Published in final edited form as:
Proteomics. 2009 July ; 9(13): 3609–3622. doi:10.1002/pmic.200800959.

CHRONIC ETHANOL FEEDING AFFECTS PROTEASOME INTERACTING PROTEINS

Marie-Pierre Bousquet-Dubouch[2,3], **Sheila Nguen**[1], **David Bouyssié**[2,3], **Odile Burlet-Schiltz**[2,3], **Samuel W. French**[1], **Bernard Monsarrat**[2,3], and **Fawzia Bardag-Gorce**[1]

[1] LA BioMed at Harbor UCLA, Torrance, CA, USA

[2] CNRS; IPBS (Institut de Pharmacologie et de Biologie Structurale); 205 route de Narbonne, F-31077 Toulouse, France

[3] Université de Toulouse; UPS; IPBS; F-31077 Toulouse, France

Abstract

Studies on alcoholic liver injury mechanisms show a significant inhibition of the proteasome activity. To investigate this phenomenon, we isolated proteasome complexes from the liver of rats fed ethanol chronically, and from the liver of their pair-fed controls, using a non-denaturing multiple centrifugations procedure to preserve Proteasome Interacting Proteins (PIPs). Isotope-Coded Affinity Tagging (ICAT) and MS/MS spectral counting, further confirmed by Western blot, showed that the levels of several PIPs were significantly decreased in the isolated ethanol proteasome fractions. This was the case of PA28α/β proteasome activator subunits, and of three proteasome-associated deubiquitinases, Rpn11, ubiquitin C-terminal hydrolase 14 (Usp14), and ubiquitin carboxyl-terminal hydrolase L5 (UCHL5). Interestingly, Rpn13 C-terminal end was missing in the ethanol proteasome fraction, which probably altered the linking of UCHL5 to the proteasome. 20S proteasome and most 19S subunits were however not changed but Ecm29, a protein known to stabilize the interactions between the 20S and its activators, was decreased in the isolated ethanol proteasome fractions. It is proposed that ethanol metabolism causes proteasome inhibition by several mechanisms, including by altering proteasome interacting proteins and proteasome regulatory complexes binding to the proteasome.

Keywords

proteasome; ICAT; MS/MS spectral counting; quantitative proteomics; mass spectrometry

1- INTRODUCTION

Proteasome is the catalytic machinery of the Ubiquitin-Proteasome system, which is involved in the degradation of most intracellular proteins [1,2]. Proteasome degrades abnormal and damaged proteins but also normal proteins with short half lives. It tightly regulates major cellular processes such as the cell cycle, NFκB activation, response to hypoxia, and transcription [3].

Corresponding Authors: Marie-Pierre Bousquet-Dubouch, PhD., IPBS - UPS/CNRS UMR5089, 205 route de Narbonne, F-31077 Toulouse, France, marie-pierre.bousquet@ipbs.fr, Tel : (33) 5 61 17 55 44, Fax : (33) 5 61 17 59 94. Fawzia Bardag-Gorce, PhD., LA BioMed at Harbor-UCLA Med. Ctr., 1124 W. Carson St., Torrance, CA 90502, fgorce@labiomed.org, Tel: (1) 310-222-1846, Fax : (1) 310-222-3614.

All authors declare no financial/commercial conflicts of interest.

Proteasomes of mammalian cells exhibit a complex heterogeneity depending on the cellular environment [4,5], the cell type [6–8] but also the sub-cellular localization [9,10]. The 20S proteasome constitutes the catalytic core for all the various proteasome isoforms. It is a relatively stable complex composed of 28 subunits, 7 different α subunits and 7 different β subunits assembled in 4 stacked heptameric rings in a α7β7β7α7 arrangement. The 20S proteasome therefore presents a cylinder-like structure, where the six proteolytic sites located on subunits β1, β2, and β5, are buried. Upon immune response or IFNγ treatment, the three catalytic subunits of the 20S proteasome, are replaced by three different subunits, β1i (LMP2),β2i (MECL-1) and β5i (LMP7), forming the immunoproteasome [11]. Several chemical compounds, proteins, and regulatory complexes, function as activators or inhibitors of the catalytic core 20S proteasome [12]. In particular, 20S proteasome binds to different activators, 19S, PA28 and PA200. These activators are termed gate-openers because they provoke the opening of the gate at the alpha-type subunits of the 20S proteasome [13], and facilitate the access of designated proteins to be degraded to the catalytic chamber formed by the beta-type subunits. The most abundant proteasome complex is the 26S proteasome formed by the axial association of one 20S complex with two 19S regulators at its two sides. The resulting complex is responsible for the ATP-dependent degradation. Proteasomes formed of the 20S core particle in association with PA28α/β and PA28γ complexes, are, among other functions, important for the generation of epitope peptides presented to the major complex of histocompatibility [14]. Those involving the 20S proteasome and PA200 activators are implicated in DNA repair [15]. Recently, the hybrid form of the 26S proteasome, containing both 19S and PA28 complexes associated at the two opposite sides of the 20S core particle, has also been characterized [16], and other activators and proteins are certainly modulating the proteasome activity, and are yet to be discovered. A growing body of evidence indicates that a number of proteins transiently associate with the proteasome complex to perform specific activity, such as substrate presentation, cleavage of the multi-ubiquitin chain from the protein substrate, and immuno-response mechanisms [17]. Such activities are essential for proteasome to efficiently fulfill its intracellular function in the protein degradation process. Therefore, proteasomes appear as highly dynamic structures, whose specific multitask functions need to be further investigated and determined.

Mass spectrometry in combination with complex purification methods and quantitative strategies has proven to be an efficient tool for the identification of protein complexes networks [18–20]. 26S proteasome can be purified by multidimensional chromatography [21], affinity-based procedures [22–25], or mass separation-based methods [26,27]. Quantitative strategies using mass spectrometry can help to distinguish specific proteasome interactors from contaminants but also labile from stable partners [25,28,29]. They can also permit to study variations of the composition of proteasome complexes in different cell types [7,30].

In experimental alcoholic liver disease, protein degradation by the ATP-ubiquitin-proteasome pathway is impaired and the levels of ubiquitin and its protein conjugates are increased in humans [31]. Failure of the proteasome to eliminate cytoplasmic proteins leads to the accumulation of oxidized and otherwise modified proteins [32–34], and increases cytochrome P450 2E1 (CYP2E1)-mediated toxicity [35,36], leading to cell death and tissue damage. Hepatic proteasome chymotrypsin-like and trypsin-like activities are significantly decreased when ethanol is administered to animals by continuous intragastric feeding [37]. The mechanism responsible for the decrease in proteasome activity caused by high blood ethanol levels is still not clear. It has been associated with an elevation in oxidative stress generated by ethanol metabolism [32]. Oxidative stress is thought to be responsible for the formation of stable 4-hydroxynonenal (4-HNE) adducts on proteins and on proteasomes [26,38]. Phosphorylation has also been reported on 20S proteasome alpha subunits in the

ethanol induced liver injury [39]. These Post-Translational Modifications (PTMs) might cause changes in the structure of proteasome subunits and thereby interfere with the association of the regulatory complexes and with the regulation by Proteasome Interacting Proteins (PIPs).

In the present study, enriched proteasomes fractions were analyzed using mass spectrometry to identify the proteasome interacting proteins, and to quantify the changes in the amount of these proteins upon chronic ethanol feeding.

2- MATERIALS AND METHODS

1- Animal Model of Alcoholic Liver Disease

Wistar male rats from Harleco (Hollister, CA), weighing 250 g and 300 g were used. The rats were fed a liquid diet intragastrically containing ethanol (13 g/kg/day) at a constant rate for 1 month [40]. Pair-fed controls were fed dextrose isocaloric to ethanol (pair-fed control group). Rats were maintained according to the Guidelines of Animal Care, as described by the National Academy of Sciences and published by the National Institute of Health (1996).

2- Proteasomes isolation and chymotrypsin-like enzyme assay

Fresh livers were used to purify the 26S proteasome fractions. The livers were homogenized in 50 mM Tris–HCl (pH 7.5), 2 mM ATP, 5 mM $MgCl_2$ and 1 mM DDT, 1 mM EGTA, 50 mM Na-Fluoride, 50 μM Na-orthovanadate, proteases inhibitors and phosphatase inhibitors cocktails (Sigma, St Louis, MO), based on the ratio 1 g/20 ml (w/v). Equal volumes of ethanol fed rat liver homogenate and of pair-fed control rat liver homogenate were used for the first centrifugation (100,000 g for 1 h) to obtain the cytosolic fraction. For the 2nd centrifugation (71,000 g for 6 h) equal volumes were used to sediment the proteasomes. The pellets were resuspended in the same volumes (1 ml of standard buffer) before they were loaded on top of the 32 ml glycerol density gradient (10–40% glycerol) and centrifuged (100,000 g for 22 h). The fractionation was performed using a peristaltic pump and 33 fractions of 1 ml were then collected. Protein concentration measurements were determined using the Bio-Rad assay (Bio-Rad Hercules, CA). The same amount of protein from each fraction analyzed was used to perform proteasome enzyme assay and Western blot analysis. The proteasome's most active fraction was identified by measurement of the chymotrypsin-like activity. These experimental procedures have been described previously [26,41].

3- Western Blots

Proteins (5 to 10 μg) from the most active proteasome fraction were separated by SDS-PAGE electrophoresis, using 12% polyacrylamide gels. Proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA) for 1 hr in 25 mM Tris-HCl (pH 8.3), glycine 192 mM and 20% methanol. Primary antibodies against 20S proteasome α subunits,β5i subunit, PA28 subunits α and β, and Rpn11 (BIOMOL, Plymouth Meeting, PA) were used for immunoblot staining. Antibodies against Usp14, UCHL5, Ecm29, and CYP2E1 were purchased, respectively from Abcam (Cambridge, MA), ABGENT (San Diego, CA), GeneTex, Inc (San Antonio, TX), and Assay Designs, Inc. (Ann Arbor, Michigan). Goat anti-mouse monoclonal and goat anti-rabbit antibodies (Bio-Rad, Hercules, CA) were used as the second antibody. Immunodetection was performed using an alkaline phosphatase kit (Bio-Rad Hercules, CA) or using the ECL Plus Kit (Amersham Biosciences Corp., Piscataway, NJ) for chemiluminescence detection. Densitometric measurements of the bands were performed using a GS-700 imaging densitometer (Bio-Rad).

Data were obtained from 3 separate biological replicates corresponding to 3 animals for each group. Bars represent mean values ± SEM. P values are determined by one way ANOVA

and Student Newman Keuls for multiple group comparisons (Sigma Stat software, San Francisco, CA). $p \leq 0.05$ was used for establishing a significant difference.

4- Identification and Differential Quantification of Proteins in Purified 26S Proteasome Fractions

The proteasome richest fraction, analyzed by measuring the chymotrypsin-like activity, was used for LC MS/MS protein identification and relative quantification.

The relative protein quantification was realized using two differential quantitative proteomic strategies, the Isotope Coded Affinity Technology (ICAT) [42,43], and a label free method, the MS/MS spectral counting approach [44], on two different biological samples. Importanly, our work was designed to look at tendencies in relative levels to identify varying proteins which could then be confirmed by Western blot, with more biological replicates and statistical validation.

- Proteins identification and relative quantification by cICAT™—After dialysis against Milli-Q water, and TCA/acetone precipitation, the washed pellets of purified proteasome samples were solubilized in 50 mM Tris, pH 8.3, containing 0.5% SDS, and 6M Urea. Then, the two different protein fractions (150 μg each), control and ethanol, were reduced, labeled with 12C- and 13C- cleavable ICAT™ (cICAT™) reagents (Applied Biosystems, Framingham, MA), respectively, combined prior to protein fractionation by SDS-PAGE, digested with modified sequencing grade trypsin (Promega, Madison, WI) and the resulting peptides were extracted before being purified by affinity chromatography on a monomeric avidin cartridge. Finally, cleavage of the cICAT™ reagent from the cICAT™-labeled peptides was achieved according to the manufacturer's instructions and the peptide mixture was dried under vacuum. These experimental procedures have been detailed previously [45].

Peptides were then analyzed by nanoLC-ESI-Q-TOF MS/MS analysis and database searching, as explained below. The automatic validation and relative quantification of the isotopically-labeled proteins were performed, using our in-house developed software, MFPaQ (version 4.0.0), as described previously [45]. After calculation of an average ratio for each protein identified, ratios were normalized, using the median ratio of the total protein population. Uncertainty levels on protein ratios could be evaluated by calculation of coefficients of variations on quantifying peptides and on two technical replicates.

- Relative Quantification of Proteins by MS/MS Spectral Counting—After dialysis against Milli-Q water, and TCA/acetone precipitation of 200 μg of purified proteasome samples, the pellets were resuspended in Laemmli buffer and separated by SDS-PAGE, using 12% polyacrylamide gels. Twenty-one homogeneous bands were excised from the gel and proteins were reduced and alkylated by successive incubations in solutions of 10 mM DTT in 100 mM NH_4HCO_3, for 35 min at 56°C, and 55 mM iodoacetamide in 100 mM NH_4HCO_3, for 30 min at room temperature, respectively. Then, proteins were in-gel digested with trypsin and identified by nanoLC-ESI-Q-TOF MS/MS analysis and database searching, as explained below. Protein hits were automatically validated if they satisfied one of the following criteria: identification with at least one top ranking peptide (bold and red) with a Mascot score of more than 65 (p-value < 0.001), or at least two top ranking peptides, each with a Mascot score of more than 50 (p-value < 0.03), or at least three top ranking peptides, each with a Mascot score of more than 45 (p-value < 0.1), as determined by the Mascot Search program, and using the automatic validation module of MFPaQ (version 4.0.0) [45]. Proteins, identified with exactly the same set of peptides, were grouped and only one member of the protein group was reported, for more clarity (the one that we considered

as the most significant, according to the functional description given in the UniProt Knowledgebase). However, detailed protein groups are shown in Supplementary Data 1. Highly homologous protein hits, *i.e.*, proteins identified with top ranking MS/MS queries also assigned to another protein hit of higher score (red, non bold peptides), were detected by the MFPaQ software, and were considered as individual hits, and included in the final list only if they were additionally assigned a specific top ranking (red and bold) peptide of score higher than 48 (p-value < 0.05).

Validated proteins were submitted to a relative quantification using MS/MS spectral counting, which has been adapted from the method described by Liu *et al*. [44]. This strategy does not require any labeling of proteins, but is based on the counting of the total number of MS/MS spectra identified for a protein. For each protein identified and validated using the MFPaQ automatic validation module, the number of spectral copies (given by Mascot) to identify this particular protein was used. When a protein was identified several times in consecutive gel slices, all the MS/MS attributed to peptides belonging to the protein were taken into account. The list of proteins, with their corresponding total number of spectral copies, was automatically generated by MFPaQ. The relative quantity of a protein present in the 2 samples was estimated as the ratio of the total number of MS/MS of the protein in each sample. Ratios were normalized, using the median ratio of the total protein population.

- Peptides analysis by NanoLC-ESI-Q-TOF MS/MS and database Searching— Tryptic peptides were resuspended with 12 μl of 2% ACN/0.05% TFA, and were subjected to nanoLC-MS/MS analysis on an ESI-Q-TOF mass spectrometer (QSTAR XL, Applied Biosystems, Foster City, CA) operating in positive mode with a 2.1 kV spray voltage. Chromatographic separation was performed onto a 75 μm ID × 15 cm PepMap C18 column (Dionex/LC Packings, Sunnyvale, CA), at a flow rate of 200 nL/min, using a linear gradient of increasing ACN in water (4.5–45%) over 60 min with 0.2% formic acid. Data were acquired in a data dependent acquisition mode with Analyst QS (version 1.1, Applied Biosystems, Foster City, CA). For each MS spectrum (1 s), the two most intense multiple charged peaks were selected for generation of subsequent CID mass spectra (3 s each). The CID collision energy was automatically adjusted, based upon peptide charge and *m/z*. A dynamic exclusion window was applied within 30 sec. Data were analyzed using Analyst QS software (version 1.1), and MS/MS centroid peak lists were generated using the Mascot Daemon software (Mascot version 2.2 - Matrix Science, Boston MA). For the analysis of cICAT™-labeled peptides, up to 10 MS/MS spectra were allowed for grouping with a precursor mass tolerance of 0.1 a.m.u. For identification and quantification of proteins using MS/MS spectral counting, no grouping of MS/MS spectra was allowed. Data were searched using the Mascot server (Mascot version 2.2.01, Matrix Sciences) against Mammalian sequences in Swiss-Prot TrEMBL database. This database consists of UniProtKB/Swiss-Prot Protein Knowledgebase Release 53.1, merged in-house with UniProtKB/TrEMBL Protein Database Release 36.1. Peptide tolerance in MS and MS/MS modes was 0.5 Da and 0.8 Da, respectively. Trypsin was designated as the protease, and up to two missed cleavages were allowed. For all searches, oxidation of methionine, amino-terminal protein acetylation, and ubiquitinylation of lysine (GG), were searched as variable modifications. For ICAT experiments, ICAT-light and ICAT-heavy on cysteines were also allowed as variable modifications. For the other MS/MS experiments, variable modifications to be considered for the search also included carbamidomethylation and propionamide on cysteines and phosphorylation on serine, threonine, and tyrosine.

To evaluate false positive rates, all the initial database searches were performed using the "decoy" option of Mascot, *i.e.*, the data were searched against a combined database containing the real specified protein sequences (target database, Swiss-Prot TrEMBL

human) and the corresponding reversed protein sequences (decoy database). MFPaQ uses the same criteria to validate decoy and target hits, calculates the False Discovery Rate (FDR = number of validated decoy hits/(number of validated target hits + number of validated decoy hits) × 100) for each gel band analyzed, and makes the average of FDR for all bands belonging to the same gel lane (*i.e.*, to the same sample). Results were considered as relevant if the false positive rate never exceeded 1%.

3- RESULTS

The purpose of the present study was to further understand the effects of chronic ethanol feeding on the environment of the proteasome, because, as complex as is the proteasome, it does not function alone. Complex protein machinery around the proteasome guides the specificity and the efficiency of the proteasome activity.

Proteasome inhibition due to chronic ethanol feeding has been shown previously but is still not fully understood [26,34,35,46,47]. This study will improve the understanding of how this inhibition is caused by ethanol feeding. The strategy used is presented on Figure 1. Proteasome complexes were isolated using zonal centrifugation that employs a glycerol gradient from 10% to 40% glycerol and separates the macromolecules of high molecular masses (MM) from the smaller proteins. This isolation is gentle and protects the structure and interactions of the proteasome interacting protein complexes. Proteasome chymotrypsin-like activity was measured and the results showed a significant decrease of proteasome activity in the most active fraction (#28) from the ethanol-fed rat proteasome compared to its counterpart fraction from the control proteasome (see supplementary data 2 and previously reported surveys [26,37]).

In order to identify and quantify the PIPs in the proteasome fractions, the most active fraction of proteasomes, assessed by proteasome ChT-L activity measurement, were analyzed by nanoLC ESI QqTOF MS/MS. A total of 322 proteins were identified in the proteasome fraction isolated from the liver of rat chronically-fed ethanol, and 495 proteins in the proteasome fraction isolated from the liver of their pair-fed control (supplementary Data 1, 3 and 4). Interestingly, all 20S proteasome subunits, including those from the immunoproteasome, as well as proteins known to associate or to be related with proteasomes [25, 29], such as proteasome activators (19S, PA28α/β and PA200 complexes), E3 ligases (ubiquitin protein ligase E3C, E3 ubiquitin-protein ligase HECTD3, S-phase kinase-associated protein 1A), elongation factor 1-alpha 1, deubiquitinating enzymes (Usp14, UCHL5), valosin containing protein (VCP), COP9 signalosome subunits, and polyubiquitin chains, were identified. One tryptic peptide of ubiquitin, ^{43}LIFAGK(GG)QLEDGR^{54} which is indicative of K^{48}-linked polyubiquitin chains, was identified in the high MM migration zone of both electrophoresis-separated samples (Supplementary Data 5). Poly-ubiquitination involving lysine at residue 48 is the major signal for protein degradation by the multicatalytic proteasome complex [48]. Other proteins identified are not related to the proteasome. These might have been co-isolated in the proteasome high molecular mass fraction.

Differential quantitative proteomic approaches were then developed to examine possible differences in the two samples which could account for the variations in the proteolytic activities observed. The goal was to identify important varying proteins which could then be confirmed by Western blot using more biological replicates. Isotope Coded Affinity Tagging (ICAT) was chosen as a good alternative of metabolic labeling (SILAC) since no cell culture could be implemented in that case [42,43]. Using isotopic labeling, overall 51 proteins were changed by at least a two fold ratio, when comparing the ethanol to the control samples (Table 1 and Supplementary Data 6). Out of these 51 proteins, 21 proteins showed

significantly lower expression in the ethanol sample, and 30 proteins showed a significant higher amount than in the control.

Three proteins belonging to the oxoglutarate dehydrogenase complex (2-oxoglutarate dehydrogenase E1 component, dihydrolipoyllysine-residue succinyltransferase and dihydrolipoyl dehydrogenase) and three proteins belonging to the pyruvate dehydrogenase complex (dihydrolipoyllysine-residue acetyltransferase, pyruvate dehydrogenase E1 component alpha subunit, and pyruvate dehydrogenase E1 component beta subunit) were found to be significantly increased (Table 1 and Supplementary Data 6). These proteins were expected to vary in the same proportion as they belong to the same complex. This therefore validates the quantitative analytical strategy using ICAT. In addition, enzymes from the glycolysis pathway, the Krebs cycle, and a cytochrome P450 enzyme, CYPIID10, were also found to be affected (Table 1). CYP2E1 known to be significantly induced upon chronic ethanol feeding [35] was confirmed by Western blotting (Figure 2).

As shown in Table 2, 13 out of the 17 subunits of 20S proteasome could be quantified by ICAT and no significant change in the amount of these proteins was observed upon chronic ethanol feeding. Although precise, the ICAT procedure is restricted to the quantification of cysteine containing proteins and the detection of their labeled cysteine-containing peptides [42,43]. Therefore, as a concern to increase the number of proteasome subunits and PIPs quantified, we applied a complementary label-free quantitative approach, based on MS/MS spectral counting, which does not require any labeling of proteins [44]. This method was also used as a biological repeat to confirm quantitative results obtained by ICAT. Overall, the spectral counting correlated nicely with the ICAT results and could also bring additional data (Table 2 and Supplementary Data 7). Western blot analysis of 20S proteasome α subunits and β5, β5i, and β1i catalytic subunits (Figure 3A) validated MS-based results. Moreover, densitometric analysis of the proteasome subunits separated by 2D gel electrophoresis, as described previously [8,49] (results not shown), was also in agreement with the two MS-based quantification methods. Interestingly, the amounts of catalytic subunits and the ratios of immunosubunits *versus* standard subunits were not affected by ethanol treatment. Therefore, the decrease in ChT-like activity observed upon ethanol feeding could not be directly attributed to changes in proteasome catalytic subunits composition. All subunits of the 19S regulator except Rpn5 could be quantified both by ICAT and spectral counting and results indicated no significant change upon chronic ethanol feeding for most of them. Only Rpn8 and Rpn11 were found to be significantly increased and decreased, respectively, by both quantification methods. Two other major proteasome-associated deubiquitinases, Ubiquitin carboxyl-terminal hydrolase 14 (Usp14) and Ubiquitin carboxyl-terminal esterase L5 (UCHL5), the rat homolog of human UCH37 (Tables 2 and Supplementary Data 7) were also shown to be decreased upon chronic ethanol feeding. Both ICAT and spectral counting quantification methods indicated a decrease of about two-fold of Usp14 in the ethanol sample. UCHL5 was quantified by label free quantification only (Table 2). However, Western blot analyses validated that these three proteasome-associated deubiquitinases, Rpn11, Usp14, and UCHL5, were significantly decreased in the ethanol sample (Figure 3B).

Moreover, ICAT experiments indicated that proteasome activator 28 subunit beta (PA28β) and proteasome-associated protein Ecm29 were decreased by ethanol feeding by a factor ranging from 2 to 5. A 3.3 fold change for the PA28β subunit was observed by ICAT. The label free method confirmed that this protein was less abundant in the proteasomes from the liver of rat-fed ethanol (Table 2 and Supplementary Data 7). PA28α was quantified only by MS/MS spectral counting, and a 2.5 fold decrease in the ethanol sample was observed (Table 2). PA28α and PA28β subunits assemble in the PA28α/β heptameric complex formed of 3 to 4 copies of each subunit [50]. They are therefore expected to vary in the same

proportion and are both decreased in the proteasome fractions isolated from the liver of rats fed ethanol. Western blot analyses confirmed that PA28α/β complex as well as Ecm29 were significantly less abundant in the proteasomes obtained from the livers of ethanol-fed rats (Figure 3C).

PA200, another activator of 20S proteasome, could not be assessed by ICAT, but the MS/MS spectral counting quantification method suggested a 10 fold decrease of this large protein after ethanol feeding. Although we consider that the number of MS/MS obtained in the control experiment (35 MS/MS) is high enough to be relevant, this result should be confirmed by other replicates or biochemical approaches.

Interestingly, MS-based quantitative results enabled to show that Adrm1, better known as Rpn13 in yeast, or hRpn13 in human, was present in at least two different forms, because part of the protein was detected in a gel slice corresponding to its native molecular mass (40 to 50 kDa), while another part was detected in a gel migration zone corresponding to a lower molecular mass (25 to 30 kDa). As obtained both by ICAT and spectral counting quantification methods, the native form of Rpn13 was significantly decreased, by a factor of 2.5, in the ethanol-treated sample, whereas the cleaved form was increased in this sample. The MS/MS analysis detected only the N-terminal sequence of the protein in the 25 to 30 kDa gel slice (cleaved form) whereas peptides distributing all along the Rpn13 protein sequence were identified by MS/MS in the 40–50 kDa gel slice (Figure 4 and Supplementary data 8), indicating that the detected protein probably corresponds to the native subunit. In particular, two peptides belonging to the 362–407 C-terminal region of Rpn13 could be identified in the high molecular mass protein only. The C-terminal part of hRpn13 (362–407 region) is involved in UCHL5 binding while the N-terminal part permits the incorporation of the subunit into the 19S complex [51].

4- DISCUSSION

Proteasome activity decrease due to chronic ethanol ingestion leads to cell death and tissue damage [52]. Failure of the proteasome to eliminate cytoplasmic proteins leads to the accumulation of oxidized and otherwise modified proteins [34]. In the present study, proteasome regulators and proteasome interacting proteins (PIPs) were investigated because of their possible contribution to the ethanol-induced proteasome inhibition. Figure 5 summarizes the results obtained on major PIPs quantification obtained by our proteomic strategy. Proteasome and its associated proteins were isolated using multiple centrifugations and a final zonal centrifugation on a glycerol gradient, which preserves the complexes PIPs. The purified proteasome fraction contained abundant PIPs that were absent in the fraction of proteasomes purified chromatographically in a previous study [41] because elevated salt concentrations used in the ion exchange columns dissociated the proteasome from its interacting proteins during the isolation.

After proteasome enrichment, two MS-based quantification methods were used in a “discovery purpose” to identify possible varying proteins. Eighty percents of the ratios obtained by ICAT exhibited a CV under 20% and therefore indicated tendencies in protein ratios with a relatively good uncertainty level. The varying proteins identified that we considered as important potential regulators of proteasome activity and/or structure were then validated by Western blotting using three different biological replicates.

Our results show for the first time that ethanol feeding affects the composition of proteasome complexes by decreasing the levels of proteasome associated proteins reported to greatly influence proteasome activity. 19S, PA28, and PA200, represent an important group of regulatory complexes involved in proteasome activity regulation. PA28α and β

subunits were significantly decreased in the proteasome most active fraction from liver of ethanol-fed rats. No difference could be observed in the cytosolic extracts before proteasome complexes isolation by glycerol gradient centrifugation (Western blot analysis, result not shown), which indicates no change in the expression of PA28α and PA28β upon chronic ethanol ingestion. It is therefore likely that the decrease in the level of PA28 subunits found in the enriched proteasome fraction from ethanol-fed rat livers is the result of a decrease of the binding of the regulatory complex PA28α/β to the 20S core particle. Crystal structure of yeast proteasome in complex with PA28 [53] revealed that the PA28 C-terminal tail inserts into pockets between the proteasomal α subunits, which provoke opening of the gate to the catalytic chamber and enhances proteasome activity. Interestingly, Ecm29, a PIP known to stabilize 20S proteasome interaction with the 19S regulatory complexes, was found to be decreased after ethanol feeding. Ecm29 tethers the proteasome core particle to the 19S regulatory particle, and enhances the stability of the 26S proteasome [54]. Proteasomes lacking Ecm29 are prone to dissociate from the 19S regulatory complex. However, we could not quantify any significant change neither in the amount of the catalytic core α and β subunits, nor in the amount of most 19S subunits, especially the base subunits of the 19S particle (Rpt 1 to 6, Rpn1, Rpn2), which also bind directly to the α subunits of the 20S catalytic core [55]. A recent study in yeast showed that interaction between the 20S core complex and the 19S regulatory particle can be stabilized by ATP, and this nucleotide was shown to efficiently replace the “natural” Ecm29 stabilizer [12]. In the present study, ATP was added in all buffers all along the purification procedure. Unlike Rpt subunits from the 19S particle, PA28α/β subunits do not exhibit ATPase activities, and the interaction between the PA28α/β complex and the 20S proteasome might, therefore, not be stabilized by ATP. The decrease in proteasome-associated Ecm29 might, therefore, have worse consequences on PA28α/β complex binding to 20S proteasome than on 19S particle binding. The same hypothesis could be suggested to explain the decrease of PA200 regulator associated to 20S proteasome upon ethanol feeding. Moreover, oxidative stress generated by ethanol metabolism is one of the mechanisms that induces PTMs such as phosphorylation, and 4HNE adducts on proteasome subunits [47]. These PTMs might cause changes in the structure of the proteasome subunits and might induce the disruption of the binding between the 20S and its regulators, thus inhibiting the ubiquitin-proteasome function.

The PA28α/β regulator is induced by IFNγ and is implicated in immune surveillance, since it favors the production of ligands for major histocompatibility complex class I molecules [56]. A recent study reports that ethanol-induced oxidative stress affects the generation of antigenic peptides in hepatoma cells [57], and blocks the expression of PA28 and β1i (LMP2) by IFNγ [34]. The decrease of proteasome-associated PA28α/β observed in this survey might therefore explain the ethanol-induced deficiency of antigen presentation in hepatoma cells. In addition, using quantitative measurements by MS, tripeptidyl-peptidase 2 (TPPII), which co-purified with proteasomes complexes, was shown to be decreased after chronic ethanol feeding. When the proteasome activity is decreased, TPPII can compensate for epitope generation and loading of MHC peptides [58]. It can also substitute for some metabolic functions of the proteasome [59]. The results reported here suggest that TPPII decrease would amplify the ethanol-induced dramatic alterations of antigenic peptides processing.

The ubiquitination system was also found to be affected by chronic ethanol feeding. This system includes i) ubiquitin activating (E1), conjugating (E2) and ligase (E3) enzymes which role is to catalyze the ubiquitination of proteins in their way to be degraded, ii) multi-ubiquitin chains binding proteins, which act as adapters to present ubiquitinated proteins to the proteasome and iii) deubiquitinating enzymes (DUBs). Proteasome-associated DUBs activities are essential for the 26S proteasome to efficiently fulfill its intracellular function in the protein degradation process [60]. Three DUBs are reported to associate with mammalian

proteasomes, Rpn11, Usp14 (the ortholog of yeast Ubp6), and UCHL5 (also known as UCH37 in human). Usp14 and UCH37 deubiquitinating activities are complementary to the one of Rpn11, a constitutive subunit of the 19S particle [61]. They fulfill two major roles, release of C-terminus ubiquitin from the conjugate and clearance of polyubiquitin chains from their binding sites on proteasomes. The three proteasome-associated DUBs were identified and quantified in proteasome purified fractions. ICAT experiment, confirmed by MS/MS spectral counting and Western blotting, showed a significant decrease of Usp14 and Rpn11 after chronic ethanol feeding. UCHL5 was MS-quantified by the label free method only but Western blot analysis confirmed its decrease in the ethanol proteasome fraction. Its decrease within proteasome complexes might be explained by the loss of the C-terminus of Rpn13 observed after ethanol treatment. Indeed, Rpn13 permits the recruitment of UCHL5 to the proteasome through its C-terminal tail while its N-terminus enables its association with Rpn2 and Rpn10 subunits of the 19S regulator [51]. As reported in the result section, a native form of the protein, exhibiting both its C-terminal and N-terminal regions, was found significantly more abundant in the control sample, whereas a cleaved form, very probably missing its C-terminal part, was increased in the ethanol sample. This might therefore explain why UCHL5 is also decreased in the proteasome complexes purified from the livers of rats fed chronically ethanol. How Rpn13 is cleaved upon ethanol treatment, and by which structural mechanism Rpn11 and Usp14 are under expressed or under incorporated into proteasome complexes of ethanol-injured livers, is still unclear. The results reported here suggest that ethanol induces a decrease in proteasome-associated DUBs, which, subsequently, could also account for the decrease in proteasome activity, mainly by saturation of the proteasome with polyubiquitin chains [62] and by accumulation of ubiquitinated proteins that need to be degraded by the proteasome [31]. Both mechanisms, deubiquitination and protein degradation, are tightly dependent [63]. Another deubiquitinating enzyme, UCH L-1, was reported as under-expressed in brain of human alcoholics [64] or inactivated through oxidation in Alzheimer's disease brain [65]. UCH-L1 is a UCH type enzyme and does not associate with proteasome. However, a decrease of activity of this enzyme is thought to dramatically affect the proteasomal degradation of damaged proteins through depleting the pool of free ubiquitin leading to accumulation of modified proteins in Alzheimer's disease brain [65] or in ethanol-induced liver pathology [33]. Ethanol consumption increases the levels of ubiquitin and its protein conjugates in human sera [31] and induces the formation of Mallory Denk bodies [66], cytoplasmic inclusions containing large amounts of bound ubiquitin and of abnormally modified proteins [67]. The accumulation of such damaged proteins is thought to be the consequence of ethanol-induced proteasome inhibition [47].

Chronic ethanol feeding therefore alters proteasome interacting proteins and proteasome regulatory complexes binding to the proteasome.

In conclusion, the effects of chronic ethanol feeding are multiple and complex, because i) as shown previously (25,37), they affect the proteasome structure by causing changes in the PTMs of the proteasome subunits, and ii) as shown in the present study, they lower the proteasome interaction with its interacting proteins, leading to a significant decrease in proteasome activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported in part by NIH/NIAAA Grants 008116 and P50-011999 Alcohol Center Grant, Liver/Pancreas. Supported in part by grants from the “Fondation pour la Recherche Médicale” (FRM–contrat “Grands Equipements”), the Génopole Toulouse Midi-Pyrénées (programme Biologie-Santé), the Région Midi-Pyrénées and the Agence Nationale de la Recherche (ANR).

Published in part at RSA meeting: Bardag-Gorce F, Bousquet M-P, Monsarrat B, French SW. Chronic ethanol feeding affects proteasome activators in rats. Alcoholism: Clin Exp Res 31: 31A, 2007.

Abbreviations

PIPs	Proteasome Interacting Proteins
ChT-like activity	chymotrypsin-like activity
Usp14	ubiquitin C-terminal hydrolase 14
UCHL5	ubiquitin carboxyl-terminal hydrolase L5
IFNγ	Interferon γ
CYP2E1	Cytochrome P450 2E1
4-HNE	4-hydroxynonenal

5- REFERENCES

1. Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem. 1998; 67:425–479. [PubMed: 9759494]
2. Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev. 2002; 82:373–428. [PubMed: 11917093]
3. Pajonk F, McBride WH. The proteasome in cancer biology and treatment. Radiat Res. 2001; 156:447–459. [PubMed: 11604057]
4. Gaczynska M, Rock KL, Spies T, Goldberg AL. Peptidase activities of proteasomes are differentially regulated by the major histocompatibility complex-encoded genes for LMP2 and LMP7. Proc Natl Acad Sci U S A. 1994; 91:9213–9217. [PubMed: 7937744]
5. Klare N, Seeger M, Janek K, Jungblut PR, Dahlmann B. Intermediate-type 20 S proteasomes in HeLa cells: “asymmetric” subunit composition, diversity and adaptation. J Mol Biol. 2007; 373:1–10. [PubMed: 17804016]
6. Gomes AV, Zong C, Edmondson RD, Li X, et al. Mapping the murine cardiac 26S proteasome complexes. Circ Res. 2006; 99:362–371. [PubMed: 16857966]
7. Froment C, Uttenweiler-Joseph S, Bousquet-Dubouch MP, Matondo M, et al. A quantitative proteomic approach using two-dimensional gel electrophoresis and isotope-coded affinity tag labeling for studying human 20S proteasome heterogeneity. Proteomics. 2005; 5:2351–2363. [PubMed: 15887188]
8. Bousquet-Dubouch MP, Uttenweiler-Joseph S, Ducoux-Petit M, Matondo M, et al. Purification and Proteomic Analysis of 20S Proteasomes from Human Cells. Methods Mol Biol. 2008; 432:301–320. [PubMed: 18370027]
9. Yan W, Hwang D, Aebersold R. Quantitative proteomic analysis to profile dynamic changes in the spatial distribution of cellular proteins. Methods Mol Biol. 2008; 432:389–401. [PubMed: 18370032]
10. Baldin V, Militello M, Thomas Y, Doucet C, et al. A novel role for PA28gamma-proteasome in nuclear speckle organization and SR protein trafficking. Mol Biol Cell. 2008; 19:1706–1716. [PubMed: 18256291]
11. Rivett AJ, Hearn AR. Proteasome function in antigen presentation: immunoproteasome complexes, Peptide production, and interactions with viral proteins. Curr Protein Pept Sci. 2004; 5:153–161. [PubMed: 15180520]

12. Kleijnen MF, Roelofs J, Park S, Hathaway NA, et al. Stability of the proteasome can be regulated allosterically through engagement of its proteolytic active sites. Nat Struct Mol Biol. 2007; 14:1180–1188. [PubMed: 18026118]
13. Rechsteiner M, Hill CP. Mobilizing the proteolytic machine: cell biological roles of proteasome activators and inhibitors. Trends Cell Biol. 2005; 15:27–33. [PubMed: 15653075]
14. Murata S, Udono H, Tanahashi N, Hamada N, et al. Immunoproteasome assembly and antigen presentation in mice lacking both PA28alpha and PA28beta. Embo J. 2001; 20:5898–5907. [PubMed: 11689430]
15. Ustrell V, Hoffman L, Pratt G, Rechsteiner M. PA200, a nuclear proteasome activator involved in DNA repair. Embo J. 2002; 21:3516–3525. [PubMed: 12093752]
16. Cascio P, Goldberg AL. Preparation of hybrid (19S-20S-PA28) proteasome complexes and analysis of peptides generated during protein degradation. Methods Enzymol. 2005; 398:336–352. [PubMed: 16275341]
17. Schmidt M, Hanna J, Elsasser S, Finley D. Proteasome-associated proteins: regulation of a proteolytic machine. Biol Chem. 2005; 386:725–737. [PubMed: 16201867]
18. Gingras AC, Gstaiger M, Raught B, Aebersold R. Analysis of protein complexes using mass spectrometry. Nat Rev Mol Cell Biol. 2007; 8:645–654. [PubMed: 17593931]
19. Kocher T, Superti-Furga G. Mass spectrometry-based functional proteomics: from molecular machines to protein networks. Nat Methods. 2007; 4:807–815. [PubMed: 17901870]
20. Mann M. Functional and quantitative proteomics using SILAC. Nat Rev Mol Cell Biol. 2006; 7:952–958. [PubMed: 17139335]
21. Hirano Y, Murata S, Tanaka K. Large- and small-scale purification of mammalian 26S proteasomes. Methods Enzymol. 2005; 399:227–240. [PubMed: 16338359]
22. Scanlon TC, Gottlieb B, Durcan TM, Fon EA, et al. Isolation of human proteasomes and putative proteasome-interacting proteins using a novel affinity chromatography method. Exp Cell Res. 2008
23. Wang X, Chen CF, Baker PR, Chen PL, et al. Mass spectrometric characterization of the affinity-purified human 26S proteasome complex. Biochemistry. 2007; 46:3553–3565. [PubMed: 17323924]
24. Verma R, Chen S, Feldman R, Schieltz D, et al. Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. Mol Biol Cell. 2000; 11:3425–3439. [PubMed: 11029046]
25. Bousquet-Dubouch MP, Baudelet E, Guerin F, Matondo M, et al. Affinity purification strategy to capture human endogeneous proteasome complexes diversity and to identify proteasome interacting proteins. Mol Cell Proteomics. 2009
26. Bardag-Gorce F, Li J, French BA, French SW. The effect of ethanol-induced CYP2E1 on proteasome activity: the role of 4-hydroxynonenal. Exp Mol Pathol. 2005; 78:109–115. [PubMed: 15713435]
27. Shibatani T, Carlson EJ, Larabee F, McCormack AL, et al. Global organization and function of mammalian cytosolic proteasome pools: Implications for PA28 and 19S regulatory complexes. Mol Biol Cell. 2006; 17:4962–4971. [PubMed: 16987959]
28. Guerrero C, Tagwerker C, Kaiser P, Huang L. An integrated mass spectrometry-based proteomic approach: quantitative analysis of tandem affinity-purified in vivo cross-linked protein complexes (QTAX) to decipher the 26 S proteasome-interacting network. Mol Cell Proteomics. 2006; 5:366–378. [PubMed: 16284124]
29. Wang X, Huang L. Identifying dynamic interactors of protein complexes by quantitative mass spectrometry. Mol Cell Proteomics. 2008; 7:46–57. [PubMed: 17934176]
30. Schmidt F, Dahlmann B, Janek K, Kloss A, et al. Comprehensive quantitative proteome analysis of 20S proteasome subtypes from rat liver by isotope coded affinity tag and 2-D gel-based approaches. Proteomics. 2006; 6:4622–4632. [PubMed: 16858736]
31. Takagi M, Yamauchi M, Toda G, Takada K, et al. Serum ubiquitin levels in patients with alcoholic liver disease. Alcohol Clin Exp Res. 1999; 23:76S–80S. [PubMed: 10235284]

32. Fataccioli V, Andraud E, Gentil M, French SW, Rouach H. Effects of chronic ethanol administration on rat liver proteasome activities: relationship with oxidative stress. Hepatology. 1999; 29:14–20. [PubMed: 9862843]
33. Donohue TM Jr, Cederbaum AI, French SW, Barve S, et al. Role of the proteasome in ethanol-induced liver pathology. Alcohol Clin Exp Res. 2007; 31:1446–1459. [PubMed: 17760783]
34. Osna NA, Donohue TM Jr. Implication of altered proteasome function in alcoholic liver injury. World J Gastroenterol. 2007; 13:4931–4937. [PubMed: 17854134]
35. Bardag-Gorce F, Yuan QX, Li J, French BA, et al. The effect of ethanol-induced cytochrome p4502E1 on the inhibition of proteasome activity by alcohol. Biochem Biophys Res Commun. 2000; 279:23–29. [PubMed: 11112412]
36. Lu Y, Cederbaum AI. CYP2E1 and oxidative liver injury by alcohol. Free Radic Biol Med. 2008; 44:723–738. [PubMed: 18078827]
37. Donohue TM Jr, Zetterman RK, Zhang-Gouillon ZQ, French SW. Peptidase activities of the multicatalytic protease in rat liver after voluntary and intragastric ethanol administration. Hepatology. 1998; 28:486–491. [PubMed: 9696015]
38. Bulteau AL, Lundberg KC, Humphries KM, Sadek HA, et al. Oxidative modification and inactivation of the proteasome during coronary occlusion/reperfusion. J Biol Chem. 2001; 276:30057–30063. [PubMed: 11375979]
39. Bardag-Gorce F, Venkatesh R, Li J, French BA, French SW. Hyperphosphorylation of rat liver proteasome subunits: the effects of ethanol and okadaic acid are compared. Life Sci. 2004; 75:585–597. [PubMed: 15158368]
40. Li J, Nguyen V, French BA, Parlow AF, et al. Mechanism of the alcohol cyclic pattern: role of the hypothalamic-pituitary-thyroid axis. Am J Physiol Gastrointest Liver Physiol. 2000; 279:G118–125. [PubMed: 10898753]
41. French SW, Mayer RJ, Bardag-Gorce F, Ingelman-Sundberg M, et al. The ubiquitin-proteasome 26s pathway in liver cell protein turnover: effect of ethanol and drugs. Alcohol Clin Exp Res. 2001; 25:225S–229S. [PubMed: 11391075]
42. Gygi SP, Rist B, Gerber SA, Turecek F, et al. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol. 1999; 17:994–999. [PubMed: 10504701]
43. Li J, Steen H, Gygi SP. Protein profiling with cleavable isotope-coded affinity tag (cICAT) reagents: the yeast salinity stress response. Mol Cell Proteomics. 2003; 2:1198–1204. [PubMed: 14506205]
44. Liu H, Sadygov RG, Yates JR 3rd. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal Chem. 2004; 76:4193–4201. [PubMed: 15253663]
45. Bouyssie D, de Peredo AG, Mouton E, Albigot R, et al. Mascot file parsing and quantification (MFPaQ), a new software to parse, validate, and quantify proteomics data generated by ICAT and SILAC mass spectrometric analyses: application to the proteomics study of membrane proteins from primary human endothelial cells. Mol Cell Proteomics. 2007; 6:1621–1637. [PubMed: 17533220]
46. Osna NA, White RL, Krutik VM, Wang T, et al. Proteasome activation by hepatitis C core protein is reversed by ethanol-induced oxidative stress. Gastroenterology. 2008; 134:2144–2152. [PubMed: 18549882]
47. Bardag-Gorce F, French BA, Nan L, Song H, et al. CYP2E1 induced by ethanol causes oxidative stress, proteasome inhibition and cytokeratin aggresome (Mallory body-like) formation. Exp Mol Pathol. 2006; 81:191–201. [PubMed: 17034788]
48. Pickart CM. Back to the future with ubiquitin. Cell. 2004; 116:181–190. [PubMed: 14744430]
49. Claverol S, Burlet-Schiltz O, Girbal-Neuhauser E, Gairin JE, Monsarrat B. Mapping and structural dissection of human 20 S proteasome using proteomic approaches. Mol Cell Proteomics. 2002; 1:567–578. [PubMed: 12376572]
50. Zhang Z, Krutchinsky A, Endicott S, Realini C, et al. Proteasome activator 11S REG or PA28: recombinant REG alpha/REG beta hetero-oligomers are heptamers. Biochemistry. 1999; 38:5651–5658. [PubMed: 10220354]

51. Qiu XB, Ouyang SY, Li CJ, Miao S, et al. hRpn13/ADRM1/GP110 is a novel proteasome subunit that binds the deubiquitinating enzyme, UCH37. Embo J. 2006; 25:5742–5753. [PubMed: 17139257]
52. Joshi-Barve S, Barve SS, Butt W, Klein J, McClain CJ. Inhibition of proteasome function leads to NF-kappaB-independent IL-8 expression in human hepatocytes. Hepatology. 2003; 38:1178–1187. [PubMed: 14578856]
53. Forster A, Whitby FG, Hill CP. The pore of activated 20S proteasomes has an ordered 7-fold symmetric conformation. Embo J. 2003; 22:4356–4364. [PubMed: 12941688]
54. Leggett DS, Hanna J, Borodovsky A, Crosas B, et al. Multiple associated proteins regulate proteasome structure and function. Mol Cell. 2002; 10:495–507. [PubMed: 12408819]
55. Rosenzweig R, Osmulski PA, Gaczynska M, Glickman MH. The central unit within the 19S regulatory particle of the proteasome. Nat Struct Mol Biol. 2008; 15:573–580. [PubMed: 18511945]
56. Sijts A, Sun Y, Janek K, Kral S, et al. The role of the proteasome activator PA28 in MHC class I antigen processing. Mol Immunol. 2002; 39:165–169. [PubMed: 12200048]
57. Osna NA, White RL, Todero S, McVicker BL, et al. Ethanol-induced oxidative stress suppresses generation of peptides for antigen presentation by hepatoma cells. Hepatology. 2007; 45:53–61. [PubMed: 17187415]
58. Wang EW, Kessler BM, Borodovsky A, Cravatt BF, et al. Integration of the ubiquitin-proteasome pathway with a cytosolic oligopeptidase activity. Proc Natl Acad Sci U S A. 2000; 97:9990–9995. [PubMed: 10954757]
59. Geier E, Pfeifer G, Wilm M, Lucchiari-Hartz M, et al. A giant protease with potential to substitute for some functions of the proteasome. Science. 1999; 283:978–981. [PubMed: 9974389]
60. Wing SS. Deubiquitinating enzymes--the importance of driving in reverse along the ubiquitin-proteasome pathway. Int J Biochem Cell Biol. 2003; 35:590–605. [PubMed: 12672452]
61. Koulich E, Li X, DeMartino GN. Relative structural and functional roles of multiple deubiquitylating proteins associated with mammalian 26S proteasome. Mol Biol Cell. 2008; 19:1072–1082. [PubMed: 18162577]
62. Kornitzer D, Ciechanover A. Modes of regulation of ubiquitin-mediated protein degradation. J Cell Physiol. 2000; 182:1–11. [PubMed: 10567911]
63. Amerik AY, Hochstrasser M. Mechanism and function of deubiquitinating enzymes. Biochim Biophys Acta. 2004; 1695:189–207. [PubMed: 15571815]
64. Alexander-Kaufman K, James G, Sheedy D, Harper C, Matsumoto I. Differential protein expression in the prefrontal white matter of human alcoholics: a proteomics study. Mol Psychiatry. 2006; 11:56–65. [PubMed: 16172612]
65. Castegna A, Aksenov M, Aksenova M, Thongboonkerd V, et al. Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part I: creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1. Free Radic Biol Med. 2002; 33:562–571. [PubMed: 12160938]
66. Zhang-Gouillon ZQ, Yuan QX, Hu B, Marceau N, et al. Mallory body formation by ethanol feeding in drug-primed mice. Hepatology. 1998; 27:116–122. [PubMed: 9425926]
67. Ohta M, Marceau N, Perry G, Manetto V, et al. Ubiquitin is present on the cytokeratin intermediate filaments and Mallory bodies of hepatocytes. Lab Invest. 1988; 59:848–856. [PubMed: 2462130]

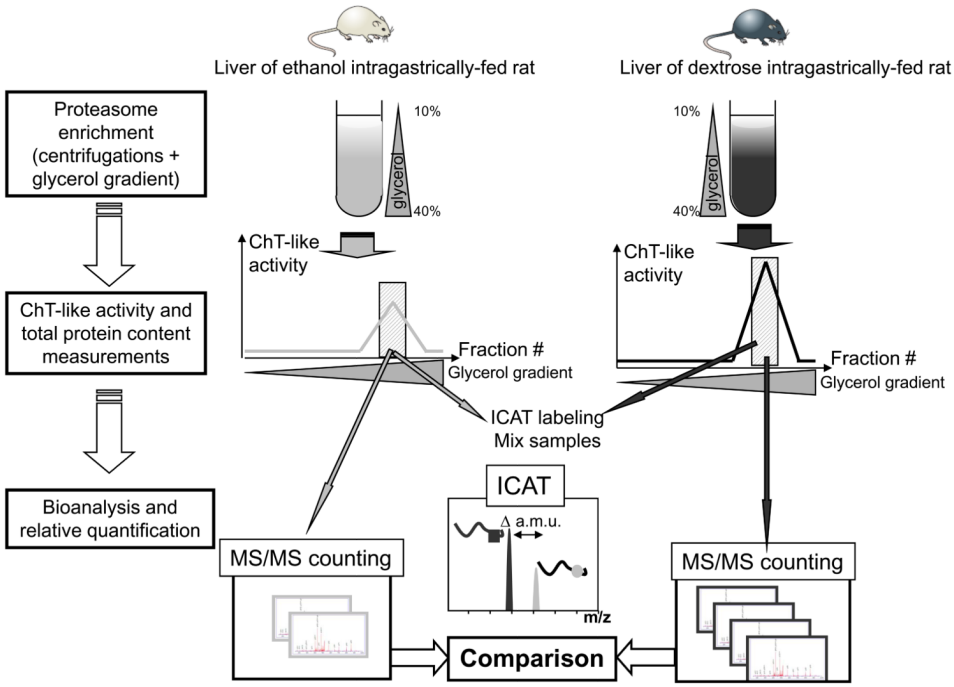


Figure 1.

Strategy to identify and differentially quantify proteasomes and PIPs from the most active proteasome fraction isolated from the liver of rats fed ethanol (fraction #28) and the liver of their pair-fed controls (fraction #28).

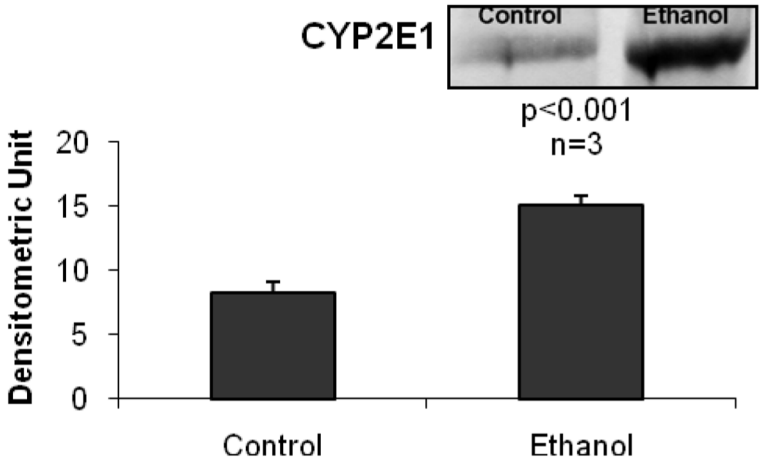


Figure 2.
Western blot analysis of cytochrome P450 2E1 (CYP2E1) in the most active proteasome fraction isolated from the liver of rats fed ethanol and the liver of their pair-fed controls. CYP2E1 is known to be significantly induced to metabolize ethanol in chronic alcoholic liver disease. Note that CYP2E1 was up regulated in the proteasome fraction in the liver of rats fed chronically ethanol.

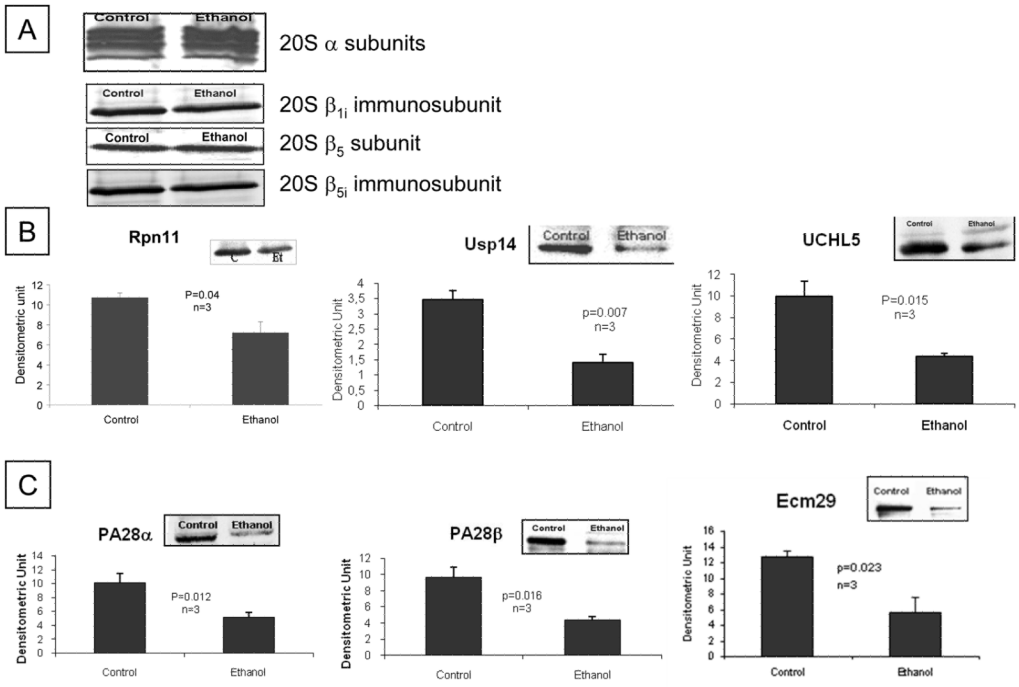


Figure 3.

Western blot analyses of 20S proteasome α subunits and β1i, β5 andβ5i catalytic subunits (A), three deubiquitinases known to associate to proteasomes, Rpn11, Usp14 and UCHL5 (B) and PA28α, PA28β and Ecm29 (C) in the most active proteasome fraction isolated from the liver of rats fed ethanol and the liver of their pair-fed controls. The same quantity of total proteins (5 to 10 μg) of both sample (ethanol-treated and control) was loaded on the gel. Note that 20S proteasome subunits were not changed upon ethanol treatment, contrary to the three DUBs as well as PA28α/β activator and Ecm29 which were significantly down regulated by ethanol feeding.

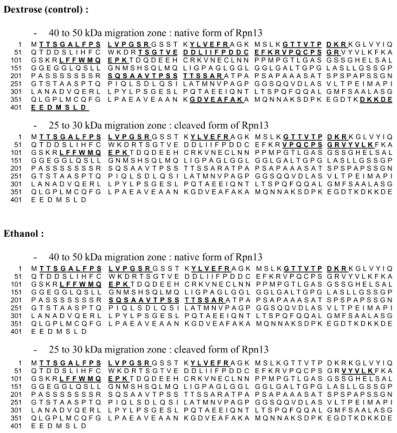
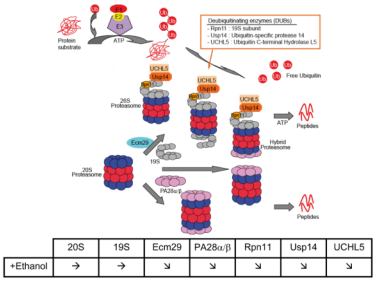


Figure 4.

Peptides from the Rpn13 protein (Q9JMB5) sequence identified by LC MS/MS in the 40 to 50 kDa and the 25 to 30 kDa migration zones of the SDS-PAGE fractionated purified proteasome complexes from the livers of rats fed chronically ethanol and their pair-fed controls. Identified peptides are underlined in the Rpn13 sequence.



	20S	19S	Ecm29	PA28α/β	Rpn11	Usp14	UCHL5
+Ethanol	→	→	↘	↘	↘	↘	↘

Figure 5.
Schematic illustration of proteasome interacting proteins changed by chronic ethanol feeding

Proteomics. Author manuscript; available in PMC 2010 July 1.

Table 1

ICAT Proteomic Analysis of zonal centrifugation-purified proteasomes from the liver of rats fed-ethanol (E) and from the liver of pair-fed controls (C). Proteins found to vary by a factor of 2 or more are reported.

Description	Accession #	ICAT quantification			
		# quantifying peptides	Ratio[a] E/C	CV (%)[b]	CV (%)[c]
Up-regulated proteins					
C9 protein	Q5BKC4	2	10.1	26.1	10.3
Alpha-1-macroglobulin	Q63041	2	9.8	8.5	20.9
Dihydrolipoyllysine-residue acetyltransferase	P08461[d]	1	7.9	/	/
Elongation factor 1-beta (EF-1-beta)	O70251	2	7.3	6.9	5.9
2-oxoglutarate dehydrogenase E1	Q5XI78[e]	9	7.3	27.7	21.0
Dihydrolipoyl dehydrogenase	Q6P6R2[e]	3	6.5	6.9	6.7
Dihydrolipoyllysine-residue succinyltransferase	Q01205[e]	2	6.1	10.2	16.8
Vitronectin	Q3KR94	2	5.2	11.8	21.2
Oxoglutarate dehydrogenase-like	Q8TAN9	3	5.0	11.6	11.2
Peptidyl-prolyl cis-trans isomerase B precursor	P24368	2	5.0	0.3	17.7
Elongation factor 1-gamma (eEF-1B gamma)	Q68FR6	3	4.8	13.2	/
Complement inhibitory factor H	Q91YB6	4	4.6	16.6	11.7
Eukaryotic translation elongation factor 1 delta	Q68FR9	1	3.8	/	/
Hemopexin precursor	P20059	1	3.3	/	/
Ribosomal protein S8	P62243	3	3.3	22	23.6
Eukaryotic translation initiation factor 3 subunit 9	Q8JZQ9	2	3.2	7.3	27.4
Ribosome-binding protein 1	Q99PL5	6	3.0	21.6	27
Vesicle amine transport protein 1	Q5BKZ7	1	2.9	/	/
Ribosomal protein S2	O55215	2	2.9	14.4	3.2
Dehydrogenase E1 and transketolase domain containing 1	Q4KLP0	9	2.8	18.4	26.1
Fructose-bisphosphate aldolase B	P00884	1	2.8	/	12
Retinol dehydrogenase 3	P50169	3	2.8	22.1	12.6
Pyruvate dehydrogenase E1	P35486[d]	2	2.6	28.3	5.2

Description	Accession #	ICAT quantification			
		# quantifying peptides	Ratio[a] E/C	CV (%)[b]	CV (%)[c]
Peroxisomal trans-2-enoyl-CoA reductase	Q9WVK3	2	2.6	15.7	6.6
Tubulin beta-3 chain	Q9ERD7	2	2.2	14.2	21
Cytochrome P450 2D10 (CYPIID10)	P12939	4	2.1	31.0	15.4
P55	Q6AYT3	2	2.1	19.6	9.9
Ribosomal protein S27a	Q6PED0	1	2	/	17.8
Pyruvate dehydrogenase E1 component beta subunit	P49432[d]	2	2	23.3	12.1
Eukaryotic translation initiation factor 3 subunit 12 (eIF-3 p25)	Q9DBZ5	2	2	6.3	16
Down-regulated proteins					
Ubiquitin carboxyl-terminal hydrolase 14	Q5U2N2	4	0.6	7.7	19.7
Lysyl-tRNA synthetase	Q5XIM7	2	0.6	1.1	12.2
Dehydrogenase/reductase SDR family member 4	Q8VID1	2	0.6	12.6	11.3
JTV1	Q32PX2	3	0.6	16.5	14.2
Formimidoyltransferase-cyclodeaminase	O88618	4	0.6	24	7
40S ribosomal protein S3	P62909	3	0.5	21.8	7.6
Ribose-phosphate pyrophosphokinase I	P60892	3	0.5	2.5	15.1
Ribose-phosphate pyrophosphokinase II	P09330	2	0.5	3.1	17.8
Glutaryl-Coenzyme A	Q6P8N6	2	0.4	5.2	/
Methyltransferase-like protein 7B [Precursor]	Q562C4	4	0.4	11.5	24.8
Peroxisomal bifunctional enzyme (PBFE)	P07896	4	0.4	25.6	17.7
LRRGT00050	Q6TXE9	7	0.4	10.6	29.5
Proteasome activator complex subunit 2 (PA28beta)	Q63798	2	0.3	7.6	1.9
40S ribosomal protein SA (p40)	P38983	1	0.4	/	21.7
Phosphoribosyl pyrophosphate synthetase-associated protein 1	Q6AYZ7	1	0.3	/	/
Tripeptidyl-peptidase 2 (TPP-II)	Q64560	2	0.3	8.1	5.8
Agmat protein (Fragment)	Q5BK25	1	0.2	/	/
40S ribosomal protein S6	P62755	2	0.2	12.4	27.1
Aldehyde dehydrogenase 16 family. member A1	Q8VD78	2	0.2	7.2	24.6
6-phosphofructokinase. liver type	P30835	1	0.2	/	33.8

Description	Accession #	ICAT quantification			
		# quantifying peptides	Ratio[a] E/C	CV (%)[b]	CV (%)[c]
Proteasome-associated protein ECM29 homolog	Q6PDI5	1	0.1	/	24.4

Equal amounts of the two different protein fractions to be quantified, zonal centrifugation purified proteasomes from the liver of rats fed ethanol and zonal centrifugation purified proteasomes from the liver of pair fed control, were reduced and labelled with ^{13}C - and ^{12}C -cleavable ICAT™ (cICAT™) reagents (Applied Biosystems), respectively, as described in the experimental section. NanoLC-ESI-QqTOF MS and MS/MS enabled proteins quantification and identification, respectively. More information on the quantification process using MFPaQ is provided in the experimental section.

a : Normalized ratio (normalization factor = 1.10).

b : coefficient of variation on quantifying peptides

c : coefficient of variation on technical replicates

d : proteins belonging to the pyruvate dehydrogenase complex

e : proteins belonging to the oxoglutarate dehydrogenase complex

Table 2

ICAT and MS/MS spectral counting quantification of zonal centrifugation-purified proteasomes from the liver of rats fed-ethanol (E) and from the liver of pair-fed controls (C): Proteasome subunits, proteasome regulators, proteasome-associated deubiquitinases and proteins from the UPP

Name	Accession #	ICAT quantification				Spectral counting quantification		
		# quantifying peptides	Ratio[a] E/C	CV (%)[b]	CV (%)[c]	# MSMS		Ratio[d] E/C
						Control	Ethanol	
20S Proteasome subunits								
α1	P60901	6	0.8	11.2	14.0	45	29	0.8
α2	P17220	3	1.2	5.6	5.9	40	28	0.8
α3	P21670	5	0.8	23.7	11.6	22	24	1.3
α4	Q9Z2U0	1	1.0	/	6.8	41	33	1.0
α5	P34064	6	0.9	7.0	2.2	34	26	0.9
α6	P18420	5	1.1	13.7	5.1	34	37	1.3
α7	P18422	1	0.9	/	0.0	27	30	1.3
β1	P28073	3	0.8	17.1	0.8	14	11	0.9
β2	Q9JHW0	1	1.0	/	0.7	20	14	0.8
β3	P40112	2	1.1	2.4	2.7	30	30	1.2
β4	P40307	2	1.0	5.6	6.9	28	20	0.9
β5	P28075	NQ	NQ	/	/	13	10	0.9
β6	P18421	3	1.0	23.4	6.9	31	24	0.9
β7	P34067	1	1.2	/	9.8	20	18	1.1
β1i	P28077	NQ	NQ	/	/	18	14	0.9
β2i	Q4KM35	NQ	NQ	/	/	15	11	0.9
β5i	P28064	NQ	NQ	/	/	38	24	0.8
19S regulator subunits								
Rpn1	Q8VDM 4	7	0.9	15.8	2.1	112	83	0.9
Rpn2	O88761	9	0.9	13.7	2.6	106	94	1.1
Rpn3	Q5U2S7	2	0.9	18.3	15.9	48	42	1.0
Rpn5	Q5XIC6	NQ	NQ	/	/	8	8	1.2
Rpn6	Q8BG32	2	1.1	18.2	1.8	44	43	1.2

Name	Accession #	ICAT quantification				Spectral counting quantification		
		# quantifying peptides	Ratio[a] E/C	CV (%)[b]	CV (%)[c]	# MSMS		Ratio[d] E/C
						Control	Ethanol	
Rpn7	Q99JI4	2	0.8	9.6	1.2	52	41	0.9
Rpn8	P26516	2	1.5	30.5	10.3	20	23	1.4
Rpn9	Q9WVJ2	2	0.9	6.4	8.0	56	43	0.9
Rpn10	O35226	7	0.8	18.4	6.8	14	15	1.3
Rpn11	O35593	1	0.7	/	3.7	58	26	0.5
Rpn12	Q9CX56	5	0.8	29.0	10.7	29	28	1.2
Adrm1 (Rpn13) native form (40 to 50 kDa) split form (25 to 30 kDa)	Q9JMB5	11 7 4	1.3 0.4 2.6	90.8 21.1 9.8	4.6 13.7 3.7	21 15 6	13 5 8	0.7 0.4 1.6
Rpt1	Q63347	7	1.1	17.3	0.2	47	36	0.9
Rpt2	P62193	4	0.9	14.2	0.2	65	73	1.3
Rpt3	Q63570	4	1.0	9.9	4.6	38	33	1.0
Rpt4	P62334	9	0.9	9.4	4.4	59	49	1.0
Rpt5	Q6P6U2	2	0.9	16.3	13.0	63	62	1.2
Rpt6	P62198	7	1.1	11.7	5.6	60	63	1.3
Other regulators								
PA28α	Q63797	NQ	NQ	/	/	8	3[c]	0.4
PA28β	Q63798	2	0.3	7.6	1.9	9	NI	<0.1
PA200	Q5SSW2	NQ	NQ	/	/	35	4[e]	0.1
Proteasome-associated Deubiquitinases								
Usp14	Q5U2N2	4	0.6	7.7	19.7	36	22	0.7
UCHL5	Q5HZY3	NQ	NQ	/	/	19	11	0.7
Other proteins from the UPP found to be varied by ICAT and MS/MS spectral counting								
TPP-II	Q64560	2[f]	0.3	8.1	5.8	6[d]	NI	<0.1
Ecm29	Q6PDI5	1[f]	0.1	/	24.4	60	8	0.1

Proteins listed in the table were purified as described in the experimental section and identified by nanoLC-ESI-QqTOF MS/MS analysis and database searching. Criteria for acceptation of proteins identification are described in the experimental part. More information on the quantification process using ICAT and MS/MS spectral counting is also provided in the experimental section. ICAT and MS/MS spectral counting quantification were obtained by independent experiments.

NQ : not quantified; NI : not identified; E: Ethanol; C: Control

[a] : Normalized ratio (normalization factor = 1.10)

[b] : coefficient of variation on quantifying peptides

[c] : coefficient of variation on technical replicates

[d] : Normalized ratio (normalization factor = 0.84)

[e] : Top ranking peptide with a Mascot expectation value of less than 0.01

[f] : Top ranking peptide with a Mascot expectation value of less than 0.05