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Comparative study of the biochemical properties of proteasomes in domestic animals

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A b s t r a c t

Information on the biochemical properties of proteasomes is lacking or, at best, only fragmentary for most species of veterinary interest. Moreover, direct comparison of the limited data available on the enzymatic features of proteasomes in domestic animals is rendered difficult due to the heterogeneity of the experimental settings used. This represents a clear drawback in veterinary research, given the crucial involvement of proteasomes in control of several physiological and pathological processes. We performed the first comparative analysis of key biochemical properties of proteasomes obtained from 8 different domestic mammals. Specifically, we investigated the three main peptidase activities of constitutive and immunoproteasomes in parallel and systematically checked the sensitivity of the chymotryptic site to three of the most potent and selective inhibitors available. Overall, there was substantial similarity in the enzymatic features of proteasomes among the species examined, although some interesting species-specific features were observed.

1. Introduction

The 26S proteasome is an ATP-dependent protease present in both the cytoplasm and the nucleus of eukaryotic cells that is responsible for degradation of the majority of cellular proteins (Voges et al., 1999). This large(2.4 MDa) and abundant multisubunit proteolytic complex consists of the 20S proteasome, in which proteins are degraded, capped at one or both ends by the 19Sregulatory particle, which is responsible for recognizing, unfolding, and translocating polyubiquitinated (and some non-ubiquitinated) substrates into the internal proteolytic cavity of the 20S particle (Glickman and Ciechanover, 2002). PA28 is

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an alternative proteasome activator that enhances hydrolysis of short peptides, but not entire proteins (Raule et al., 2014a). The 20S proteasome is a 700 kDa barrel-shaped structure composed of four stacked heptameric rings. The two outer rings consist of α subunits, while the two central rings are made up of β-subunits (Coux et al., 1996). Three of the subunits in the a rings (_1, _2, and _5) contain the proteolytic active sites that are positioned on the interior face of the cylinder. When measured with short fluorogenic substrates, the proteolytic activities of proteasomes have three distinct cleavage preferences: b1 has caspase activity (i.e. cleavage after acidic residues); b2 possesses tryptic activity (i.e. cleavage after basic residues); and b5 displays chymotryptic activity (i.e. cleavage after hydrophobic residues). However, lymphoid cells and cells exposed tocytokines such as IFN-g or TNF-a express three homologous subunits (b1i/LMP2, b2i/MECL-1, and b5i/LMP7) that replace the constitutive ones in newly assembled, so-called immunoproteasome particles (Cascio, 2014). A variety of studies have demonstrated that incorporation of these IFN-gamma induced subunits quantitatively modifies the preferences of proteasomal cleavage in a way that enhances production of a significant number of antigenic peptides (Sijts and Kloetzel, 2011). Additionally, immunoproteasomes have been reported to be important for efficient cytokine production (Groettrup et al., 2010) and have recently been shown to hydrolyze basic proteins, such as histones, at greatly increased rates compared with constitutive proteasomes. This suggests that they have a potential role in regulation of transcription (Raule et al., 2014b). The crucial role of the ubiquitin proteasomes system in controlling several physiological (e.g. heat shock and unfolded protein responses, apoptosis, transcription, aging, B-cell differentiation, cell cycle regulation, MHC-I antigen presentation) and pathological (e.g. neoplastic transformation, inflammation, neurodegenerative and autoimmune diseases) processes was unambiguously established during the last decade (Lecker et al., 2006). Moreover, proteasome inhibitors have emerged as a promising new class of anticancer agents due to their ability to selectively induce apoptosis in tumor cells, especially in those of hematological origin (Adams, 2004; Cenci et al., 2012). It is, therefore, somehow surprising that there is a distinct lack of data regarding the biochemical properties of proteasomes in domestic animals, and especially the absence of studies that systematically compare the functional and structural characteristics of these proteases indifferent species. For two mammalian species (i.e. cattle and rabbit) there is a significant amount of data regarding the enzymatic activities of both constitutive and

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immunoproteasomes, sensitivity to proteasome inhibitors, subunit composition, and overall structure of both 20S and 19S particles that has been acquired during the last two decades(mainly because these animals were chosen as experimental models by several groups performing basic studies on the cell biology and biochemistry of proteolysis) (Eleuteriet al., 1997; Cascio et al., 2001). However, for other species there is only scanty information available. Furthermore, direct comparison between species is further rendered difficult due to the extreme heterogeneity of the experimental settings used, including differences in protocols for proteasome extraction and purification, assay conditions for peptidase activity, and chemical structure and concentration of the fluorogenic substrates and inhibitors tested.

2. Materials and methods

2.1. Proteasome purification

Constitutive and immunoproteasomes were purified as described (Cerruti et al., 2007) with minor modifications. Briefly, samples of muscle and spleen from dog and cat were derived from surgical resections, rabbit muscle and spleen were purchased from Pel Freez Biologicals; mouse muscle and spleen were obtained from animals maintained in the animal facility unit of the SPAE (Universityof Torino), in conformity with European laws and policies and with the approval of the Ethical Committee of the University of Torino and the Italian Ministry of Health; horse, swine, goat, sheep and cattle samples were collected at the local slaughterhouse. Tissues were homogenized in ice-cold extraction buffer (50 mM Tris-HCl pH 7.5, 1 mM dithiothreitol, 250 mM sucrose, 5 mM MgCl2, 0.5 mM EDTA, and 2 mM ATP) using an Ultraturax DIAX900 homogenizer (Heidolph Instruments, Kelheim, Germany), centrifuged twice at 21,000 × g for 45 min at 4◦C to remove cell debris, and then ultracentrifuged at 300,000 × g for 3 hours. Pellets obtained were dissolved in ice cold extraction buffer and further centrifuged at 21,000 ×g for 15 min at 4◦C. The protein concentration in supernatants was determined with the Bradford reagent (Sigma-Aldrich) using a standard curve constructed with BSA. Samples were stored at −80◦C until use. Human proteasomes were recovered from the human cell lines HeLa and RPMI8226 that express at high level only constitutive (Nathan et al., 2013) or immunoproteasomes (Cenci et al., 2012) respectively. Cellular extracts and proteasome purification were performed as described (Kisselev and Goldberg, 2005).

2.2. Immunoblot analyses

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Immunoblot analyses of proteasomal non-catalytic sub-units b3, b4, b5, and a6, were performed as previously described (Favole et al., 2012). Briefly, 75 ug of proteins recovered after ultracentrifugation of muscle extracts were separated on a 12% SDS-PAGE gel, and transferred on aImmobilon®-P transfer membrane (Millipore). The membrane was then incubated in blocking buffer (5% BSA, 0.1%Tween-20 in 1 × PBS), followed by incubation with primary monoclonal antibodies (MCP196, MCP106, MCP79,MCP257, Enzo Lifesciences). Bound antibodies were visualized using the ECL technique.

2.3. Proteasome activity assays and susceptibility to inhibitors

Peptidase activities of partially purified proteasomes and immunoproteasomes were measured using 100 uM Suc-LLVY-amc (for chymotrypsin-like activity), 100 uM Z-YVAD-amc (for caspase-like activity), and 100 uM Bz-VGR-amc (for trypsin-like activity) (Bachem) in 20 mM Tris–HCl pH 7.5, 1 mM ATP, 2 mM MgCl2, and 0.2% BSA. The fluorescence of released amc (excitation, 380 nm; emission, 460 nm) was monitored continuously at 37◦C with a Carry Eclipse spectrofluorometer (Varian). Assays were calibrated using standard solutions of the free fluorophore, and the reaction velocities were calculated from the slopes of the initial linear portions of the curves. Substrate consumption at the end of incubation never exceeded 1%. To evaluate the effects of proteasome inhibitors, aliquots of constitutive proteasomes from different species were pre-incubated at room temperature for 20 minutes in the presence or absence (vehicle alone) of 10 uM MG132 (Calbiochem), 5 uM clasto-lactacystin-b-lactone (Biomol), 2 uM or 20 uM epoxomicin (Sigma-Aldrich), 100 uM leupeptin (Sigma-Aldrich) and immediately used for proteasome activity assays as described above.

2.4. Statistical analysis

To compare average measurements of peptidase proteasomal activities among domestic animals, we adopted a Mann-Whitney test. Data were graphically visualized using box plots.

3. Results and Discussion

Peptidase activities of proteasomes obtained from several species of veterinary interest were assessed according to a protocol widely used for human and experimental animal cells and tissues (Kisselev and Goldberg, 2005). Herein, the enzyme was partially purified by ultracentrifugation from homogenates of skeletal muscle that exclusively contain

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constitutive proteasomes (Van Kaer et al., 1994;Kisselev et al., 1999; Cascio et al., 2001), which were characterized with various fluorogenic peptides. Fig. 1A shows the comparative quantification of the chymotrypsin-like, caspase-like and trypsin-like activities of constitutive proteasomes in different species. In agreement with data for humans and mouse reported in literature (Chu-Ping et al.,1994; Akaishi et al., 1996; Coux et al., 1996) that we confirmed in our study (Fig. S1A), chymotryptic activity was higher in all species analyzed, followed by the tryptic and caspase-like activities (Fig. 1A). Consequently, the ratios among the three main proteasomal peptidase specificities were similar in all species assessed, including human and mouse (Table 1). On the other hand, the specific activities of constitutive proteasomes varied among the domestic animals analyzed. Specifically, swine, sheep, horse, and rabbit had significantly lower values of the three activities than those in cat, goat, cattle, and dog (Fig. 1A), that appear more similar to those measured in parallel experiments for human and mouse (Fig. S1A).To further define the biochemical properties of the proteasomal chymotryptic site in domestic animals, we assessed its sensitivity to MG132, clasto-lactacystin b-lactone, and epoxomicin, three of the most potent and specific proteasome inhibitors available. As shown in Fig. 2A, proteasomes from all species analyzed were highly sensitive (inhibition > 90%) to the inhibitory effects of these three compounds, thus demonstrating substantial conservation of the chymotryptic b5 site in mammals. More importantly, these conclusions were further corroborated by the results of an accurate titration of the three compounds that didn't highlight any difference in their inhibitory effects at any of the concentrations assessed (Fig. 2B). This further provides the rationale for the use of these chemicals in research involving domestic animals, as well as their potential therapeutic application. Sensitivity to inhibitors of the other two catalytic b-subunits was also assessed by means of leupeptin (a potent and specific inhibitor of the b2 tryptic subunit) (Kisselev et al., 2006) and epoxomicin (that at the concentration used efficiently suppresses the activity of the b1 caspase subunit) (Kisselevet al., 2006). As shown in Fig. 2A, both inhibitors proved highly effective in suppressing the activity of the target subunit in all species analyzed, thus indicating a substantial conservation in mammals also of b1 and b2 proteasome subunits. A similar analysis of peptidase activities was subsequently performed for immunoproteasomes. To this purpose, the enzyme was purified by ultracentrifugation from the spleen, which constitutively and exclusively expresses this form of the proteasome (Eleuteri et al., 1997;Cascio et al., 2001). As shown in Fig. 1B, this analysis revealed that,

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compared with constitutive proteasomes, immunoproteasomes present enhanced trypsin-like and reduced caspase-like activities, in agreement with the data reported in literature for human enzymes (Gaczynska et al.,1993) that we confirmed in our study (Fig. S1B). Notwithstanding, some had slightly enhanced (cat, rabbit but also human) or reduced (dog and mouse) specific activities. Furthermore, similar to what has already been established for constitutive proteasomes, the general trend of the relative ratios among activities of immunoproteasomes were maintained in all the species analyzed, including human and mice (Table 1). Of interest, those from dog, goat, and cattle had a trypsin-like activity that, at least in absolute terms, was even higher than the chymotryptic-like activity (Fig. 1Band S1B). Additionally, conservation of the a-subunits was preliminary investigated by assessing the cross-reactivity of proteasomes with monoclonal antibodies specific for the a3, a4, a5, and a6 subunits of the human enzyme. As shown in Fig. 3, anti- a5 and anti-a6 antibodies recognized their target subunits in all species with equal efficiency. Anti- a4 cross-reacted in all animals, although with slightly different affinities, and one anti- a3 failed to identify the protein in dog and cat. Thus, these results are consistent with an overall conservation of sequences and structures among proteasomal a-subunits in the domestic mammals analyzed, although some species-specific differences are present that warrant further investigation. There is limited data on the biochemical properties and the structural features of proteasomes in domestic animals. Furthermore, the genomic information on proteasomal genes present in international databases are only partial and fragmentary for most species of veterinary interest (www.uniprot.org). To fill this gap, we undertook the first systematic comparative characterization of proteolytic active sites of constitutive and immunoproteasomes in 8 widely different domestic mammals. It was demonstrated that the general biochemical features of the proteasomal machinery are conserved among all animals analyzed. In fact, we found that the chymotrypsin-like activity of constitutive proteasomes is always higher than the other two cleavage specificities. Moreover, the caspase-like activity was consistently the lowest, while the values for trypsin-like activity were situated in the middle between the values of the other two activities. Consequently, the relative ratios among the three cleavage specificities of constitutive proteasomes are similar in all species analyzed. Remarkably, however, some animals (i.e. dog, cat, goat and cattle) displayed values of the three proteasome peptidase activities that were higher than those in the other species. Although the precise molecular mechanisms of this enhanced catalytic capacity is unknown, species-specific differences

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in the relative ratios and stimulatory capacities of various proteasome activators (e.g. 19S, PA28, PA200) appear to be the most likely explanation. Considering constitutive proteasomes, in the case of immunoproteasomes the general enzymatic properties of the proteolytic particle were conserved among the species analyzed. Specifically, immunoproteasomes were consistently characterized by enhanced trypsin-like and a reduced caspase-like activities in accordance with what has already been established for the human enzyme (Gaczynska et al., 1993). Interestingly, for immunoproteasomes the absolute specific activities were not the same in all species, with cat and rabbit presenting slightly enhanced values, and dog had slightly reduced peptidase activities. Moreover, compared with constitutive proteasomes, the relative ratios of enzymatic activities were more variable for immunoproteasomes mainly due the unexpected finding that dog, goat, and cattle had a trypsin-like activity that is even higher than the chymotryptic-like activity in absolute terms. Collectively, our results demonstrate that there is over-all conservation of the general enzymatic properties of proteasomes in the species analyzed, as shown by the similarities in the relative ratios of peptidase cleavage specificities. Our study, therefore, justifies the widely established practice in the field of protein degradation research to use proteasomal particles purified from one specie to perform in vitro studies on another species. In this regard, it is worth noting that the vast majority of human in vitro immunological studies investigating the process of MHC class I epitope processing have been performed with proteasomes and immunoproteasomes obtained from rabbit or cattle (Goldberg et al., 2002). However, our data also indicate that some degree of caution should be taken when translating in vitro results on proteasomal degradation from one species to another. In fact, our data showed that proteasomes from some species are characterized by overall enhanced enzymatic activity. Although the biochemical mechanisms and the biological relevance of these differences in peptidase properties are unknown, the observation that they are not related to the evolutionary proximity of the species investigated is of interest. In fact, among ruminants, sheep has a chymotrypsin-like activity of constitutive proteasomes that is around four-fold lower than goat and cattle, but very similar to rabbit and swine. Moreover, the two carnivores (cat and dog) show the same enhanced chymotryptic activity of constitutive proteasomes as the two ruminants (goat and cattle). A similar lack of correlation between differences in enzymatic activity levels and phylogenetic relationships was also evident for immunoproteasomes. In this case, cat displays a chymotryptic activity that is much higher than dog, but similar to rabbit

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which, by itself, has values that are higher than those of other herbivorous animals. At the moment, it is unclear if the differences in the levels of proteasomal activities actually reflect some species-specific requirements for an enhanced proteolytic intracellular pathway or merely indicate substantial redundancy of the proteasomal apparatus in some animals. Further studies will be required to clarify this intriguing issue. To further define the biochemical properties of proteasomes from domestic animals, we tested the sensitivity of the chymotryptic site of the b5 subunit to the effects of three highly specific and widely used proteasome inhibitors. Chymotrypsin-like is the main proteasomal activity, and is rate limiting for the turnover of the vast majority of proteins in vivo (Chen and Hochstrasser,1996; Arendt and Hochstrasser, 1997; Heinemeyer et al.,1997). For this reason, most compounds used for scientific and therapeutic purposes were developed to exclusively or preferentially target the active site of b5 (Kisselevand Goldberg, 2001). In our assays, when employed at concentrations that quench the human enzyme, MG132,clasto-lactacystin b-lactone, and epoxomicin efficiently inhibited proteasomes from all animals tested, with a level of inhibition that was always higher than 90%. There were, moreover, no clear differences among species. Importantly, these inhibitors are known to inactivate chymotryptic activity by accommodating into the substrate-binding groove of _5 subunits and selectively blocking the N-terminal catalytic threonine of the enzymatic site either reversibly (MG132) or irreversibly (clasto-lactacystin b-lactone and epoxomicin). Consequently, the uniform and strong effect of these three inhibitors clearly demonstrates substantial conservation of the catalytic mechanism and active site structure of the b5 subunit in these mammals. Moreover, our results demonstrate for the first time the high sensitivity of proteasomes of several domestic animals to three of the most potent and widely available proteasome inhibitors, thus validating their use in the veterinary field as important research tools and as potential therapeutic agents. Finally, we also performed a preliminary investigation of the non-catalytic a subunits that form the two outer rings connecting the internal proteolytic chamber with different proteasome activators (e.g. 19S, PA28, PA200). For this purpose, the cross-reactivity of the four different proteasomal a subunits was tested in western blot analysis using commercial antibodies specific for the corresponding human proteins. Remarkably, two antibodies recognized their specific target with equal, high affinity in all species; one detected its subunit in all animals, although with slight differences in affinity and one reacted the protein equally well in six species, but unexpectedly did not give any signal in dog or cat. Altogether, our study

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demonstrates that there is substantial overall conservation of the a and catalytic b subunits of proteasomes in all the domestic animals investigated, although there are some minor species-specific differences that warrant further investigation. Moreover, our study demonstrates that the inhibitors and antibodies widely utilized in research in humans are also applicable in veterinary research as valuable scientific tools and potential therapeutic agents.

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Appendix A. Supplementary dataSupplementary data associated with this article can befound, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2015.04.005.

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Table 1 Relative ratios of activities of constitutive and immunoproteasomes in domestic animals, mouse and human.

	Constitutive proteasome		Immuno-proteasome	
	CL/CTL (%)	TL/CTL (%)	CL/CTL (%)	TL/CTL (%)
DOG	4.9	25.2	7.8	174.4
CAT	5.5	ND	5.2	ND
RABBIT	7.2	40.7	3.0	94.4
HORSE	7.8	51.9	2.4	82.0
SWINE	12.8	31.1	3.3	66.4
GOAT	4.9	20.8	3.7	133.4
SHEEP	10.8	33.2	3.3	82.5
CATTLE	5.1	28.8	2.9	199.5
MOUSE	10.5	46.0	9.4	145.8
HUMAN	4.21	51.34	2.5	92.9

Caspase-like (CL) and trypsin-like (TL) activities of constitutive and immunoproteasomes were assessed as described in Section 2 and indicated as percentage of chymotryptic-like (CTL) activity. ND, not determined.

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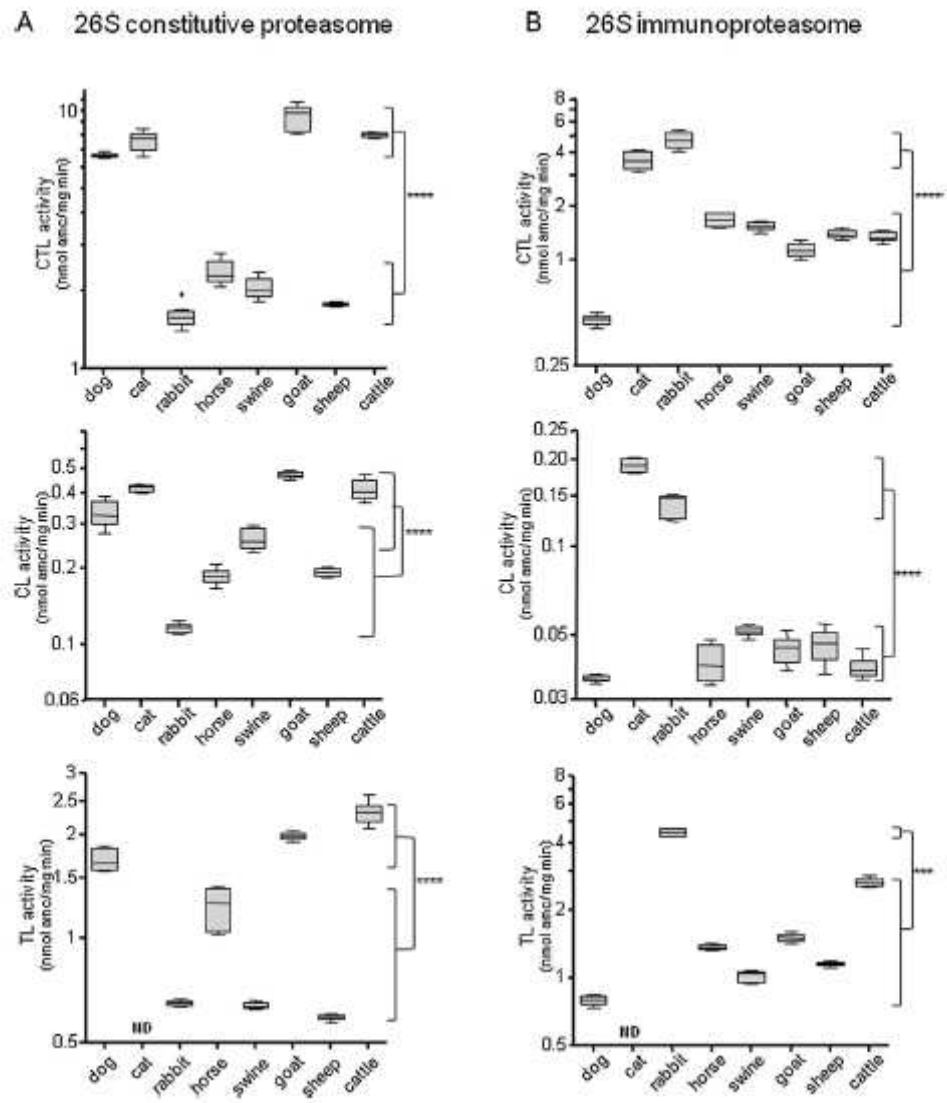

Fig. 1. Peptidase activities of constitutive and immune- proteasomes in domestic animals. Chymotrypsin-like, caspase-like, and trypsin-like activities of constitutive (A) and immune-proteasomes (B) were assessed as described in Section 2. Data are the mean of nine independent measurements ± SEM. ****p < 0.0001. CTL, chymotrypsin-like; CL, caspase-like; TL, trypsin-like; ND, not determined. Reference values for human and mouse enzymes are shown in figure S1.

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A

	CHYMOTRYPSIN-LIKE ACTIVITY (% inhibition)			TRYPSIN-LIKE ACTIVITY (% inhibition)	CASPASE-LIKE ACTIVITY (% inhibition)
	Clasto-lactacystin β-lactone	MG-132	Epoxomicin	Leupeptin	Epoxomicin
DOG	96.3 ± 0.4	98.1 ± 0.3	98.2 ± 0.4	97.8 ± 0.7	90.2 ± 0.8
CAT	93.4 ± 0.7	97.6 ± 0.4	98.0 ± 0.7	ND	89.3 ± 1.2
RABBIT	93.8 ± 2.0	96.6 ± 0.4	93.3 ± 2.7	95.2 ± 1.6	88.8 ± 2.2
HORSE	90.4 ± 1.1	95.8 ± 1.8	94.6 ± 1.0	95.0 ± 1.2	89.0 ± 1.6
SWINE	95.1 ± 0.1	97.5 ± 0.4	96.9 ± 0.2	95.7 ± 2.1	93.1 ± 3.6
GOAT	97.1 ± 0.2	98.9 ± 0.2	98.5 ± 0.2	95.5 ± 1.1	95.8 ± 1.0
SHEEP	92.5± 0.4	95.1 ± 0.3	94.3 ± 0.2	97.2 ± 1.7	93.2 ± 3.4
CATTLE	96.2 ± 0.5	98.5 ± 0.2	98.6 ± 0.2	97.8 ± 1.2	92.7 ± 2.2
MOUSE	95.0 ± 1.2	98.3 ± 0.3	98.9 ± 0.1	98.0 ± 0.1	93.8 ± 2.1

B

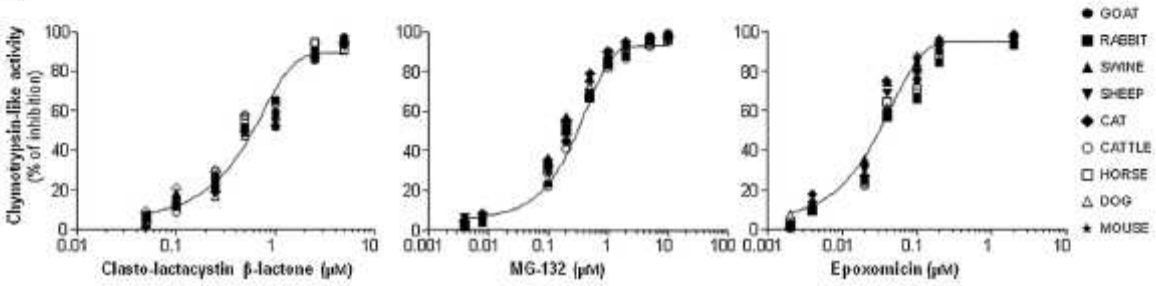

Fig. 2. Effect of inhibitors on peptidase activities of constitutive proteasomes in different animals. (A) The inhibitory effects of clasto-lactacystin b-lactone(5 uM), MG-132 (10 uM), and epoxomicin (2 uM) on the chymotrypsin-like activity, of leupeptin (100 uM) on the trypsin-like activity and of epoxomicin (20 uM) on the caspase-like activity of constitutive proteasomes were assessed as described in Section 2 and indicated as percentage of inhibition relative to vehicle-treated controls. Data are the means of three independent measurements ± S.D. (B) Titration of the three inhibitors of the chymotrypsin-like activity.

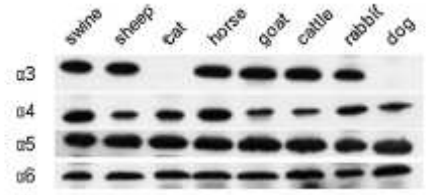

Fig. 3. Western blot analysis of proteasome a-subunits in domestic animals. Western blots of the a3, a4 a5, and a6 proteasomal subunits were performed with specific monoclonal antibodies as described in Section 2.

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