METHODS & TECHNIQUES

Pitfalls in invertebrate proteasome assays

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SUMMARY

The ubiquitin-proteasome system controls a variety of essential intracellular processes through directed protein turnover. The invertebrate proteasome has recently gained increasing interest with respect to central physiological processes and pathways in different taxa. A pitfall in proteasome activity assays, represented by the trypsin-like, chymotrypsin-like or caspase-like site, lies in the fact that most commonly used experimental substrates are susceptible to degradation by non-proteasomal proteolytic enzymes, which can lead to erroneous interpretation of activity data obtained. Through the use of a proteasome-specific inhibitor, epoxomicin, we showed that the shares of proteasomal and non-proteasomal activities in the degradation of a model polypeptide substrate for chymotrypsin-like activity vary considerably between invertebrate taxa. Crustacean muscle tissue and hemocytes showed almost exclusively proteasomal activity. In yeast, approximately 90% of total proteolytic activity can be attributed to the proteasome. In contrast, proteasomal activity comprises only 20–60% of the total proteolytic activity in bivalve tissues. These results reveal that, without verification of the shares of proteasomal and non-proteasomal activities in crude extracts through the use of highly specific inhibitors, common proteasomal enzyme assays should be used and interpreted with caution.

Key words: proteasome, bivalve, Crustacea, epoxomicin, protein catabolism.

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INTRODUCTION

The ubiquitin-proteasome system is present in all living organisms. It catalyzes the degradation of intracellular proteins and is involved in the control of a variety of important cellular processes such as cell division, cell differentiation and apoptosis. More specifically, the proteasome exhibits multiple peptidase activity referred to as trypsin-like, chymotrypsin-like and caspase-like activity. It targets damaged or non-functional proteins that have been tagged for trypsin-like, chymotrypsin-like and caspase-like activity. It catalyzes the degradation of intracellular proteins and is involved in the fact that most commonly used experimental substrates are susceptible to degradation by non-proteasomal proteolytic enzymes, which can lead to erroneous interpretation of activity data obtained. Through the use of a proteasome-specific inhibitor, epoxomicin, we showed that the shares of proteasomal and non-proteasomal activities in the degradation of a model polypeptide substrate for chymotrypsin-like activity vary considerably between invertebrate taxa. Crustacean muscle tissue and hemocytes showed almost exclusively proteasomal activity. In yeast, approximately 90% of total proteolytic activity can be attributed to the proteasome. In contrast, proteasomal activity comprises only 20–60% of the total proteolytic activity in bivalve tissues. These results reveal that, without verification of the shares of proteasomal and non-proteasomal activities in crude extracts through the use of highly specific inhibitors, common proteasomal enzyme assays should be used and interpreted with caution.

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In recent years, the proteasomes of marine invertebrates have gained greater research attention, reflecting increasing recognition of their importance for protein turnover and degradation in the physiology of these organisms (Mykles, 1998). For example, the proteasome is thought to play an important role in claw muscle degradation during the molting process of adult and larval lobsters (Mykles, 1999b; Götze and Saborowski, 2011a). Bivalves were established as new model organisms to investigate physiological processes such as stress tolerance and ageing (Cherkasov et al., 2006; Ungvari et al., 2011). Besides the comprehensive work on the structure and function of arthropod proteasomes (Haire et al., 1995; Mykles, 1999a), limited information exists on the basic characteristics and properties of proteasome systems of most marine invertebrate taxa. Knowledge is particularly scarce with respect to the cross-reactivity of non-proteasomal proteases with the substrates used for proteasome activity determination in these animals. This knowledge, however, is crucial for choosing appropriate analytical methods and for the interpretation of the resulting data. Therefore, the aim of the present study was to investigate the chymotrypsin-like proteasomal activities in crude tissue extracts of different crustacean and bivalve species and to distinguish between proteasomal and non-proteasomal activities using epoxomicin, a proteasome-specific inhibitor (Meng et al. 1999; Kisselev and Goldberg, 2001). We focused our study on the chymotrypsin-like activity, which may serve as an indicator for the overall proteasomal capacity and which reacts among the three activity sites most sensitive towards the inhibitor epoxomicin. This study shows a considerable diversity in the relative shares of proteasomal and non-proteasomal activities between taxa, which has to be considered in future proteasome studies in marine invertebrates.

MATERIALS AND METHODS

Total chymotrypsin-like proteolytic activity was measured in the bivalves Arctica islandica (foot), Mytilus edulis (foot, gill), Mercenaria mercenaria (gill) and Crassostrea virginica (gill), and the crustaceans Homarus gammarus (claw muscle), Cancer pagurus (claw muscle) and Cragon crangon (hemocytes). Cell extracts of yeast, Saccharomyces cerevisiae, were used as a reference. The

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mussels were placed on ice for 15 min and then opened by incisions through the adductor muscles. The foot and the gills were dissected and immediately flash-frozen in liquid nitrogen. The remains of the mussel were immediately shock-frozen as well. The crustaceans were not killed but were induced to autotomize one claw by carefully bending the claw away from the body and breaking it at the basichium joint. The shell of the autotomized claws was crushed and the muscle tissue was dissected and frozen in liquid nitrogen. Hemocytes were isolated from the hemolymph as described previously (Götze and Saborowski, 2011b). The tissues were homogenized by mortar and pestle in liquid nitrogen and resuspended in fourfold volume (v/w) of assay buffer [0.05 mol l\(^{-1}\) Tris(hydroxymethyl) aminomethane, 0.025 mol l\(^{-1}\) KCl, 0.01 mol l\(^{-1}\) NaCl, 0.001 mol l\(^{-1}\) MgCl\(_2\), pH 8.0]. The homogenates were centrifuged for 35 min at 4°C and 13,000 g. The supernatant was collected and stored at −80°C until further analysis.

Concentration-dependent response to a specific proteasome inhibitor, epoxomicin, was determined for each species in 96-well plates with a total volume of 50 µl per assay. The assay contained 40 µl assay buffer [0.05 mol l\(^{-1}\) Tris(hydroxymethyl) aminomethane, 0.025 mol l\(^{-1}\) KCl, 0.01 mol l\(^{-1}\) NaCl, 0.001 mol l\(^{-1}\) MgCl\(_2\), and 0.002 mol l\(^{-1}\) adenosine triphosphate, pH 8.0 at 30°C], 5 µl of sample extract and 2.5 µl of dimethylsulfoxide (DMSO, as vehicle control) with epoxomicin at concentrations between 0 and 100 µmol l\(^{-1}\). The reactions were started by addition of 2.5 µl of Suc-Leu-Leu-Val-Tyr-AMC (I-1395, Bachem, Bubendorf, Switzerland; 10 mmol l\(^{-1}\) dissolved in DMSO, final concentration in assay 0.5 mmol l\(^{-1}\)) and incubated for 1 h at 30°C in the dark. Fluorescence was measured in a Fluoroscan Ascent Microplate Fluorometer (Thermo Scientific, Dreieich, Germany) at 355 nm excitation and 460 nm emission wavelengths.

In a separate set of experiments, the total chymotrypsin-like proteolytic activities of tissue homogenates were measured in the absence and presence of 50 µmol l\(^{-1}\) of epoxomicin. The share of activity inhibited by epoxomicin represents the proteasomal activity while the difference between the total activity and the inhibited share describes the non-proteasomal activity. The results were calculated and presented as relative amounts (%) of the total proteolytic activity. The percent data were arcsin square-root transformed and then analyzed with a one-way ANOVA and Tukey’s honestly significant different post hoc test (SPSS ver. 15.0, IBM, Armonk, NY, USA).

**RESULTS**

The chymotrypsin-like proteasome activities of the crustacean and bivalve tissues were notably inhibited in the presence of 5 µmol l\(^{-1}\) of epoxomicin (Fig. 1). The concentration-dependent curve for epoxomicin inhibition was similar in shape across all taxa, although it differed in the degree of inhibition, indicative of species-specific proteasomal contributions to total proteolytic activity (Fig. 1). At 10 µmol l\(^{-1}\) epoxomicin, most proteasome-like activity was inhibited with no further change at higher inhibitor concentrations up to 100 µmol l\(^{-1}\). The total proteolytic activity in the crustacean and the yeast samples could be almost exclusively attributed to proteasome-like activity (Fig. 2). In the hemocytes of *C. crangon*, activity was completely inhibited by epoxomicin and thus was exclusively of proteasomal origin (100±0%). In the muscle tissues of *H. gammarus* and *C. pagurus*, proteasomal activity accounted for 96.6±3.6% and 96.6±3.4% of the total proteolytic activity, respectively. The fraction of total proteolytic activity that could be explained by proteasomal catalysis was not significantly different among the three studied crustacean species (*P* = 0.4). The share of proteasome activity was slightly lower in the whole-cell lysates of *S. cerevisiae* (90.3±7.8%), but not significantly different from the muscle extracts of *H. gammarus* and *C. pagurus* (*P* = 0.8). All bivalve samples had a significantly lower content of proteasomal activity than the crustacean and the yeast samples. The gill tissue of *C. virginica* (64.2±5.5%) and the foot tissue of *A. islandica* (62.8±11.4%) showed the highest proteasomal contribution to the total proteolytic activity among the studied bivalves. Slightly, but not significantly, lower shares were detected in the gill tissue of *M. edulis* (44.2±10.2%). The lowest proteasome-based activity fractions were observed in the foot tissue of *M. edulis* (32.7±10.1%) and the gill tissue of *M. mercenaria* (23.2±16.1%). In these last two species, the proteasomal contribution to total proteolytic activity was significantly lower than in *C. virginica* and *A. islandica*.

**DISCUSSION**

This is the first study to report that cross-reactions, caused by non-proteasomal proteases, in crude extract assays differ significantly between marine invertebrate taxa. These findings are highly relevant for future studies on the proteasome in these organisms.

Our results reveal that when using a common substrate for testing proteasomal activities with muscle and hemocyte samples of crustaceans, the risk of obtaining erroneous results is actually quite low. More than 95% of the total chymotrypsin-like activity was inhibited by epoxomicin at concentrations as low as 5 µmol l\(^{-1}\), indicating that proteasome-like activity is a near-total contributor to the chymotrypsin-like activity of these crustacean tissue extracts. In contrast, measure of chymotrypsin-like activities in crude tissue extracts of marine bivalves cannot provide a reliable estimate...
of proteasomal activity unless the relative contributions of proteasomal and non-proteasomal pathways are determined. Therefore, verification of the cross-reactivity via inhibition with epoxomicin or other highly proteasome-specific inhibitors (Dick et al., 1996; Kisselev and Goldberg, 2001; Mykles, 2001) is an essential analytical step when working with bivalve samples. The inhibitor enables distinction between proteasomal and non-proteasomal activities and is particularly important when new species or tissue types are under investigation.

Epoxomicin has been shown to inhibit protease-related proteolytic activities in mammalian tissues, leaving calcium-dependent or lysosomal proteases unaffected. It is a natural epoxyketone with a unique mechanism to inhibit the proteasome but not serine or cysteine proteases. Therefore, it is the most selective inhibitor of proteasome known (Kisselev and Goldberg, 2001). The specific inhibition has been demonstrated in the proteasome of various tissues and cells, including yeast. Because the proteasome has a very conservative structure, we assume that epoxomicin acts in invertebrate proteasomes in the same way as in vertebrate proteasomes, and therefore is the most suitable inhibitor for studies on marine invertebrate proteasomes.

The main reason why proteasome assays can be susceptible to other proteinases is the lack of commercially available proteasome-specific substrates, such as labeled ubiquitinated proteins, which are exclusively degraded by the proteasome. Instead, short fluorophore-labeled peptides are commonly used, which are less specific and can therefore also be targeted by other proteolytic enzymes (Rosser et al., 1993; Garcia et al., 1998; Michael et al., 2005). Of particular importance when preparing crude tissue extracts is avoiding contamination with digestive enzymes from the gut or digestive glands of the animals, which may entirely mask the innate proteasomal activity of the sample (Götze and Saborowski, 2011a; A.B., personal observation). So, when starting work with new species the degree of cross-reactivity needs to be determined in crude extracts of uncontaminated samples.

Commercial proteasome assay kits provide a fast and easy way of screening proteasome activities. However, because these kits were designed for use with purified proteasome samples they do not include proteasome inhibitors. They are therefore not suitable for distinguishing between proteasomal and non-proteasomal activities without modifications to the assay protocol. For example, in a study by Ungvari et al. (Ungvari et al., 2011), proteasomal activities were determined in crude extracts of gill tissues from the bivalves *A. islandica* and *M. mercenaria* according to the manufacturer’s instructions with a Proteasome-Glo assay kit (Promega, Mannheim, Germany). The authors reported similar levels of chymotrypsin-like activities in both species. Our results, however, reveal that the northern quahog, *M. mercenaria*, possesses the lowest share of chymotrypsin-like proteasomal activity, accounting for approximately 23% of total activity, whereas the chymotrypsin-like proteasomal activity in the ocean quahog, *A. islandica*, contributes to roughly 60% to the total proteolytic activity. Thus, gill tissues of *A. islandica* actually have higher chymotrypsin-like proteasomal activities than *M. mercenaria*; however, this cannot be correctly assayed with common commercial test kits. This led the authors to the wrong conclusion, namely that chymotrypsin-like activity is lower in the long-lived *A. islandica* compared with the relatively shorter-lived *M. mercenaria* and, consequently, that there is no relationship between proteasomal activity and longevity.

In conclusion, verification of the cross-reactivity via inhibition with epoxomicin or other highly specific proteasome inhibitors (Dick et al., 1996; Kisselev and Goldberg, 2001) is an essential analytical step that enables the distinction between proteasomal and non-proteasomal activities as described here with the example of the chymotrypsin-like activity. This procedure is particularly important when new species or tissues are under investigation.

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**AUTHOR CONTRIBUTIONS**

S.G. and A.B. performed the sampling of tissue and the biochemical analysis of proteasomal activities, analyzed the data and drafted the manuscript. D.A., I.M.S. and R.S. designed and supervised the study, performed the statistics, created the graphs and contributed to the writing and editing of the text. All authors read and approved the final version of the manuscript.

**COMPETING INTERESTS**

No competing interests declared.

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