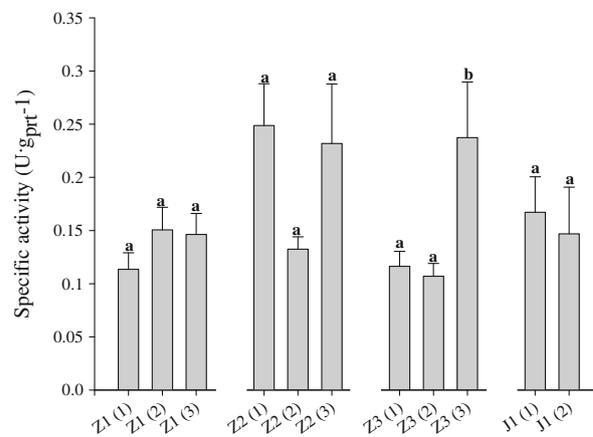
Fig. 7 Chymotrypsin-like activities of the 26S proteasome within the larval and juvenile stages ($n = 6$, mean ± SEM). *Different letters* indicate significant differences within the single stages ($p < 0.05$)

Fig. 8 Ratio between the specific protesomal activities of the trypsin-like site and chymotrypsin-like site. Values are expressed as mean ratio ($n = 10$, mean ± SEM) and *different letters* indicate significant differences ($p < 0.05$) within the respective stages

Discussion

Our results indicate that proteasome plays an important role during the larval development of the European lobster. All catalytically active sites of the 20S proteasome as well as the chymotrypsin-like site of the 26S proteasome showed high activities within all larval stages. However, in the first juvenile stage, the activities decreased remarkably. Compared to the claw muscle tissue of adult European lobsters, the proteasomal activities of the larvae were several times higher than in adults. Accordingly, it is very likely that these high activities are indicative for the extensive physiological and anatomical changes which the larvae encounter during their development (Charmantier et al. 1991).

It is commonly accepted that the 26S proteasome represents the physiological relevant form of the proteolytic multi-enzyme complex (e.g.,Coux et al. 1996; Baumeister et al. 1998; Voges et al. 1999; Galastegui and Groll 2010). However, recent research revealed that the 20S unit is active under physiological conditions as well. The 20S proteasome seems capable of degrading protein with unstructured regions or oxidative modification in an ubiquitin-independent manner (Davies 2001; Grune et al. 2003; Liu et al. 2003; Orlowski and Wilk 2003; Asher et al. 2006, Jariel-Encontre et al. 2008). We observed in our studies quite similar pattern of 20S and 26S chymotrypsin-like activities during the larval development of lobsters. Accordingly, it seems feasible that both the 20S and the 26S forms of the proteasome may be active during the molt cycles. This observation is in agreement with Mykles 1998, 1999a, b, who suggested that 20S and 26S proteasome as well as intracellular Ca^{2+}-dependent proteinases may be involved in the complex pathways of muscle protein catabolism.

The activities varied distinctly between stages and within stages. While within Z1 larvae the activities continuously increased, the activity patterns in Z2 and Z3 seemed to be more related to the molt stage. It is likely that in the first larval stage, general anabolic processes happen, which may include the formation of effective cellular regulation capacities. The rising activities may reflect the continuous formation of the proteasome after the hatch of the larvae. Lower activities in the inter-molt individuals of Z2 and Z3 may indicate a period of lower protein turnover and, therefore, of lower proteasomal activities due to a down-regulation of enzyme expression. In contrast, the increasing activities particularly of the trypsin-like and the chymotrypsin-like sites of the pre-molt Z2 may indicate a phase of enhanced protein turnover closely before molt. The increase of activities was most distinct in the pre-molt Z2. In pre-molt individuals of the Z3, the activities followed a similar trend but the increase of activities was not statistically significant.

The lessened increase of activities in pre-molt Z3 may be explained by the difficult stage determination of the individuals during sampling. The only possibility we had to identify pre-molt larvae was to take other larvae as a clue and to interpolate the moment of sampling. Larvae of the first two zoea stages pass their development on average within 4 and 8 days, respectively. Furthermore, larvae of these stages molted into the next stage almost simultaneously, which made it relatively easy to collect them at a defined time shortly before molt. In contrast, Z3 larvae needed almost 13 days to reach the next stage and, moreover, they did not molt as simultaneously as the earlier stages did. Thus, individuals which might have been sampled too early caused an underestimation of the enzyme activities.

Similar to the 20S proteasome, which was isolated from adult lobster muscle tissue (Mykles 1997), the proteasome of the larvae possessed highest trypsin-like activities and lower chymotrypsin-like and PGPH-like activities. Remarkably, besides the overall activities, also the ratios between the proteasomal activities changed during the larval development. While in freshly hatched larvae and in juvenile inter-molt individuals the trypsin-like site was dominant, the chymotrypsin-like site seemed to gain relevance in the Z2 and Z3 stages.

It was shown in several studies that the proteasome is characterized by high plasticity and, moreover, distinct tissue allocation and specificity (Cardozo et al. 1995; Mykles 1999b; Dahlmann et al. 2000). All catalytic sites of mammal proteasomes are closely interlinked and may allosterically influence each other in a complex way (Kisselev et al. 1999, 2002, 2003, 2006). Drews et al. (2007) reported on proteasome subpopulations with distinct molecular compositions and proteolytic activities. Gomes et al. (2009) compared the murine cardiac and hepatic 20S proteasomes and found an organ-specific organization of the 20S proteasome, which was concomitant with a functional heterogeneity in either organ. Similarly, differences in the relative abundance of proteasomal subunits in several mammalian tissues were revealed by Pelletier et al. (2010).

Moreover, ontogenetic variations of proteasomal activities were reported in protists, invertebrates and vertebrates. De Diego et al. (2001) assumed that the enzyme plays an important role during the remodeling phase of *Trypanosoma cruzi* and indicated a significant role of the proteasome during the development of organisms. In the arthropod *Drosophila melanogaster*, a cell-specific accumulation of proteasome was reported during the early development (Klein et al. 1990). During embryogenesis, proteasomes were present in proliferating cells and accumulation and turnover were differentially regulated. Klein et al. (1990), moreover, suggested that the proteasomes

may serve different proteolytic processes and may be involved in cell-specific proteolytic events. Haass and Kloetzel (1989) raised the possibility that both in vivo proteolytic activity and the in vitro substrate specificity of the proteasome may be controlled by modifications of the subunit composition during the development. Ádám et al. (2004) studied the sub-cellular distribution of the 26S proteasome in ovarian tissues of *D. melanogaster* and concluded that also the sub-cellular distribution of the 26S proteasome is strictly tissue- and developmental stage dependent. Verras et al. (2008) showed that the expression of an α5-subunit of the 20S proteasome is up-regulated before mid-embryogenesis and at the onset of metamorphosis in the fruit fly *Ceratitis capitata*.

Ahn et al. (1991) described tissue-specific changes in the enzymatic activities and the subunit pattern of the 20S proteasome during the embryonic development of chick. In the muscle tissue, overall peptide hydrolysis decreased between day 8 and day 20 of the embryonic development but, exceptionally, chymotrypsin-like activity increased between day 8 and day 12. Moreover, proteasomal casein degradation increased during the experiment. The authors concluded that proteasomal activities were under developmental regulation. Distinct changes in proteasomal activities also appeared in the liver tissue, but were not present in the brain tissue. Hong et al. (1994) found significantly different subunit pattern of proteasomes, which the authors isolated from the muscles, liver and the brain of chicken. Moreover, the proteasomes from embryonic tissues and adult tissues differed from each other indicating that proteasomal activities and subunit patterns are tissue specific and under developmental control.

Our results on lobster larvae complement remarkably the above listed findings in invertebrates and vertebrates. Accordingly, the changes in the enzymatic activities and variations in the ratios of trypsin-like and chymotrypsin-like activities of the 20S proteasome seems indicative of functional variations of the proteasome during the larval development of lobsters. During the molt cycle and, particularly, during ecdysis, the claw muscle tissue undergoes substantial changes. The enormous reduction of claw muscle tissue prior to each molt confront adult lobsters with serious degradation- and remodeling processes. Although the muscle mass has to be reduced so that the tissue fits through the narrow joint particularly between the ischium and the merus, the muscle has to maintain its contractile function. This is achieved through a distinct remodeling of the myofibrillar structures (Mykles and Skinner 1981; Ismail and Mykles 1992; Covi et al. 2010). Surprisingly, the protein synthesis in the claw tissues is not reduced in pre-molt crustaceans, but instead significantly increased (El Haj et al. 1996; Mykles 1997). The reason for the steady protein synthesis is a complex mechanism to re-

modulate the myofibrillar structures and to reduce the muscle tissue while the contractile function remains unimpaired (Mykles and Skinner 1981). We assume that this mechanism, as it represents a period of enhanced protein turnover, will strongly depend on the balanced interplay of proteasomal activities. The proteasome is one of the main regulators of the cell-cycle (Richter-Ruoff and Wolf 1993; Naujokat and Hoffmann 2002), differentiation processes such as remodeling (De Diego et al. 2001) and growth. Therefore, it seems likely that especially in the developmental stages which are characterized by high mitotic activity, growth and high transcription rates might demand an active and regulating proteasome.

In conclusion, elevated 20S and 26S proteasome activities clearly indicate an important contribution of the proteasome pathway in the larval development of clawed lobsters, most likely in the re-organization of muscle tissue during molt and growth. Beside the general increase of the proteolytic enzyme activities, the proteasome seems adjusted probably to better cope with higher protein turnover rates during the entire larval development. The detailed mechanisms and particularly the interplay between the Ca^{2+}-dependent calpain proteases and the proteasome need further investigation.

References

Ádám G, Gausz J, Noselli S, Kurucz E, Andó I, Udvardy A (2004) Tissue and developmental stage-dependent changes in the subcellular localization of the 26S proteasome in the ovary of *Drosophila melanogaster*. Gene Expr Patterns 4:329–333

Ahn JY, Hong SO, Kwak KB, Kang SS, Tanaka K, Ichihara A, Ha DB, Chung CH (1991) Developmental regulation of proteolytic activities and subunit pattern of 20S proteasome in chick embryonic muscle. J Biol Chem 266:15746–15749

Asher G, Reuven N, Shaul Y (2006) 20S proteasome and protein degradation "by default". BioEssays 28:844–849

Baumeister W, Walz J, Zühl F, Seemüller E (1998) The proteasome: paradigm of a self-compartmentalizing protease. Cell 92:367–380

Bradford M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248–256

Cardozo C, Eleuteri AM, Orlowski M (1995) Differences in catalytic activities and subunit pattern of multicatalytic proteinase complexes (proteasomes) isolated from bovine pituitary, lung and liver. J Biol Chem 270:22645–22651

Charmantier G, Charmantier-Daures M, Aiken DE (1991) Metamorphosis in the lobster *Homarus* (Decapoda): a review. J Crust Biol 11:481–495

Coux O, Tanaka K, Goldberg AL (1996) Structure and function of the 20S and the 26S proteasomes. Annu Rev Biochem 65:801–847

Covi JA, Bader BD, Chang ES, Mykles DL (2010) Molt cycle regulation of protein synthesis in skeletal muscle of the blackback land crab, *Gecarcinus lateralis*, and the differential

expression of a myostatin-like factor during atrophy induced by molting or unweighting. J Exp Biol 213:172–183

Dahlmann B, Ruppert T, Kuehn LO, Merforth S, Kloetzel PM (2000) Different proteasome subtypes in a single tissue exhibit different enzymatic properties. J Mol Biol 303:643–653

Davies KJA (2001) Degradation of oxidized proteins by the 20S proteasome. Biochimie 83:301–310

De Diego JL, Katz JM, Marshall P, Gutierrez B, Manning JE, Nussenzweig V, Gondalez J (2001) The ubiquitin-proteasome pathway plays an essential role in proteolysis during *Trypanosoma cruzi* remodeling. Biochemistry 40:1053–1062

De Mot R, Nagy I, Walz J, Baumeister W (1999) Proteasomes and other self-compartmentalizing proteases in prokaryotes. Trends Microbiol 7:88–92

Drews O, Wildgruber R, Zong C, Sukop U, Nissum M, Weber G, Gomes AV, Ping P (2007) Mammalian proteasome subpopulations with distinct molecular compositions and proteolytic activities. Mol Cell Proteomics 6:2021–2031

El Haj A, Clarke S, Harrison P, Chang E (1996) In vivo muscle protein synthesis rates in the American lobster *Homarus americanus* during the molt cycle and in response to 20-hydroxyecdysone. J Exp Biol 199:579–585

Frisan F, Levitsky V, Polack A, Masucci MG (1998) Phenotype-dependent differences in proteasome subunit composition and cleavage specificity in B cell lines. J Immunol 160:3281–3289

Galastegui N, Groll M (2010) The 26S proteasome: assembly and function of a destructive machine. Trends Biochem Sci 35:634–642

Gomes AV, Young GW, Wang Y, Zong C, Eghbali M, Drews O, Lu H, Stefani E, Ping P (2009) Contrasting proteome biology and functional heterogeneity of the 20S proteasome complexes in mammalian tissues. Mol Cell Proteomics 8:302–315

Götze S, Saborowski R (2011) NanoDrop fluorometry adopted for microassays of proteasomal enzyme activities. Anal Biochem 413:203–205. doi:10.1016/j.ab.2011.02.023

Grune T, Merker K, Sandig G, Davies KJA (2003) Selective degradation of oxidatively modified protein substrates by the proteasome. Biochem Biophys Res Comm 305:709–718

Haass C, Kloetzel PM (1989) The drosophila proteasome undergoes changes in its subunit pattern during development. Exp Cell Res 180:243–252

Hochstrasser M (1995) Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. Curr Op Cell Biol 7:215–223

Hong SO, Ahn JY, Lee CS, Kang MS, Ha DB, Tanaka K, Chung CH (1994) Tissue-specific expression of the subunits of chick 20S proteasomes. Biochem Mol Biol Int 32:723–729

Husom AD, Peters EA, Kolling EA, Fugere NA, Thompson LV, Ferrington DA (2003) Altered proteasome function and subunit composition in aged muscle. Arch Biochem Biophys 421:67–76

Ismail SZM, Mykles DL (1992) Differential molt-induced atrophy in the dimorphic claws of the male fiddler crabs, *Uca pugnax*. J Exp Zool 263:18–31

Jariel-Encontre I, Bossis G, Piechaczyk M (2008) Ubiquitin independent degradation of proteins by the proteasome. Biochem Biophys Acta Rec Cancer 1786:153–177

Kisselev AF, Goldberg AL (2001) Proteasome inhibitors: from research tools to drug candidates. Chem Biol 8:739–758

Kisselev AF, Akopian TN, Castillo V, Goldberg AL (1999) Proteasome active sites allosterically regulate each other, suggesting a cyclical bite–chew mechanism for protein breakdown. Mol Cell 4:395–402

Kisselev AF, Kaganowich D, Goldberg AL (2002) Binding of hydrophobic peptides to several non-catalytic sites promotes peptide hydrolysis by all active sites of 20S proteasomes. Evidence for peptide-induced channel opening in the α-rings. J Biol Chem 277:22260–22270

Kisselev AF, Garcia-Calvo M, Overkleeeft HS, Peterson E, Pennington MW, Ploegh HL, Thornberry NA, Goldberg AL (2003) The caspase-like sites of proteasomes, their substrate specificity, new inhibitors and substrates, and allosteric interactions with the trypsin-like sites. J Biol Chem 278:35869–35877

Kisselev A, Callard A, Goldberg AL (2006) Importance of the different proteolytic sites of the proteasome and the efficacy of inhibitors varies with the protein substrate. J Biol Chem 281:8582–8590

Klein U, Gernold M, Kloetzel P-M (1990) Cell-specific accumulation of *Drosophila* proteasome (MCP) during early development. Journal Cell Biol 111:2275–2282

Koenders A, Yu X, Chang ES, Mykles DL (2002) Ubiquitin and actin expression in claw muscles of land crab, *Gecarcinus lateralis*, and American lobster, *Homarus americanus*: differential expression of ubiquitin in two slow muscle fiber types during molt-induced atrophy. J Exp Zool 292:618–632

Liu C-W, Corboy MJ, DeMartino GN, Thomas PJ (2003) Endoproteolytic activity of the proteasome. Science 299:408–411

Meng L, Mohan R, Kwork BHB, Elofsson M, Sin N, Crews CM (1999) Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo anti-inflammatory activity. Proc Natl Acad Sci USA 96:10403–10408

Mykles DL (1990) Calcium-dependent proteolysis in crustacean claw closer muscle maintained in vitro. J Exp Zool 256:16–30

Mykles DL (1996) Differential effects of bovine PA28 on six peptidase activities of the lobster muscle proteasome (MCP). Arch Biochem Biophys 325:77–81

Mykles DL (1997) Biochemical properties of insect and crustacean proteasomes. Mol Biol Rep 24:133–138

Mykles DL (1998) Intracellular proteinases of invertebrates: calcium-dependent and proteasome/ubiquitin-dependent systems. Int Rev Cytol 184:157–289

Mykles DL (1999a) Proteolytic processes underlying molt-induced claw muscle atrophy in decapod crustaceans. Am Zool 39:541–551

Mykles DL (1999b) Structure and functions of arthropod proteasomes. Mol Biol Rep 26:103–111

Mykles DL, Haire MF (1991) Sodium dodecyl sulfate and heat induce two distinct forms of lobster muscle multicatalytic proteinase: the heat-activated form degrades myofibrillar proteins. Arch Biochem Biophys 288:543–551

Mykles DL, Skinner DM (1981) Preferential loss of thin filaments during molt-induced atrophy in crab claw muscle. J Ultrastruc Res 75:314–325

Naujokat C, Hoffmann S (2002) Role and function of the 26S proteasome in proliferation and apoptosis. Lab Invest 82:965–980

Orlowski M, Wilk S (2003) Ubiquitin-independent proteolytic function of the proteasome. Arch Biochem Biophys 415:1–5

Pelletier S, Schuurman KG, Berkers CR, Ovaa H, Heck AJR, Raijmakers R (2010) Quantifying cross-tissue diversity in proteasome complexes by mass spectrometry. Mol BioSyst 6:1450–1453

Richter-Ruoff B, Wolf DH (1993) Proteasome and cell cycle: evidence for a regulatory role of the proteasome on mitotic cyclins in yeast. FEBS Lett 336:34–36

Schmalenbach I, Fanke HD (2010) Potential impact of climate warming on the recruitment of an economically and ecologically species, the European lobster (*Homarus gammarus*) at Helgoland, North Sea. Mar Biol 157:1127–1135

Shean BS, Mykles DL (1995) Polyubiquitin in crustacean striated muscle: increased expression and conjugation during molt-induced claw muscle atrophy. Biochim Biophys Acta 1264:312–322

Skinner DM (1966) Macromolecular changes associated with the growth of crustacean tissues. Am Zool 6:235–242

Verras M, Gourzi P, Kalosaka K, Zacharopoulou A, Mintzas AC (2008) cDNA cloning, characterization, and developmental expression of the 20S proteasome α5 subunit in the Mediterranean fruit fly *Ceratitis capitata*. Arch Insect Biochem Physiol 67:120–129

Voges D, Zwickel P, Baumeister W (1999) The 26S proteasome: a molecular machine designed for controlled proteolysis. An Rev Biochem 68:1015–1068