Comparative Biochemistry and Physiology, Part C 162 (2014) 62–69

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

The proteasomes of two marine decapod crustaceans, European lobster (*Homarus gammarus*) and Edible crab (*Cancer pagurus*), are differently impaired by heavy metals

Sandra Götze [a], Aneesh Bose [a], Inna M. Sokolova [b], Doris Abele [a], Reinhard Saborowski [a,*]

[a] Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Functional Ecology, 27570 Bremerhaven, Germany
[b] Department of Biology, University of North Carolina at Charlotte, Charlotte, NC, USA

ARTICLE INFO

Article history:
Received 20 January 2014
Received in revised form 23 February 2014
Accepted 31 March 2014
Available online 8 April 2014

Keywords:
Proteasome
Lobster
Crab
Metal pollution
Cadmium
Mercury
Copper
Zinc

ABSTRACT

The intracellular ubiquitin-proteasome system is a key regulator of cellular processes involved in the controlled degradation of short-living or malfunctioning proteins. Certain diseases and cellular dysfunctions are known to arise from the disruption of proteasome pathways. Trace metals are recognized stressors of the proteasome system in vertebrates and plants, but their effects on the proteasome of invertebrates are not well understood. Since marine invertebrates, and particularly benthic crustaceans, can be exposed to high metal levels, we studied the effects of *in vitro* exposure to Hg^{2+}, Zn^{2+}, Cu^{2+}, and Cd^{2+} on the activities of the proteasome from the claw muscles of lobsters (*Homarus gammarus*) and crabs (*Cancer pagurus*). The chymotrypsin like activity of the proteasome of these two species showed different sensitivity to metals. In lobsters the activity was significantly inhibited by all metals to a similar extent. In crabs the activities were severely suppressed only by Hg^{2+} and Cu^{2+} while Zn^{2+} had only a moderate effect and Cd^{2+} caused almost no inhibition of the crab proteasome. This indicates that the proteasomes of both species possess structural characteristics that determine different susceptibility to metals. Consequently, the proteasome-mediated protein degradation in crab *C. pagurus* may be less affected by metal pollution than that of the lobster *H. gammarus*.

1. Introduction

Industrial processes are major sources of anthropogenic metal pollution in the environment (Järup, 2003; Rainbow, 2007). Pollution often occurs locally, and variation among regions may depend on the composition and quantity of industrial discharges, as well as on the distance to the point source of pollution (Soto-Jiméneza and Páez-Osuna, 2001; Yuan et al., 2012; Gao et al., 2013). Trace metals such as cadmium (Cd), mercury (Hg), lead (Pb), nickel (Ni), or copper (Cu) are common persistent pollutants in estuarine and coastal waters and sediments. Over the last several decades considerable increases in the concentrations of these metals have been reported in marine environments alongside severe impacts on ecosystems. For example, metal concentrations may reach ~80 μg · L^{-1} cadmium, more than 1 mg · L^{-1} copper, or 500 ng · L^{-1} mercury in marine surface waters depending on the site (US Department of Health and Human Services, Toxicological profiles, http://www.atsdr.cdc.gov/). These levels are often hundreds of times more concentrated than those from pristine control regions.

Metals are not naturally degradable in the marine environment and hence bio-accumulate and/or biomagnify along the food chains, often with severe health consequences for higher trophic level organisms (Mance, 1987; Storelli and Marcotrigiano, 2003; Jung and Zauke, 2008; Bánfalvi, 2011; Ramakritinan et al., 2012). Toxic effects of metals mainly depend on the metal speciation and bioavailability, but also on the means of uptake, accumulation, and excretion rates of the organisms. The toxicity of borderline and class B metals (such as Cu, Zn, Cd and Hg) is linked to their high affinity to sulfur- and nitrogen-rich compounds including cysteinyl and histidyl residues of proteins (Vallee and Ulmer, 1972; Eichhorn, 1976; Nieboer and Richardson, 1980). Hence, intracellular proteins are vulnerable to binding by metal ions, which may damage these proteins and impair their functions. However, essential trace metals such as Cu or Zn also act as important constituents of metalloenzymes (Lehninger, 1950) and only become toxic at high concentrations.

Cellular homeostasis requires the accurate regulation of cellular processes, and is crucial for cell proliferation, apoptosis, and even immune or stress responses. One key regulator of these processes is the ubiquitin–proteasome system (Fig. 1). This system is responsible for the precise degradation of more than 80% of short-living or malfunctioning proteins (Baumeister et al., 1998; Voges et al., 1999; Ciechanover, 2005). A disruption of this system has been linked with severe cellular dysfunctions and diseases (Ciechanover and Brundin, 2003; Powell et al., 2005). Class B metals are known stressors of the

* Corresponding author. Tel.: +49 471 4831 2220; fax: +49 471 4831 1776.
E-mail address: Reinhard.Saborowski@awi.de (R. Saborowski).

http://dx.doi.org/10.1016/j.cbpc.2014.03.012
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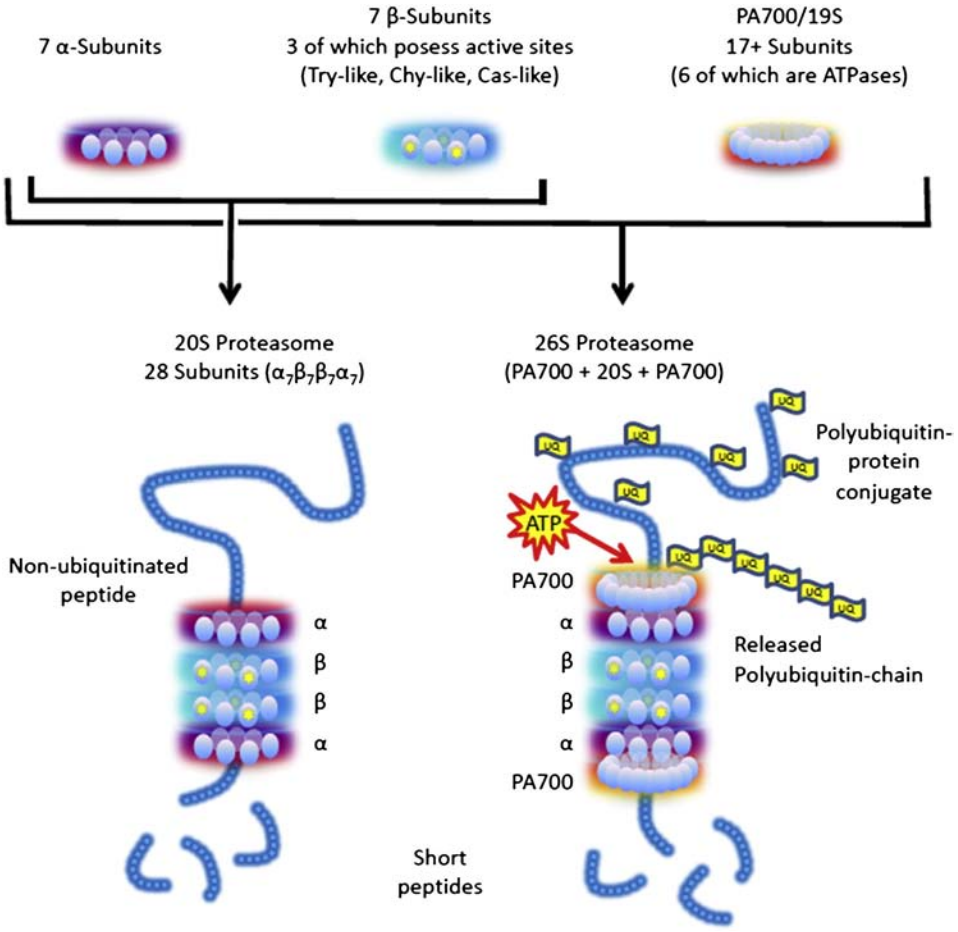

Fig. 1. Structure and function of the 20S and the 26S proteasome. The barrel-like structure consists of the catalytic core (20S proteasome) to which up to two regulator units may be bound forming the 26S proteasome. The core complex comprises 28 subunits organized in four stacked rings. In eukaryotes the two outer rings consist of 7 α-subunits while the two inner rings consist of 7 ß-subunits. The two inner ß-rings form a central chamber carrying in total six proteolytic active sites (indicated by yellow stars). Each of the two catalytic sites possesses the same cleavage preference after which they are named: chymotrypsin-like (Chy-like), trypsin-like (Try-like) and caspase-like (Cas-like) activities. *In-vivo*, the 20S core complex degrades mainly oxidatively damaged proteins in an ubiquitin-independent manner while the 26S proteasome degrades only short-living ubiquitinated proteins.

proteasome system. Metal ions can impair proteasome function directly or indirectly by respectively binding to the proteasome complex or by the generation of oxidative stress (Figueiredo-Pereira and Cohen, 1999; Grune, 2000; Pena et al., 2008).

Marine invertebrates, such as bivalves and crustaceans, are of high ecological and economic importance (*e.g.* Costello et al., 2010; Boudreou and Worm, 2012; FAO, 2012). Many of these species serve as sentinel organisms for monitoring environmental pollution in estuaries and coastal areas (Najle et al., 2000; Mirza et al., 2012). However, studies investigating the effects of class B metals on proteasome activities in marine crustacean species are sparse. To address this gap in our knowledge, we studied the *in-vitro* effects of Cu^{2+}, Zn^{2+}, Hg^{2+} and Cd^{2+} on the proteasome activity in two common decapods species, European lobster (*Homarus gammarus*) and edible crab (*Cancer pagurus*). The results demonstrate species-specific sensitivity of the proteasome of lobsters and crabs to metals, and provide a basis for future contributions to our understanding of sub-cellular effects of metals and their toxicity in marine decapods.

2. Materials and methods

2.1. Animal collections

European lobsters (*H. gammarus*) were purchased from a seafood merchant (CuxFisch Ditzer GmbH, Cuxhaven, Germany). The animals were captured off the Danish North Sea coast. Body lengths and masses of the animals were 15 to 20 cm and 500 to 550 g, respectively. Edible crabs (*C. pagurus*) were collected from bottom trawls with R/V Uthörn off the island of Helgoland (German Bight, North Sea). Carapace widths and body masses ranged from 10 to 15 cm and 250 to 450 g, respectively.

2.2. Preparation of extracts

Muscle tissues were dissected from the chelae of adult *H. gammarus* and *C. pagurus*, shock frozen in liquid nitrogen, ground to a fine powder by mortar and pestle, and stored at −80 °C until further analysis. About 1.2 g of the powdered tissue samples were resuspended in 4 mL of assay buffer (0.05 M Tris–(hydroxymethyl) aminomethane, 0.025 M KCl, 0.01 M NaCl, 0.001 M $MgCl_2$, pH 8.0). The suspensions were homogenized on ice with an ultrasonic cell disruptor (Branson, Sonfier B15) and centrifuged for 35 min at 13,000 *g* (4 °C). The supernatants were used directly for ultracentrifugation on a glycerol density gradient.

2.3. Glycerol density ultra-centrifugation

The proteasome fraction was partly cleaned up by glycerol density ultracentrifugation. Linear glycerol gradients of 20 mL glycerol (ranging from 10% to 40%) were prepared in assay buffer (50 mM Tris, 40 mM KCl, 5 mM $MgCl_2$, 2 mM ATP) with a gradient maker (Pharmacia Biotec, 80-1315-58) using polycarbonate tubes (Beckman Coulter, 355618). Crude muscle extracts (4 mL) were applied onto the gradients and

centrifuged for 15 h at 67,000 *g* at 4 °C with an ultracentrifuge (Beckman Coulter L7-80) equipped with a 70 Ti fixed-angle rotor. Fractions of 2 mL each were taken along the density gradient from the top to the bottom of the tubes and transferred into reaction tubes. The pellets were resuspended in 300 μL assay buffer and also transferred into reaction tubes. All fractions were placed on ice.

2.4. Measurements of proteasome activities

The chymotrypsin-like, trypsin-like, and caspase-like activities of the 20S/26S proteasome were determined with the fluorogenic substrates *Suc–Leu–Leu–Val–Tyr–AMC* (Bachem, I-1395), *Boc–Leu–Arg–Arg–AMC* (Bachem, I-1585), and *Gly–Pro–Leu–Asp–AMC* (Enzo Life Science, BML-AW9560), respectively. Verification that solely proteasome activity contributed to the measured proteolytic activity was achieved by running parallel assays containing the highly specific proteasome inhibitor epoxomicin (PeptaNova, 4381) (Meng et al., 1999; Götze et al., 2013). All assays were performed in 96-well plates in a total volume of 50 μL per assay, containing 40 μL of assay buffer (0.05 M Tris(hydroxymethyl)-aminomethane, 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, and 2 mM adenosine triphosphate (ATP), pH 8.0 at 30 °C) and 5 μL of sample extract. Reactions measuring total proteolytic activity also contained 2.5 μL of dimethylsulfoxide (DMSO), while the reactions measuring the proteasome-inhibited proteolytic activity contained 2.5 μL of 1 mM epoxomicin dissolved in DMSO (final concentration in the assay 50 μM). The reactions were initiated by the addition of 2.5 μL of substrate (10 mM; final concentration in the assay 0.5 mM). The initial fluorescence values were immediately read in a microplate fluorometer at 355 nm excitation and 460 nm emission (Fluoroscan Ascent, Thermo Scientific). The reaction was allowed to proceed for 1 h in the dark at 30 °C in a microplate incubator (CAT, SH26) before the plate was read again. The difference in the relative fluorescence units (RFU) between the final and the initial reads served as a measure of the enzyme activity. The amount of the released product, 7-amino-4-methylcoumarin (AMC), was calculated on the basis of a standard curve, and expressed as $U \cdot g^{-1}{}_{protein}$ ($U = \mu mol\ AMC \cdot min^{-1}$). The protein content was determined after Bradford (1976) with a commercial dye reagent (BioRad, 500-0006) and bovine serum albumin as standard.

To test the sensitivity of the proteasome to trace metals (Hg^{2+}, Zn^{2+}, Cu^{2+}, and Cd^{2+}), the chymotrypsin-like proteasome activities were measured as described above in the proteasome-enriched pellet fraction obtained by the gradient ultracentrifugation. The assays were conducted in the metal-free assay buffer or in the buffer supplemented with 10 to 100 μM of Cu^{2+}, Zn^{2+}, Hg^{2+} and Cd^{2+} (added as the respective chloride salts). The chymotrypsin-like proteasome activities in the presence of metals were calculated relative to the corresponding control assays (without metal additions) and expressed as mean activity (%) ± the standard error of the mean (SEM) of three replicates ($n = 3$).

2.5. Statistics and graphs

The percent data were *arcsin* square root transformed and analyzed with a one-way ANOVA and Tukey post-hoc test. Significant differences between species, and/or the metals are indicated in graphs by different letters when p values were below 0.05. For each metal and species the half maximal inhibitory concentration (IC_{50}) was calculated by applying the sigmoidal function:

$$y = \frac{\min + (\max - \min)}{1 + \left(\frac{x}{EC_{50}}\right)^{Hillslope}}.$$

Calculations and graph were done with the computer program SigmaPlot/Sigma Stat. 12.0.

3. Results

3.1. Proteasome activities in lobsters and crabs

The proteasome (20S/26S) of both species was effectively purified by glycerol-density ultracentrifugation. Proteasome activity was present in the last quarter of the gradient with increasing activities towards the pellet (Fig. 2). The highest activities towards all three substrates (representing the chymotrypsin-, trypsin-, and caspase-like activity) were found in the pellets, in accordance with the calculated sedimentation coefficient of the 20S/26S proteasome at the applied conditions. Furthermore, all three activities were completely (100%) inhibited by epoxomicin proving that solely proteasome activity was measured.

H. gammarus had higher average activities of all catalytic sites of the proteasome than *C. pagurus* (Fig. 3). However, a significant difference between the two species was only found for the trypsin-like activity ($p = 0.002$). For both crustaceans trypsin-like activities (TRY) were the highest, followed by chymotrypsin-like (CHY) activities and last by caspase-like (CAS) activities. In *H. gammarus* the trypsin-like activity amounted to $0.0058 \pm 0.0022\ U \cdot g^{-1}{}_{prt}$, the chymotrypsin-like activity to $0.0250 \pm 0.0011\ U \cdot g^{-1}{}_{prt}$, and the caspase-like activity to $0.0010 \pm 0.0006\ U \cdot g^{-1}{}_{prt}$ (all mean ± SEM; Fig. 3a). The trypsin-like activity was significantly higher than the two other activities (TRY to CHY: $p = 0.04$ and TRY to CAS: $p = 0.004$). The chymotrypsin-like activity and the caspase-like activity did not differ from each other. In *C. pagurus* the trypsin-like activity amounted to $0.0009 \pm 0.0003\ U \cdot g^{-1}{}_{prt}$, the chymotrypsin-like activity amounted to $0.0006 \pm 0.0002\ U \cdot g^{-1}{}_{prt}$, and the caspase-like activity amounted to $0.\ 0002 \pm 0.0001\ U \cdot g^{-1}{}_{prt}$ (Fig. 3b). In the crab, activities were not significantly different from each other.

3.2. Effects of metals

The effect of metal ions was determined for the chymotrypsin-like activity of the proteasome since this catalytic site has a prominent role for the overall protein breakdown (Rock et al., 1994; Heinemeyer et al., 1997). Accordingly, inhibition of this site will affect cells (organisms) more severely than inhibition of the two other catalytic sites. Our study revealed that metal ions inhibited this catalytic site in a species- and concentration-dependent manner (Fig. 4). The lobster proteasome was more sensitive towards all investigated metals than the crab proteasome. In *H. gammarus*, the activity was strongest inhibited by mercury (Hg^{2+}) with a complete inhibition at 100 μM Hg^{2+}. The other three tested metal ions, Cu^{2+}, Zn^{2+}, and Cd^{2+} caused 76.0–78.6% inhibition of the chymotrypsin-like proteasomal activity at 100 μM (Figs. 4, 5). The degrees of inhibition by Cu^{2+}, Zn^{2+}, and Cd^{2+} were not significantly different from each other, while inhibition by Hg^{2+} was significantly stronger than by any of the other three metals ($p < 0.04$).

Similar to the lobster, the proteasome of *C. pagurus* was the strongest inhibited by Hg^{2+} (100% inhibition at 100 μM). The second most potent inhibitor was copper with a 66.3% ± 3.5% reduction of the chymotrypsin-like proteasomal activity at 100 μM. Zinc had only a weak effect on the proteasome activity with 39.1% ± 8.5% at 100 μM. Cadmium had no significant effect on the chymotrypsin-like activity of the crab proteasome (Figs. 4, 5).

3.2.1. Effect of Hg^{2+}

Mercury caused the strongest inhibition of the chymotrypsin-like proteasome activity in both species (Fig. 4a and b). In the lobsters, 10 μM Hg^{2+} significantly decreased the activity to 43% residual activity (Fig. 4a; $p = 0.03$). At 25 μM Hg^{2+} only 8.2% ± 5.8% of the initial activity remained. Concentrations of more than 25 μM completely inhibited all activity ($p = 0.001$). The chymotrypsin-like activity of *C. pagurus* was not significantly inhibited below 25 μM Hg^{2+} (Fig. 4b). At 25 μM the activity decreased to 16% ± 16% of the initial activity ($p < 0.001$). At more

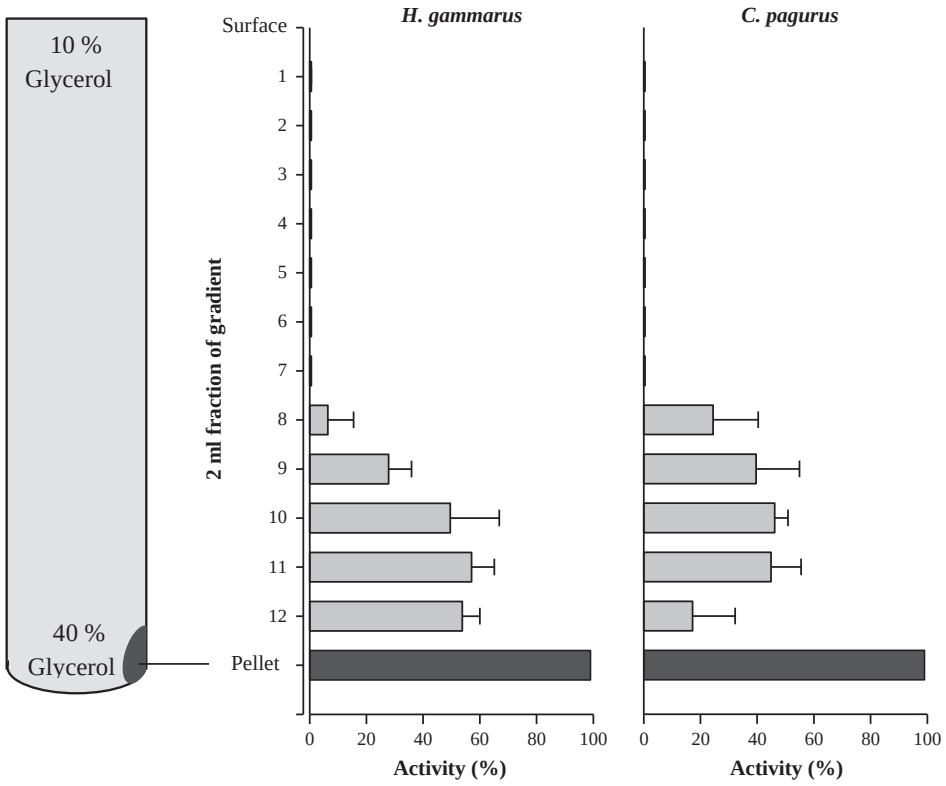

Fig. 2. Proteasome activity along the glycerol-density gradient after ultracentrifugation of extracts from the lobster *H. gammarus* and the crab *C. pagurus*. Means + SEM, n = 3.

than 25 μM Hg^{2+} no activity remained. The half maximal inhibitory concentration (IC_{50}) of mercury on the chymotrypsin-like activity was reached at 10.8 μM ± 0.7 μM in the lobster and at 13.4 μM ± 3.4 μM Hg^{2+} in the crab.

3.2.2. Effect of Zn^{2+}

Zinc ions affected the chymotrypsin-like activity of either species in a different way (Fig. 4c and d). In lobsters, the activity decreased significantly to 57.7 ± 11.3% in the presence of 10 μM Zn^{2+} (Fig. 4c; $p = 0.002$), and only 21.4% ± 11.7% of the residual activity remained in the 100 μM Zn^{2+} treatment. The proteolytic activity at 100 μM Zn^{2+}, however, was not statistically different from the activity measured at 10 μM Zn^{2+}. The chymotrypsin-like activity of *C. pagurus* was less affected by Zn^{2+} than the activity of *H. gammarus* (Fig. 4d). The activity remained unaffected up to a concentration of 25 μM Zn^{2+}. There was a slight reduction in activity at 50 μM Zn^{2+}, and a significant decrease at 100 μM Zn^{2+} (69.3% ± 8.5%; $p = 0.003$). In *H. gammarus*, the IC_{50} of zinc was reached at 11.3 μM ± 9.0 μM Zn^{2+}, and in *C. pagurus* at 56.1 μM ± 19.4 μM Zn^{2+}.

3.2.3. Effect of Cu^{2+}

The chymotrypsin-like activity of *H. gammarus* decreased significantly to 66.2% ± 9.6% in the presence of 10 μM Cu^{2+} (Fig. 4e; $p = 0.02$). Higher Cu^{2+} concentrations caused a stronger, although not significant,

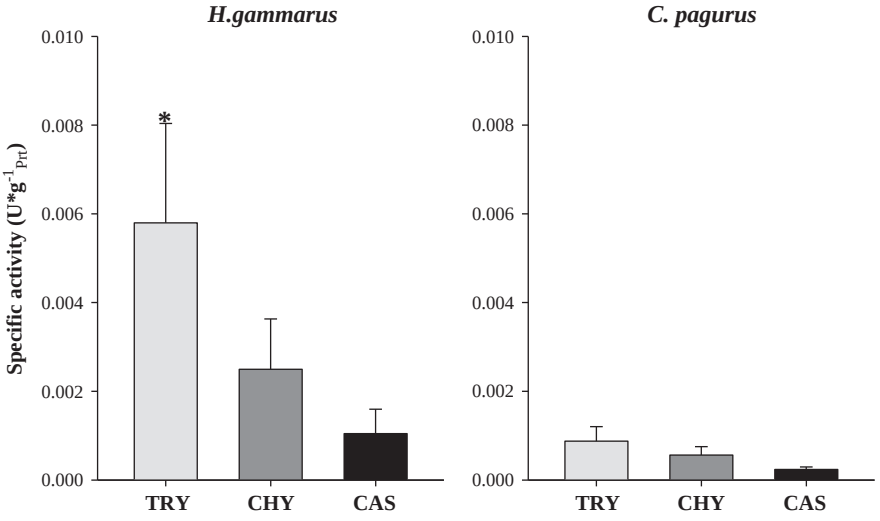

Fig. 3. Trypsin (TRY), chymotrypsin (CHY), and caspase (CAS) like proteasome activities of *H. gammarus* and *C. pagurus*. Values represent the mean specific activity ($U \cdot g^{-1}_{Prt}$) ± SEM. Significant differences between species are indicated by the asterisks when $p < 0.05$ ($n = 3$).

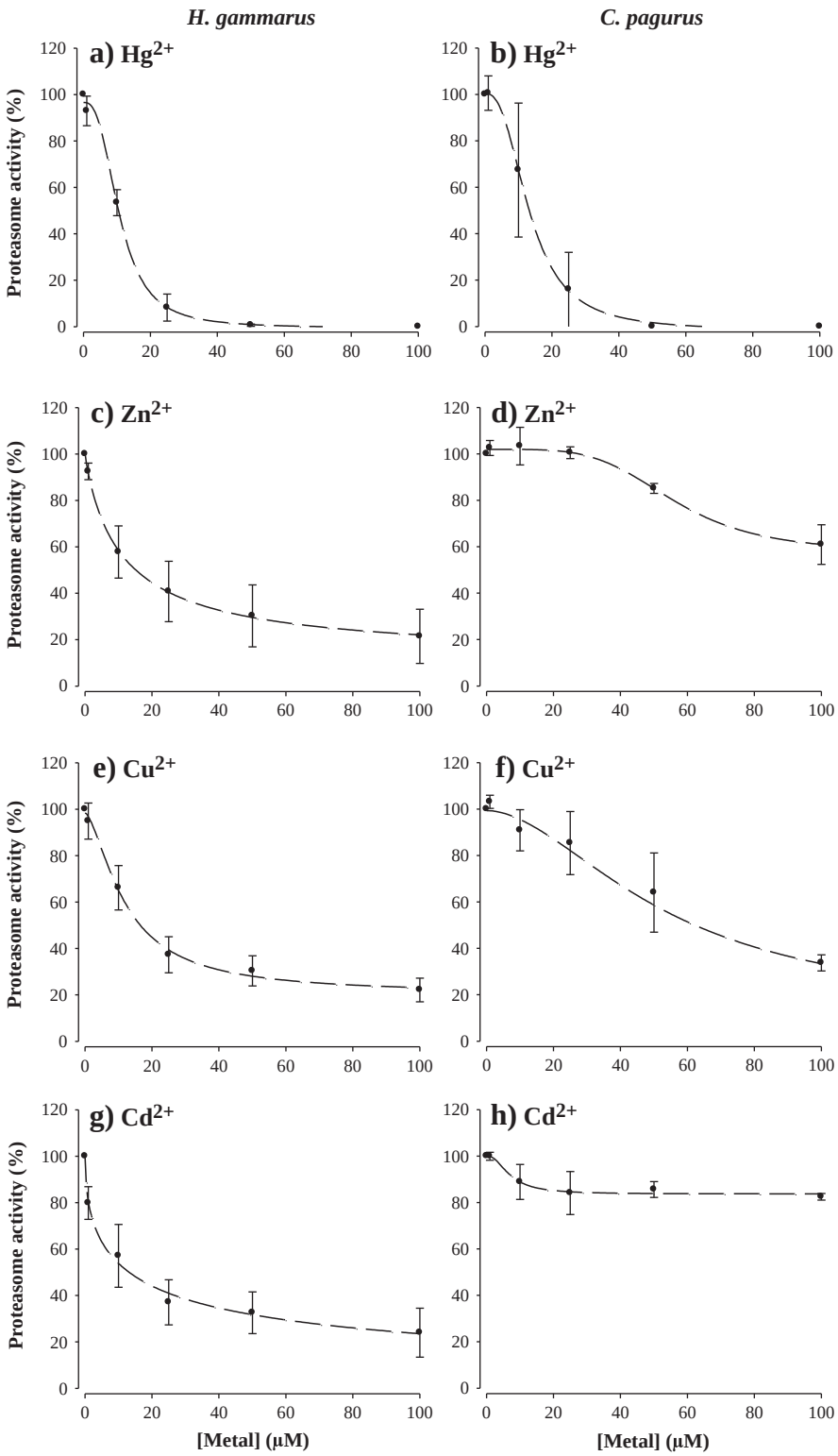

Fig. 4. Inhibitory effects of the four metals on the chymotrypsin-like activity (% related to an untreated control) of the 20S/26S proteasome. a), c), e), and g) represent the effects of Hg^{2+}, Zn^{2+}, Cu^{2+}, and Cd^{2+} on the proteasome of *H. gammarus*. b) d), f), and h) represent the effects of Hg^{2+}, Zn^{2+}, Cu^{2+}, and Cd^{2+} on the proteasome of *C. pagurus*.

decrease of activity. At 100 µM Cu^{2+} only 22.1 ± 5.09% of the initial activity was left. In contrast, the chymotrypsin-like activity of *C. pagurus* decreased slowly but not significantly up to 25 µM Cu^2 (Fig. 4f). At 25 µM Cu^2 the activity decreased significantly to 64.1% ± 17.1% ($p = 0.02$). The IC_{50} in *H. gammarus* was reached at 12.3 µM ± 3.2 µM Cd^{2+} and in *C. pagurus* at 40.0 µM ± 11.1 µM Cd^{2+}.

3.2.4. Effect of Cd^{2+}

Cadmium had different effects on the chymotrypsin-like activity of the lobster and crab. (Fig. 4g and h). In lobsters the activity decreased significantly in the presence of 10 µM Cd^{2+} to 57% ± 13.6% ($p = 0.001$). Higher concentrations up to 100 µM Cd^{2+} continuously reduced the activity down to 22% ± 13.6% at 100 µM Cd^{2+}. The chymotrypsin-like

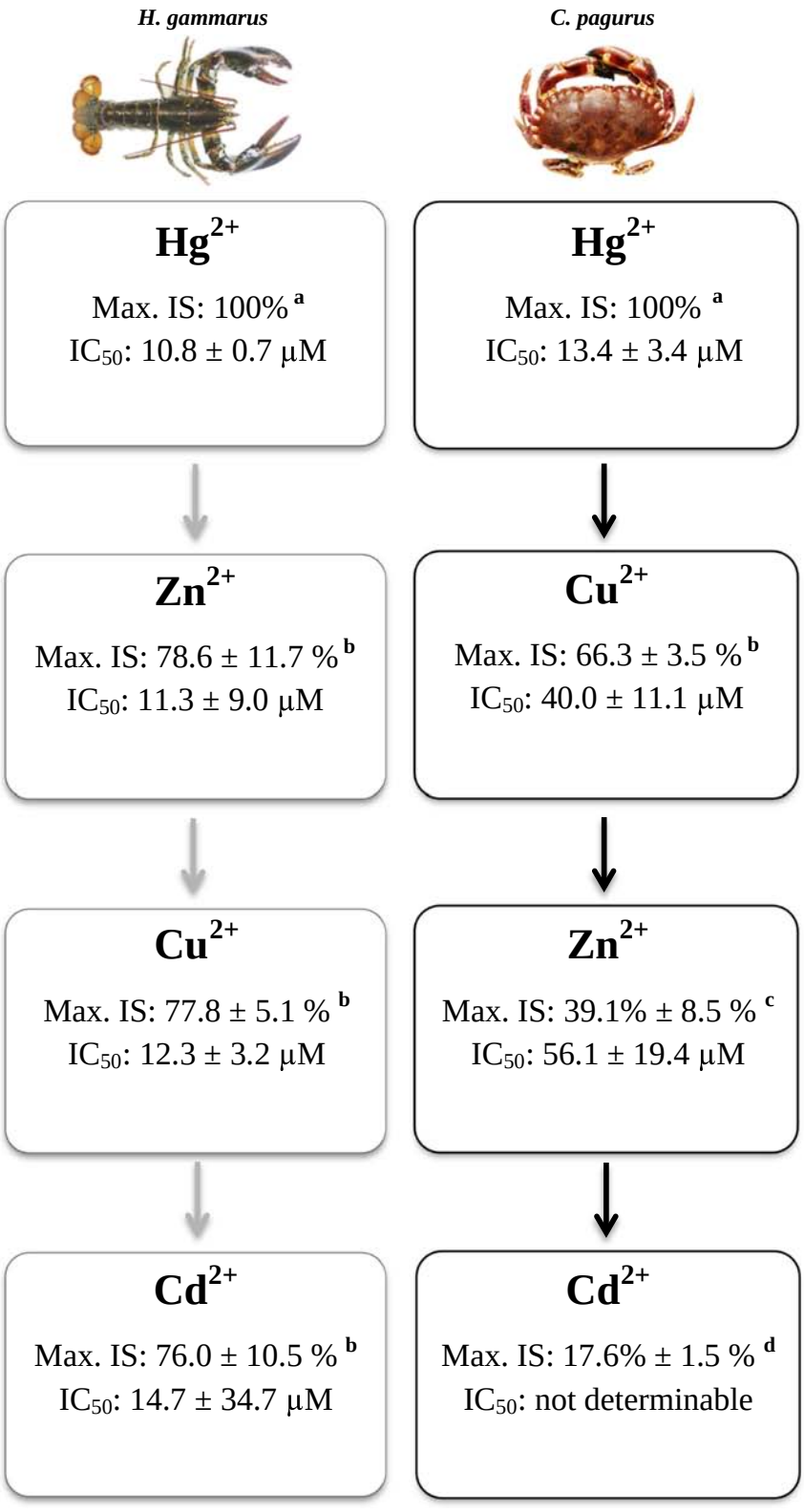

Fig. 5. Calculated maximum inhibitory strength (Max. IS) and IC_{50} of Hg^{2+}, Zn^{2+}, Cu^{2+}, and Cd^{2+} for the lobster *H. gammarus* and the crab *C. pagurus*. Significant differences between the max. IS of metals within the same species are indicated by the different letters when $p < 0.05$ ($n = 3$).

activity of *C. pagurus* was not significantly inhibited by any of the applied Cd^{2+} concentrations ($p > 0.05$). At 100 μM Cd^{2+} 82.5% ± 1.44% of the initial activity remained. The IC_{50} for cadmium of *H. gammarus* was reached at 14.7 μM ± 34.7 μM Cd^{2+}.

4. Discussion

The proper operation of the proteasome and associated pathways is crucial for cellular function and stress resistance. Intracellular excess of

borderline and class B metals has been shown to damage and modulate these pathways *in vivo* with severe negative effects for organisms (Amici et al., 2002; Kim et al., 2004; Pena et al., 2008; Yu et al., 2010; Kanthasamy et al., 2012). Here we show for the first time that the *in-vitro* functionality of crustacean proteasomes is impaired by metal ions. The fraction that we separated by glycerol-gradient centrifugation contained most likely both, the 20S and the 26S population of the proteasome. Previous studies on crustaceans showed that both forms of the proteasome are active under physiological conditions (Mykles, 1998, 1999a,b; Götze and Saborowski, 2011). Therefore, the use of a mixed population of the proteasome appears suitable for our study. The observed effects on the proteasomes were surprisingly different between species, despite the fact that both crustaceans originate from unpolluted benthic habitats of the North Sea and share similar life styles and feeding modes. The proteasome of the crab *C. pagurus* displayed wide variation in responses to different metal ions. It was severely inhibited by Hg, while being less sensitive to Cu and Zn, and almost unaffected by Cd. In contrast, all metals (Hg, Zn, Cu, and Cd) inhibited the proteasome of the lobster, *H. gammarus*, to similar degrees.

Metal ions can bind to proteins of the regulatory complexes, as well as to the core proteins of the proteasome complex (Fig. 1). In the core proteins, especially the terminal threonine amino acids in the ß-sites, and the Mg^{2+}-containing catalytic centers, were identified to have high affinities for various metal ions (Jover et al., 2008). Both essential metals, Cu and Zn, showed different and species-specific inhibitory effects on the crustacean proteasomes. Copper is one of the most effective metal proteasome inhibitors known for vertebrate species (Kim et al., 2004; Milacic et al., 2008; Pena et al., 2008). This metal attacks the 20S core complex at the chymotrypsin-like catalytic site (ß5) and affects both the 20S and the 26S proteasome populations (Daniel et al., 2004). Zinc interferes mainly with the JAMM domain of the 19S regulatory complex, and inhibits predominantly the 26S proteasome (Cvek et al., 2008). One possible explanation for the observed differences in the crustacean proteasome sensitivity towards metals may be due to adaptive or regulatory mechanisms. Especially the concentrations of essential metals can be regulated within appropriate intracellular levels, for example during elevated metal exposure (Stoeppler and Nürnberg, 1979; Rainbow and White, 1989). This regulatory capacity is often species-specific and depends on physiological traits such as osmoregulatory capacity, excretion rates, the ability to eliminate metals through ecdysis, and induction of detoxification pathways (Engel, 1987; Viarengo and Nott, 1993; Engel et al., 2001; Ahearn et al., 2004; Rainbow, 2007). For example, the rockpool prawn, *Palaemon elegans*, is capable of maintaining its internal concentrations of ~76 μg Zn g^{-1}dry mass and ~125 μg Cu g^{-1} dry mass when exposed to up to 315 μg · L^{-1} Zn and 100 μg · L^{-1} Cu in seawater before a net accumulation starts (Rainbow and White, 1989).

H. gammarus has a natural concentration of ~10 μg Cu and 26 μg Zn per gram wet mass of the muscle tissue (Barrento et al., 2008). *C. pagurus* has similar Cu concentrations in its muscle tissue (~8 μg Cu g^{-1} wet mass), but more than double the concentration of Zn (~60 μg g^{-1} wet mass) (Berge and Brevik, 1996). These Zn concentrations correspond to ~0.5 mM in lobster and ~1.2 mM Zn in crab and are much higher than the *in-vitro* concentrations investigated in our study. However, only a small fraction of the intracellular metals (including Zn) occur in the free ionic form, with the vast majority being incorporated into enzymes or bound by metal chelators such as metallothioneins and glutathione (Andersen and Baatrup, 1988; Finney and O'Halloran, 2003). Nevertheless, the low sensitivity of the crab proteasome towards Zn may reflect a possible physiological adaption to the naturally high concentrations of Zn in the crab muscle *in vivo*. Given the high biochemical similarity between Zn^{2+} and Cd^{2+} and similar affinity of these ions to intracellular targets (Zalups and Koropatnick, 2010), adaptation to high intracellular levels of Zn may also explain the considerably higher tolerance of the crab proteasome to Cd compared to that of the lobster. The mechanisms of the effects of Zn or Cd on proteasome activity are not yet fully understood and may involve direct binding to the proteasome as well as indirect interactions caused by metal-induced oxidative stress, release of essential metals from catalytic sites or intracellular storage, and/or disruption of associated pathways (Funk et al., 1987; Figueiredo-Pereira and Cohen, 1999; Pena et al., 2006). Our *in-vitro* study indicates that both, Cd and Zn, may affect the chymotrypsin-like proteasome activity directly, at least in lobsters. Differences in susceptibility of the proteasome to Zn and Cd between crabs and lobsters indicate potential structural differences at or around the binding sites for these metals on the proteasome of the two species, and require further investigation.

Of the four studied metals, mercury was the strongest inhibitor of the proteasome activity in lobsters and crabs. More than 90% of activity was suppressed at concentrations above 25 μM, which matches well with the acute sublethal dose of water-borne mercury (LC_{50}: 6 μM to 30 μM) for adult crustaceans (Connor, 1972; Eisler, 1981). Mercury has been shown to interfere with the proteasome activities in other organisms, but the precise mechanisms of the binding of Hg and Hg-induced inhibition of the proteasome are unknown (Yu et al., 2010). Moreover, the effects of Hg on the proteasome depend on the mode of exposure and differ between inorganic Hg^{2+} and organic mercury complexes. Hg was shown to highly inhibit the proteasome in plants and mammalian cell lines (Pena et al., 2008; Yu et al., 2010). However, under *in-vivo* conditions toxic effects of MeHg may be counterbalanced by a proteasome-mediated protection. Hwang et al. (2002) and Hwang (2011) demonstrated in yeast and human cell lines that enhanced resistance is mediated through enhanced expression of Cdc34 or Rad23 proteins which are important for ubiquitination, and accordingly, proteasomal degradation. Our study indicates that in crustaceans the exposure to inorganic mercury may suppress also *in-vivo* significantly the intracellular protein degradation. It is worth noting that, with the exception of cadmium, the relative inhibitory strengths of these metals on the crustacean proteasome correspond well to their respective degrees of toxicity measured for the whole organism. Marine crustaceans are highly sensitive to mercury, copper, and cadmium, and only to a lesser extent towards nickel or zinc (Eisler and Hennekey, 1977; Devi, 1987). This separation in sensitivity reflects distinct physiological traits and characteristics of species for regulating internal metal concentrations through uptake, excretion, or induction of detoxification pathways (Eisler, 1981; Amiard et al., 1987; Viarengo and Nott, 1993; Rainbow, 1995, 2007; Ahearn et al., 2004). Furthermore, abiotic or ecological factors, such as temperature, salinity, lifestyle, feeding mode, metal uptake route, or metal speciation influence rates of absorption and internal metal sequestration. Since metals act at different physiological levels, detailed studies on intracellular biochemical mechanisms can contribute to a better understanding of the complex interaction of environmental pollution and toxic effects in marine invertebrates. Our present study shows that suppression of proteasomal activity may represent a significant mechanism of trace metal toxicity in marine crustaceans, affecting rates of protein degradation and turnover which are essential for proper cell functioning and rapid response to stress (Bayne, 2004).

Acknowledgments

We are grateful to the crew of R/V Uthörn for providing crabs. I.M.S. was partially supported by Charlotte Research Institute and UNC Charlotte's Faculty Research Grant during the work on this manuscript.

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