

**Protein Synthesis and Degradation:  
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## Non-Repair Pathways for Minimizing Protein Isoaspartyl Damage in the Yeast *Saccharomyces cerevisiae*\*

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\*Running title: *Processing of L-isoaspartyl-containing proteins in yeast*

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**Background:** Isoaspartyl damage is a common spontaneous protein modification repaired by the protein isoaspartyl methyltransferase (PCMT).

**Results:** Although *Saccharomyces cerevisiae* is one of the few organisms that lack PCMT, isoaspartyl-damaged polypeptides slowly accumulate except in extracts incubated with EDTA.

**Conclusions:** Isoaspartyl-containing polypeptides are degraded by a metalloprotease.

**Significance:** The mechanism of isoaspartyl control in *S. cerevisiae* may also be used in higher organisms.

### SUMMARY

The spontaneous degradation of asparaginyl and aspartyl residues to isoaspartyl residues is a common type of protein damage in aging organisms. Although the protein L-isoaspartyl (D-aspartyl) O-methyltransferase (PCMT, EC 2.1.1.77) can initiate the repair of L-isoaspartyl residues to L-aspartyl residues in most organisms, no gene homolog or enzymatic activity is present in the budding yeast *Saccharomyces cerevisiae*. Therefore, we used

biochemical approaches to elucidate how proteins containing isoaspartyl residues are metabolized in this organism. Surprisingly, the level of isoaspartyl residues in yeast proteins (50-300 pmol isoaspartyl residues/mg extract protein) is comparable to organisms with PCMT, suggesting a novel regulatory pathway. Interfering with common protein quality control mechanisms by mutating and inhibiting the proteasomal and autophagic pathways *in vivo* did not increase isoaspartyl residue levels compared to wild type or uninhibited cells. However, the inhibition of metalloproteases in *in vitro* aging experiments by EDTA resulted in an approximately three-fold increase in the level of isoaspartyl-containing peptides. Characterization by mass spectrometry of these peptides identified several proteins involved in metabolism as targets of isoaspartyl damage. Further analysis of these peptides revealed that many have an N-terminal isoaspartyl site and originate from proteins with short half-lives. These results suggest that one or more metalloproteases participate in limiting isoaspartyl formation by robust proteolysis.

Protein function can be impaired by spontaneous posttranslational modifications as a result of exposure to oxygen and nitrogen reactive species, sugars, aldehydes, and even water (1). Because many of these modifications irreversibly lead to nonfunctional and aggregated proteins, biological pathways can be disrupted and result in disease (2-5). To prevent the accumulation of modified proteins, organisms have developed repair mechanisms, such as the reductase and isomerase repair enzymes associated with methionine sulfoxide- and *cis*-proline-containing damaged proteins, respectively (1,4,6). We have been particularly interested in the widespread isomerization damage to aspartyl and asparaginyl residues that is also associated with a repair enzyme (2,6,7).

Isoaspartyl damaged polypeptides result from the spontaneous deamidation and isomerization of asparaginyl and aspartyl amino acids, respectively, and is one of the most common types of protein damage under physiological conditions (8). Asparaginyl and aspartyl residues are hot spots for damage due to the favorable nucleophilic attack on the side chain carbonyl group by the peptide-backbone nitrogen atom of the following residue (Fig. 1). This reaction leads to the formation of an unstable succinimidyl intermediate that nonenzymatically hydrolyzes into either L-aspartyl or, to a greater extent, the abnormal L-isoaspartyl residue (8-11). The latter kinks the protein by rerouting the polypeptide backbone through the  $\beta$ -carbonyl instead of the  $\alpha$ -carbonyl moiety (12). Altered proteins containing isoaspartyl residues have been linked to changes in the pharmaceutical efficacy of antibodies (13), p53 tumor suppressor regulation (14), autoimmunity disorders (15-19), Alzheimer's disease (20,21), and cataract formation (22-24). Because no known protease cleaves isoaspartyl linkages, aging cells can accumulate these dysfunctional proteins to unfavorable levels if left unrepaired or undegraded (25).

The protein repair L-isoaspartyl (D-aspartyl) *O*-methyltransferase (PCMT, EC 2.1.1.77) prevents the accumulation of isoaspartyl-damaged polypeptides in cells. Using the cofactor *S*-adenosylmethionine, PCMT methylates abnormal isoaspartyl residues and initiates a process that results in the formation of normal L-aspartyl residues as the major product (Fig. 1, (26,27)).

This repair of isoaspartyl-containing polypeptides by PCMT is important for a diverse array of organisms. In *Escherichia coli*, strains deficient in PCMT are more susceptible to oxidative stress, high salt, and elevated temperature in stationary phase (28,29). Overexpressing PCMT in *E. coli* (30) or *Drosophila melanogaster* (31) results in an increase in longevity under stress conditions. Similarly in *Caenorhabditis elegans*, PCMT mutant L1 larvae have decreased survival in minimal media (32) and developmental defects with oxidative stress (33), while PCMT overexpressing animals upregulate Daf-16-dependent stress response genes and have increased heat stress survival (34,35). Finally, and most striking, homozygous PCMT knockout mice rapidly accumulate isoaspartyl-damaged polypeptides in the brain, heart, testis, and erythrocyte cells, and have fatal tonic-clonic seizures at approximately 42 days of age (7,36-40). Despite being well characterized in the organisms described above, and less thoroughly in other animals, fungi, plants, bacteria, and archaea, PCMT appears to be absent in a few organisms, including the budding yeast *Saccharomyces cerevisiae* (Fig. 2).

Although PCMT is important for the repair of isoaspartyl-damaged polypeptides, there is also evidence supporting a compensatory proteolytic degradation pathway. In *C. elegans*, proteins of aged dauer stage animals have increased isoaspartyl content at normal and elevated temperatures (41). However, when nonviable animals are removed and only living nematodes analyzed, no difference in isoaspartyl damage is observed, suggesting an alternative regulation pathway is keeping damage low in mutant strains. When the isoaspartyl-containing species were characterized, the majority of damage was found in peptides, rather than proteins (41). Similarly in mice, PCMT knockout animals exhibit an increase in isoaspartyl damage in urinary peptides over time (36,42). These data suggests one or more proteolytic pathways can participate in the degradation and excretion of isoaspartyl-containing species in parallel with the methylation repair pathway. Thus far, only the isoaspartyl dipeptidase (IadA) in bacteria (43) and the isoaspartyl aminopeptidase (IaaA) in bacteria, cyanobacteria, plants, and animals (44-47), have been associated with the proteolytic degradation of

isoaspartyl-containing di- and tri-peptides. Although further evidence is lacking from other organisms, data from *C. elegans* and mice suggest an isoaspartyl-specific protease targeting large polypeptides and proteins, and not simply short peptides, may also be responsible for removing isoaspartyl damage.

Here we show that even though a PCMT homolog or equivalent activity is not present in *S. cerevisiae*, isoaspartyl damage is low in this organism. We show that the proteasome and autophagy pathways are not significantly involved in limiting damage. In this work, we characterize the isoaspartyl-containing polypeptides found in *S. cerevisiae* and provide evidence for a metalloprotease that limits isoaspartyl damage.

## EXPERIMENTAL PROCEDURES

*Strains and Growth Conditions*—The *S. cerevisiae* strains used in this study are listed in Table 1. Overnight cultures (5 ml) were grown in YPD (BD Difco, catalog # 242810, 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) dextrose) or CSM (complete synthetic media, 0.07% (w/v) CSM powder (MP Biomedicals, catalog # 114500012), 0.17% (w/v) yeast nitrogen base without amino acids or ammonium sulfate, 0.5% (w/v) ammonium sulfate, 2% (w/v) dextrose) and used to inoculate 30 ml flasks of YPD or CSM. After culturing cells at 30 °C in a 250 rpm shaker, the cultures were transferred to 50 ml polypropylene tubes and centrifuged at 2,000 x g for 5 min at 4 °C. The supernatant was removed and the cell pellet resuspended in phosphate buffered saline (PBS, 13.7 mM NaCl, 0.3 mM KCl, 0.4 mM Na<sub>2</sub>HPO<sub>4</sub>, and 0.1 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4), and the cells were centrifuged at 2,000 x g for 5 min at 4 °C. After repeating the wash three times, the cells were transferred to 1.5 ml polypropylene microcentrifuge tubes and centrifuged at 2,000 x g for 5 min at 4 °C. The supernatant was aspirated and the pellet stored at -80 °C until lysis.

*Proteasome and Autophagy Inhibition*—To determine the relationship between protein isoaspartyl content and the proteasome and autophagy pathways, *pdr5Δ* yeast overnights in 5 ml of YPD were used to inoculate 30 ml flasks of YPD to a starting OD600 of 0.1. After culturing for 8 or 24 h, cells were washed three times in

PBS as described above and resuspended in 30 ml of water with 20 μM MG132 (Calbiochem, catalog # 80053-196) and/or 5 mM 3-methyladenine (Sigma, catalog # M9281). The cultures were incubated for an additional 24 h at 30 °C and 250 rpm before the cultures were centrifuged and the pellet washed three times with PBS. Cell pellets were stored at -80 °C until lysis. To analyze the effect of combining proteasome mutations with chemical inhibition, 5 ml overnight cultures of *PUP1PRE3pdr5Δ* and *pup1pre3pdr5Δ* cells (obtained as a gift from the William Tansey laboratory at Vanderbilt University)(48) were prepared in YPAD (YPD with 0.002% adenine hemisulfate (Sigma, catalog # A9126)) and used to inoculate 30 ml of YPAD with or without 50 μM MG132. The cells were cultured for 8h (to 0.6-0.8 OD600) or 24 h (to >5 OD600) before centrifugation at 2,000 x g for 5 min at 4 °C. After washing three times with PBS, the pellets were stored at -80 °C until lysis. Finally, to test the affect of other autophagy inhibitors, BY4741 or BY4742 overnights were used to inoculate 5 ml YPD cultures supplemented with 10 nM or 1 μM concanamycin A (Sigma, catalog # 80890-47-7), or 10 mM, 100 mM, and 200 mM ammonium chloride, to a starting OD600 of 0.1. Cells not incubated with the inhibitors served as the control. After culturing for 7 d at 30 °C and 250 rpm, the cells were spun down and washed as described above before the pellets were stored at -80 °C until lysis.

*Cytosolic Protein Preparation*—Yeast pellets were resuspended in 500 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 0.7 mM phenylmethylsulfonyl fluoride (PMSF) and one Roche Complete Protease Inhibitor tablet with EDTA (catalog # 11836145001) per 25 ml of buffer) and approximately 1 ml of 0.5 mm diameter glass beads (BioSpec, catalog # 11079105). The cells were vortexed at room temperature for 1 min on setting 10 (Fisher Scientific analog vortex mixer, catalog # 02-215-365) and subsequently placed on ice for 1 min. After repeating this cycle seven times, the extract was removed from the glass beads and centrifuged at 12,000 x g for 10 min at 4 °C. The supernatant was removed and centrifuged at 18,000 x g for 10 min at 4 °C. Finally, the supernatant was transferred to a new microcentrifuge tube and stored at -20 °C until analysis.

Protein extracts were also prepared of OP50 *E. coli*, N2 *C. elegans*, and PCMT-deficient mice. Briefly, overnight cultures of *E. coli* grown in Luria Broth (BD Difco, catalog # 241410) were centrifuged at 5,000 x g for 5 min at 4 °C, and the pellet was washed five times with PBS as described above. *C. elegans* samples were obtained by washing plates of mixed populations of worms with M9 media (3 g/l KH<sub>2</sub>PO<sub>4</sub>, 6 g/l Na<sub>2</sub>HPO<sub>4</sub>, 5 g/l NaCl, 1 ml/l 1 M MgSO<sub>4</sub>), performing a sucrose float to separate the worms from *E. coli*, and finally washing the worms 3 times with M9. Both the *E. coli* and *C. elegans* pellets were resuspended in the lysis buffer described above, freeze-thawed with liquid nitrogen, and lysed for a total of 5 min with a Sonifier cell disrupter W-350 (Smith-Kline Corp., 5 s sonication and 25 s on ice, continuous, 50% output, setting 4). The extracts were centrifuged at 20,000 x g for 10 min at 4 °C, and the supernatant was stored at -20 °C until analysis. Protein extracts from mouse brains deficient of PCMT were obtained as previously described (36).

Protein concentrations were determined by Lowry assays after precipitation with 10% trichloroacetic acid. The assays were performed in duplicate with 2-5 µl of extract and bovine serum albumin served as the standard.

**Quantification of Isoaspartyl Protein Damage**—We used the human recombinant PCMT protein as an analytical reagent to quantitate isoaspartyl residue damage in yeast proteins and peptides. This enzyme has a high activity with peptides and proteins containing L-isoaspartyl residues and a lower activity for ones containing D-aspartyl residues (9). In a volume of 100 µl, 4-130 µg of cytosolic *S. cerevisiae* extract was incubated in 135 mM Bis-Tris-HCl, pH 6.4, 5 µg of recombinant human PCMT (purified from *E. coli* as described in Dai *et al.* (42), approximate concentration of 1 mg/ml, specific activity of 9,682 pmol of methyl esters formed on ovalbumin at 37 °C /min /mg protein), and 10 µM [<sup>14</sup>C]AdoMet (*S*-adenosyl-L-[methyl-<sup>14</sup>C]methionine; PerkinElmer Life Sciences, 47 mCi/mmol, 20 µCi/ml in 10 mM H<sub>2</sub>SO<sub>4</sub>: ethanol (9:1 v/v)) for 2 h at 37 °C. After the incubation, 10 µl of 2 M sodium hydroxide was added to the reaction, and 100 µl of sample was immediately transferred to a 9 x 2.5-cm piece of thick filter paper (Bio-Rad, catalog # 1650962) suspended

above 5 ml of scintillation reagent (Safety Solve, Research Products International, catalog # 111177) in a sealed 20 ml scintillation vial at room temperature (Research Products International, catalog #121000). After a 2 h incubation, the filter paper was removed and each individual sample counted 3 times for 3 min each in a Beckman LS6500 scintillation counter to determine the average radioactivity. Each sample was assayed in duplicate, and the reported data represents an average of the two radioactive measurements. Background radioactivity was determined by incubating recombinant human PCMT with lysis buffer, 135 mM Bis-Tris-HCl buffer, and 10 µM [<sup>14</sup>C]AdoMet as described above, and was subtracted from the value obtained in experimental samples.

**Characterization of Isoaspartyl-Damaged *S. cerevisiae* proteins by SDS-PAGE**—In a volume of 30 µl, 5 µg of protein extract was incubated with 1 µg of recombinant human PCMT, 0.3 µM [<sup>3</sup>H]AdoMet (*S*-adenosyl-L-[methyl-<sup>3</sup>H]methionine; PerkinElmer Life Sciences, 75-85 Ci/mmol, 0.55 mCi/ml in 10 mM H<sub>2</sub>SO<sub>4</sub>: ethanol (9:1 v/v)) and 80 mM Bis-Tris-HCl buffer, pH 6.4, for 2 h at 37 °C. The reaction was quenched by adding 5 µl of SDS-PAGE sample buffer (250 mM Tris-HCl, pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, and 0.05% (w/v) bromophenol blue). The samples were heated at 100 °C for 3 min and separated on a 4-12% Bis-Tris NuPAGE gel (Life Technologies, catalog # NP0323BOX) with NuPAGE MES SDS running buffer (Life Technologies, catalog # NP0002) at 200V for 1 h. The gels were stained with Coomassie (0.1% (w/v) Brilliant Blue R-250, 10% (v/v) glacial acetic acid, and 50% (v/v) methanol) for 1 h, destained with 10% (v/v) acetic acid and 5% (v/v) methanol, and the protein bands were imaged using an Alpha Imager 2200 (Alpha Innotech Corp). To visualize the location of isoaspartyl-damaged proteins by fluorography, the gels were treated with EN<sup>3</sup>HANCE (PerkinElmer, catalog # 6NE9701) for 1 h, incubated in water for 30 min, and dried before the gels were exposed to film (Denville Scientific, 8-in x 10-in Hyblot Cl) for 5-7 d at -80 °C.

**In Vitro Aging of *S. cerevisiae* Extracts**—Extracts of BY4742 yeast cultured for 1 and 7 d in YPD were prepared as described above. Yeast extract (25 µg protein) was diluted with 50 mM

Tris-HCl, pH 7.5 to a total volume of 10  $\mu$ l. To this, 40  $\mu$ l of one of the following buffers was added: (1) 50 mM Tris-HCl, pH 7.5, (2) 50 mM Tris-HCl, pH 7.5, and 2 mM sodium EDTA, pH 7.5 (3) 50 mM Tris-HCl, pH 7.5, and 0.7 mM PMSF, (4) 50 mM Tris-HCl, pH 7.5, and 2  $\mu$ g/ml aprotinin from bovine lung (Sigma, catalog # A1153, 10.7 TIU/mg solid), (5) 50 mM Tris-HCl, pH 7.5, and 21  $\mu$ M leupeptin hemisulfate salt (Sigma, catalog # L2884), (6) 50 mM Tris-HCl, pH 7.5, and 100  $\mu$ g/ml soybean trypsin inhibitor (STI, Calbiochem, catalog # 65035), (7) 50 mM Tris-HCl, pH 7.5, and a Roche protease inhibitor tablet (1 tablet per 25 ml), and (8) 50 mM Tris-HCl, pH 7.5, a Roche protease inhibitor tablet (1 tablet per 25 ml), and 0.7 mM PMSF. The samples were placed in a 37 °C incubator for specific periods of time. The reactions were stopped by storing the samples at -80 °C until use.

*Trifluoroacetic Acid Precipitation of S. cerevisiae Extracts*—To compare isoaspartyl damage in the peptides and proteins, 5 d *in vitro* aged *S. cerevisiae* extracts obtained from 1 or 7 d cultures were incubated with a final concentration of 5% trifluoroacetic acid for 20 min at room temperature. The samples were centrifuged at 20,000  $\times$  g for 10 min and the supernatant was transferred to a new vial. Both the supernatant and the pellet fractions were dried under vacuum centrifugation. 25  $\mu$ l of 0.2 M Bis-Tris-HCl, pH 6.4 buffer was added to each sample and isoaspartyl content was quantified by the vapor diffusion assay described above.

*HPLC Analysis of In Vitro Aged S. cerevisiae Extracts*—1 and 7 d *in vitro* aged extracts were prepared and precipitated with trifluoroacetic acid as previously described, except that instead of vacuum centrifugation, 100  $\mu$ l of the supernatant fractions were transferred to HPLC vials and separated using an HP 1090 II liquid chromatograph and a Grace Vydac 218 MS polymeric C18 analytical HPLC column (5  $\mu$ m, 4.6-mm inner diameter, 250-mm length) at room temperature and a flow rate of 1 ml/min. Solvent A consisted of 0.1% trifluoroacetic acid in water and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The HPLC gradient was as follows: isocratic for 5 min at 100% A, a linear gradient for 30 min from 100% A to 5% A, 5 min at 5% A, a linear gradient for 1 min from 5% A to 100% A, and isocratic for 19 min at 100% A. 1 ml fractions

were collected throughout the run, and 500  $\mu$ l of each fraction was dried by vacuum centrifugation. These samples were resuspended in 25  $\mu$ l of 0.2 M Bis-Tris-HCl, pH 6.4, and analyzed for isoaspartyl content by the vapor diffusion assay as described above.

*Nano-Liquid Chromatography with Tandem Mass Spectrometry (nLC-MS/MS) Analysis*—nLC-MS/MS with collision induced dissociation (CID) was performed on an Orbitrap XL (Thermo Fisher, Waltham, MA) integrated with an Eksigent nano-LC. A reverse-phase column (75  $\mu$ m  $\times$  200 mm containing 5- $\mu$ m C18 resin with 300 Å pores (AcuTech, San Diego, CA)) was used for peptide chromatography. ESI conditions using the nano-spray source (Thermo Fisher) for the Orbitrap are set as follows: capillary temperature at 220 °C, tube lens at 110 V and a spray voltage of 2.3 kV. The flow rate for reverse-phase chromatography is 500 nl/min for loading and analytical separation (buffer A: 0.1% formic acid, 3% acetonitrile; buffer B: 0.1% formic acid, 100% acetonitrile). Peptides are loaded onto the column for 30 min and resolved by a gradient of 0–40% buffer B over 180 min. The Orbitrap is operated in data-dependent mode with a full precursor scan at high-resolution (60,000 at m/z 400) and ten MS/MS experiments at low resolution in the linear trap with a scan range of 350–2000 m/z. For CID, the intensity threshold was set to 500 counts and a collision energy of 40% was applied. Dynamic exclusion was set with a repeat count of 1 and exclusion duration of 30 s.

Database searches of the acquired spectra were analyzed with MASCOT (Matrix Science, v2.4). The NCBI non-redundant database (20131226, 35,149,712 sequences, 12,374,887,350 residues) were used and limited to *S. cerevisiae*. The search parameters used were: no enzyme cleavage, no missed cleavages, no fixed modifications, deamidated (NQ) and acetyl (N-term) variable modifications, monoisotopic mass values, unrestricted protein mass, +/- 50 ppm peptide mass tolerance, and +/- 0.8 Da fragment mass tolerance. With these parameters, an overall peptide false discovery rate of 9.35%, or 10 decoy peptide matches above identity threshold versus 107 matches in the NCBIInr, were obtained against a decoy database.

Proteins were confirmed from the Mascot entries if they had at least one peptide designated

in bold and red with an ion score of at least 20 and ppm error no greater than 13. Peptides were analyzed for asparagine deamidation if they had an ion score of at least 20 and ppm error no greater than 13. The N- and C-termini of both non-isoaspartyl and isoaspartyl-containing peptides were compared using the criteria established for peptides. Four amino acids from both the N and C-termini were inputted into WebLogo (<http://weblogo.berkeley.edu/logo.cgi>) for each unique peptide and a frequency plot was obtained.

## RESULTS

*S. cerevisiae* Does Not Have a PCMT Homolog or Equivalent Activity—To determine if alternative mechanisms exist to remove isoaspartyl protein damage other than by PCMT methylation repair, we first wanted to identify an organism that lacked PCMT. We identified the closest protein match to human PCMT in a diverse array of organisms by BLAST searching and phylogenetic tree analysis (Fig. 2). Although PCMT activity and homologous proteins have been previously observed in many animals, plants, fungi, archaea, and bacteria, PCMT was found to be absent in *Amphidinium carterae* (dinoflagellate) (49), *Bacillus subtilis* (Gram-positive bacteria) (50), *Mycoplasma genitalium* (Gram-positive bacteria) (51), *Synedra ulna* (diatom) (49), and *S. cerevisiae* (Fig. 2). In these organisms, the closest match to PCMT is not a protein repair methyltransferase, but rather an unrelated protein such as elongation factor 2 (*A. carterae*), 2-heptaprenyl-1,4-naphthoquinone methyltransferase (*B. subtilis*), DNAJ-like protein (*M. genitalium*), photosystem II reaction center protein (*S. ulna*), and the sterol 24-C-methyltransferase (*S. cerevisiae*). These data confirm previous studies that also found a lack of PCMT homolog and/or enzymatic activity in these organisms (6,49-51).

Because *S. cerevisiae* is a frequently used model organism and knowledge of isoaspartyl damage in fungi is lacking, we were interested in determining if *S. cerevisiae* had endogenous PCMT activity despite lacking a homologous enzyme. We incubated an isoaspartyl-containing peptide, KASA(L-isoD)LAKY (52), with *S. cerevisiae* extract to determine if it would be methylated by an enzyme with PCMT activity. When incubating the peptide with extracts derived

from *S. cerevisiae* cultured for 1, 3, or 7 days, representing young, middle-aged, and old stage cells, respectively, no isoaspartyl methylation was observed compared to controls (Fig. 3A). However, methylation was observed when reacting these extracts with recombinant human PCMT, confirming the presence of isoaspartyl residues in *S. cerevisiae* polypeptides and indicating the absence of inhibitory factors (Fig. 3A). When *S. cerevisiae* extracts were incubated with [<sup>3</sup>H]AdoMet, separated by SDS-PAGE, and analyzed by fluorography, no radioactive bands found (Fig. 3B). However, when these extracts were reacted with recombinant human PCMT, a limited number of protein bands were present, confirming again the existence of isoaspartyl-damaged species (Fig. 3B).

*S. cerevisiae* Has Low Isoaspartyl Damage Levels Despite Lacking PCMT—To quantify the amount of isoaspartyl residues in cells, we cultured *S. cerevisiae* for 1, 3, or 7 days in either YPD or CSM media and used recombinant human PCMT as an analytical probe to determine the number of methyl-accepting isoaspartyl substrates. The amount of damage observed ranged from 165 to 295 pmol of isoaspartyl residues per mg of total protein extract for the 1 and 7-day cultures, respectively, with similar levels for cells cultured in either YPD or CSM media (Fig. 4). Other rich and minimal media conditions were also tested, including media based on lactate or lacking ammonium salts, but no significant differences were observed in isoaspartyl levels (data not shown). When compared to extracts of other organisms that contain PCMT, such as *E. coli*, *C. elegans*, and *M. musculus*, the levels of isoaspartyl damage observed in *S. cerevisiae* are strikingly low. For example, isoaspartyl damage in *E. coli* cultured for 1 or 11 days in Luria Bertani broth, have approximately 450 and 2000 pmol of isoaspartyl residues/mg protein, respectively, and the removal of PCMT and its cotranscribed *surE* gene results in an increase to 3500 pmol of isoaspartyl residues/mg protein in 11 day cultures (28). In *C. elegans*, 14 and 31 day old dauer cultures have 453 to 1115 pmol of isoaspartyl residues/mg extract, respectively, and animals deficient in PCMT have 511 and 1890 pmol of isoaspartyl residues/mg protein, respectively (53). Finally, in mice, isoaspartyl damage in the brain is approximately 200 and 1100 pmol/mg protein in

wild type and PCMT-deficient animals, respectively (36).

We hypothesized that isoaspartyl damage could be limited in *S. cerevisiae* due to a lower content of deamidation/isomerization-susceptible asparagine and aspartate residues. We thus analyzed the frequency of each amino acid in *S. cerevisiae*. We found that the frequency of these two amino acids in *S. cerevisiae* is comparable, if not greater, than that seen in *H. sapiens*, *M. musculus*, and *E. coli* (Fig. 5A). Next, we asked whether there was a decrease in the amount of four sequences known to rapidly convert to isoaspartyl residues - asparagine-glycine (NG), asparagine-serine (NS), asparagine-cysteine (NC), and asparagine-alanine (NA) (10). In *S. cerevisiae*, the percentage of proteins containing at least one NG, NS, NC, or NA was 68.9, 75.0, 26.9, and 65.9 percent, respectively, and was comparable to other organisms (Fig. 5B).

*The Proteasome and Autophagy Pathways do not Control Isoaspartyl Damage in S. cerevisiae*—The proteasome and the autophagy pathways are two of the most important protein quality control mechanisms in the cell. The proteasome, which is responsible for 80-90 percent of protein degradation in mammals (54), is associated with the removal of 40-60 percent of short lived proteins (55), in addition to the regulation of proteins (56,57) and removal of denatured and oxidatively damaged proteins (58-60). On the other hand, the autophagy pathway largely degrades long-lived proteins (61,62) and protein aggregates (63,64). Because isoaspartyl-damage kinks proteins and may result in protein aggregation, we hypothesized that the proteasome and/or autophagy pathways could remove this type of damage in *S. cerevisiae*, and reducing the activity of these pathways would result in an increase in isoaspartyl-damaged species. To test this hypothesis, we analyzed the effects of proteasome and autophagy inhibitors on *pdr5Δ S. cerevisiae*, which are deficient in an ATP-binding cassette multidrug transporter involved in removing drugs from inside the cell (65,66). *pdr5Δ* cells treated with the proteasome peptide aldehyde inhibitor MG132 (10) and/or the autophagy inhibitor 3-methyladenine (67) showed no significant increase in isoaspartyl damage when compared to the controls (Fig. 6A). Recent studies have shown that although the inhibition of

proteasome activity by MG132 targets mainly the chymotryptic activity of the proteasome, the tryptic and caspase-like activities imparted by the Pup1 and Pre3 proteases, are still present (48). Therefore, we treated *pup1pre3pdr5Δ S. cerevisiae* with MG132 to see if there was an increase in isoaspartyl damage. Consistent with our previous results, there was no statistically significant increase in isoaspartyl residue levels when incubating the cells for 8 h or 24 h with the inhibitor (Fig. 6B). Likewise, although 3-methyladenine is a commonly used autophagy inhibitor, recent research has shown that it can also promote autophagy when cells are incubated in rich conditions (68). Therefore, we tested two other autophagy inhibitors, concanamycin A and ammonium chloride (69), to see if these inhibitors would increase isoaspartyl levels (Fig. 6C). Once again, no significant increase in isoaspartyl damage was observed when compared to the controls even when the cells were incubated with the inhibitors for 7 days (Fig. 6C). Finally, we tested mutants associated with the proteasome and autophagy pathways to further verify our inhibition studies (Fig. 6D). For the proteasome, we tested cells deficient in Rpn4, a transcription factor responsible for the expression of proteasome genes (70,71), Ubr2, an E3 ligase that regulates Rpn4 (72,73), and Doa4, a deubiquitinating enzyme associated with recycling the ubiquitin pool in cells (74). We also analyzed cells deficient in Atg8, Atg18, and Mon1, proteins necessary for autophagy (75,76). Once again, comparing the mutant strains to the control BY4742 cells indicated no increase in isoaspartyl damage after 7 days of culture (Fig. 6D).

*In vitro Aged S. cerevisiae Extracts Accumulate Isoaspartyl Residues at Lower Rates Than Those of Other Organisms*—We next investigated *in vitro* formation of isoaspartyl residues in *S. cerevisiae* extracts. We incubated extracts derived from cells cultured 7 days for various periods of time and found that isoaspartyl residue content increased linearly from approximately 89 to 849 pmol of isoaspartyl residues/mg protein over the course of 11 days (Fig. 7). Although the initial amount of isoaspartyl damage is similar to that observed in extracts of a mixed population of *C. elegans* and in stationary phase *E. coli*, the rate of isoaspartyl residue accumulation in stationary phase extracts

of *S. cerevisiae* is lower than that found for the worm and bacterial extracts. We also found a lower rate of accumulation in brain extracts derived from PCMT-deficient mice (Fig. 7). These results suggest that an active mechanism, such as a proteolytic pathway, may be limiting the amount of isoaspartyl residues in *S. cerevisiae*.

*In Vitro Aged S. cerevisiae Extracts Incubated with EDTA Rapidly Accumulate Isoaspartyl Residues in Low Molecular Weight Polypeptides*—To test if proteolysis limits isoaspartyl damage in *S. cerevisiae*, both 1- and 7-day culture extracts were *in vitro* aged in Tris buffer alone or Tris buffer with protease inhibitors of different types, including cysteine- (leupeptin), serine- (PMSF, aprotinin, leupeptin, soybean trypsin inhibitor), and metallo- (EDTA) inhibitors. Additionally, samples were incubated with a commercial protease inhibitor mixture from Roche. When we quantified the level of isoaspartyl residues in extracts of 1 day cultures *in vitro* aged over the course of 6 days, no difference in the rate of accumulation was observed when comparing the control buffer sample to those incubated with PMSF, aprotinin, leupeptin, and soybean trypsin inhibitor (Fig. 8A). However, extracts aged in the presence of EDTA or the Roche inhibitor mixture resulted in an increased accumulation of isoaspartyl-containing species (Fig. 8A). Analyzing these isoaspartyl species using SDS-PAGE (Fig. 8C) and fluorography (Fig. 8E), showed an increase in isoaspartyl residue damage in polypeptides of about 6 kDa and smaller for only the samples aged with EDTA and the Roche inhibitor mixture. These results were reproduced using *S. cerevisiae* cultured for 7 days and *in vitro* aged under the same conditions as for the 1 day cultures (Fig. 8B, 8D, 8F).

Interestingly, the Roche inhibitor tablets contain EDTA, and only extracts *in vitro* aged in the presence of EDTA had an increase in isoaspartyl residues. Because EDTA is a metalloprotease inhibitor, we hypothesized that a metalloprotease is either specifically degrading isoaspartyl sites in damaged polypeptides or that a nonspecific metalloprotease is degrading polypeptides before isoaspartyl residues have a chance to form. Inhibiting the metalloprotease would therefore result in an increase in damaged peptides. To confirm the presence of an increased amount of isoaspartyl-damaged peptides, protein

extracts derived from 1- and 7- day *S. cerevisiae* cultures were *in vitro* aged for 5 days, and a trifluoroacetic acid precipitation was used to separate peptides from proteins. Although there was a slight increase in isoaspartyl-damaged proteins in the presence of EDTA for both the 1 and 7 day extracts (less than 2-fold), a large increase (about 15-fold) in isoaspartyl-species was observed in the peptide fraction of samples incubated with the metalloprotease inhibitor (Fig. 9).

*Isoaspartyl Damage in S. cerevisiae is Ubiquitous and N-terminal Isoaspartyl-Containing Peptides Accumulate in the Presence of EDTA*—To isolate isoaspartyl-damaged peptides, the trifluoroacetic acid supernatant of 1- and 7-day *S. cerevisiae* extracts *in vitro* aged for 5 days were separated by reverse phase HPLC. In the absence of EDTA, both the extracts from the 1- (Fig. 10A) and 7-day (Fig. 10B) cultures did not show a significant amount of isoaspartyl damage in the HPLC fractions. However, in the presence of EDTA, an increase in damaged polypeptides eluting between approximately 16 and 20 min was observed for the 1- (Fig. 10C) and 7- (Fig. 10D) day culture extracts. To identify the isoaspartyl-species isolated by HPLC, the material in fractions 17, 18, and 19 was analyzed by tandem mass spectrometry. In the samples not treated with EDTA, no peptides were found that matched yeast proteins under our stringent criteria. However, for the samples incubated with EDTA, a number of peptides derived from yeast proteins were detected (Tables 2, 3). In the aged extract from the 1-day culture, peptides derived from pyruvate kinase, enolase, hexokinase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate decarboxylase, and the Ran GTPase were found to have deamidated asparagine sites consistent with the presence of isoaspartyl residues (Table 2). In the aged extract from the 7-day culture, a similar set of proteins were found to contain deamidated asparagine residues, with the addition of Pol1, a subunit of the DNA polymerase 1 alpha-primase complex (Table 3). With the exception of Pol1, all the deamidated proteins observed were in the top 10 percent of abundant proteins in *S. cerevisiae* as calculated by the comprehensive protein abundance database PaxDB (77). Additionally, many of the deamidated peptides observed were from proteins with relatively short half-lives, such as enolase,

hexokinase, pyruvate decarboxylase, and glyceraldehyde-3-phosphate dehydrogenase, which have half-lives of 27, 42, 29, and 85 minutes, respectively, as previously determined by Belle *et al.* (78) (Tables 2 and 3).

To determine if there is a metal ion-dependent isoaspartyl-specific protease activity in yeast, we analyzed the amino acid sequences of the peptides identified by mass spectrometry in the extracts aged in the presence of EDTA (Fig. 11). Peptides showing evidence for deamidation at coded asparagine residues are expected to contain largely isoaspartyl residues at that site, although aspartyl residues may also be found in lower amounts. For peptides with no deamidation sites, nonpolar aliphatic amino acids were predominant at the N- and C-termini (Fig. 11A, B). For peptides with evidence for deamidation of asparagine residues, the C-terminal amino acid sequences were similar to those of the non-deamidated peptides. However, we found that their N-terminal residues contained a disproportionate frequency of deamidated asparagine residues compared to the non-damaged proteins, suggesting that these may represent isoaspartyl residues that are not cleaved in the absence of one or more metallopeptidases (Fig. 11C, D). Of the 13 deamidated peptides identified from the 1-day culture, 6 demonstrated deamidation at the N-terminal position (Table 2, Fig. 11C). Likewise, of the 10 deamidated peptides identified from the 7-day culture, 5 peptides were deamidated at the N-terminal position (Table 3, Fig. 11D). Combined, these data suggest that metallopeptidase activity is required to fully degrade peptides containing isoaspartyl damage. Such activity thus represents a crucial part of the proteolytic pathway to limit the accumulation of isoaspartyl residues in yeast cells that do not have the repair pathway.

## DISCUSSION

Previous work investigating the importance of isoaspartyl repair focused on several key model organisms, including *E. coli* (28-30), *A. thaliana* (79-81), *C. elegans* (32-35,41,53), *D. melanogaster* (31,82), and mice (7,36-39). However, little is known about the role of PCMT in fungi, other than in the observation of PCMT activity in mushroom (83) and the presence of

deamidated asparagine in glutamate dehydrogenase isoenzymes (84). This study is the first to quantify isoaspartyl residues in a fungus, confirm the lack of PCMT in *S. cerevisiae*, and shed light on how isoaspartyl residues could potentially be removed in an organism lacking repair methyltransferase activity. Because isoaspartyl levels are generally lower in *S. cerevisiae* compared to other organisms with PCMT, we hypothesized that alternative mechanisms to limit this ubiquitous damage must exist. In *C. elegans* and mice, the presence of isoaspartyl-containing peptides supports the hypothesis that isoaspartyl damage may not only be repaired but also degraded (36,41,42). However, no biochemical study has further searched for such proteolytic activity in other organisms. In this work, we showed that the proteasome and autophagy pathways, two common mechanisms to remove altered proteins from cells, do not appear to be involved in limiting isoaspartyl damage. We now provide evidence for the participation of one or more metallopeptidases with the ability to cleave peptides containing N-terminal isoaspartyl residues. These latter enzymes can ensure the full hydrolysis of isoaspartyl-containing proteins to free amino acids.

In *S. cerevisiae*, there are approximately 47 enzymatically active metalloproteases (85). Since EDTA is a broad-spectrum inhibitor of metalloprotease activity, more than one metalloenzyme may play a role in reducing the accumulation of isoaspartyl residue damage, including enzymes that both insure the rapid overall degradation of proteins as well as those that specifically recognize isoaspartyl sites (Fig. 12). The deamidation and isomerization of asparaginyl and aspartyl residues to isoaspartyl damage is a relatively slow process that depends on protein sequence. Asparagine-glycine and asparagine-serine form isoaspartyl residues most rapidly, with half of the asparagine residues in these sequences deamidating to isoaspartyl residues in 1.2 or 6.8 days after formation, respectively (10). Additionally, sites in sequences such as asparagine-valine and aspartate-histidine are some of the slowest to form isoaspartyl residues, with half-lives of 107 and 266 days, respectively (10). In comparison to these formation rates, most proteins in *S. cerevisiae* are

robustly turned over and have a half-life of approximately 43 minutes (78). In the presence of EDTA, many of the peptides we identified with isoaspartyl residues were derived from proteins with turnover rates ranging between 27 and 85 minutes (Table 2 & 3). Consequently, repair mechanisms may not be needed by *S. cerevisiae* because proteins are typically degraded before they are allowed to age a sufficient time to generate isoaspartyl damage.

A comparison of peptides identified by mass spectrometry in our *in vitro* aging experiments show that peptides with and without isoaspartyl residues have similar C-terminal sequences, but the N-terminus of isoaspartyl-peptides contain mostly deamidated asparagine in the first position. We hypothesize that this N-terminal isoaspartyl site blocks degradation of the peptide by typical proteases. In the presence of EDTA, the degradation of isoaspartyl-species by an isoaspartyl-specific metalloprotease is inhibited. Future studies will look further into these 47 metalloproteases to see if one or a combination of proteases is required to limit isoaspartyl residues in *S. cerevisiae*. We note that N-terminal isoaspartyl residues can be cleaved in peptides by the IaaA isoaspartyl aminopeptidase present in a variety of organisms (44-47), but BLAST searches against this non-metalloprotease do not identify any homolog in yeast or other fungi.

Protein oxidation and isoaspartyl formation are comparable types of protein damage and it is reasonable to hypothesize both modifications are limited by similar mechanisms. As with PCMT and isoaspartyl residues, repair enzymes restore methionine and cysteine oxidation sites, in addition to protein thiols (86). In non-repairable protein oxidative damage involving methionine sulfones and carbonyl derivatives, the proteasome and vacuole systems have increased activity to degrade damaged proteins (86-90). The present study shows that neither the proteasome or autophagy pathways regulate isoaspartyl damage significantly. However, we were interested in seeing if the vacuole was important for isoaspartyl control and tested *S. cerevisiae* strains deficient in the key vacuole proteases Pep4 and Prb1. In both

cases, the amount of isoaspartyl residues was no different when compared to controls (data not shown). Interestingly, when we analyzed other vacuole mutants, the Fab1 protein was consistently found to have at least a two-fold increase in isoaspartyl residues (data not shown). Fab1 is a 1-phosphatidylinositol-3-phosphate 5-kinase involved in vacuole sorting and homeostasis, and Fab1-deficient cells have deacidified vacuoles (91). However, this increase in isoaspartyl damage was only observed in the MAT $\alpha$  strain of *S. cerevisiae*, and no change was observed in the MAT $\alpha$  and diploid mutant strains (data not shown). Nevertheless, this result brings attention to the fact that at least 8 metalloproteases are located in the vacuole and could participate in isoaspartyl degradation (85).

The only previous report that investigated isoaspartyl residues in *S. cerevisiae* was performed by DeLuna *et al.* (84), who showed that a specific asparagine-glycine sequence in Gdh3 was deamidated *in vitro* and resulted in protein instability. We did not observe the presence of Gdh3 in our mass spectrometry data, which may be due to the relative low abundance of the protein (PaxDB abundance of 32.2 ppm) or because it is not deamidated under physiological conditions. It is important to note that our mass spectrometry analysis is only analyzing isoaspartyl formation due to asparagine deamidation. A proteomic approach using electron transfer dissociation mass spectrometry techniques would provide a more thorough characterization of not only asparagine deamidation but also isoaspartyl formation due to aspartyl isomerization (42). Nevertheless, our analysis provided the first in-depth analysis of isoaspartyl damage in fungi. Of the proteins identified by tandem mass spectrometry, many of the isoaspartyl-damaged species were found to originate from abundant proteins involved in metabolism and gene regulation (Table 2 & 3). The present study also provides evidence for metalloproteases that may limit the amount of isoaspartyl damage in other organisms, such as *C. elegans*, mice, and humans.

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**FOOTNOTES:**

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<sup>2</sup>The abbreviations used are: PCMT, Protein-L-isoaspartyl (D-aspartyl) *O*-methyltransferase; AdoMet, S-adenosylmethionine; [<sup>3</sup>H]AdoMet, S-adenosyl-L-[*methyl*-<sup>3</sup>H]-methionine.

## Figure Legends

**Figure 1. Pathways for the formation and metabolism of isoaspartyl residues in peptides and proteins.** The deamidation or isomerization of L-asparaginyl or L-aspartyl residues, respectively, results in the formation of an L-succinimidyl intermediate. Although approximately 15-40% of this intermediate will nonenzymatically hydrolyze into the normal L-aspartyl residue, the majority product is the abnormal L-isoaspartyl residue that reroutes the polypeptide backbone through the side chain. To repair this protein damage, most organisms rely on PCMT, which uses the cofactor *S*-adenosylmethionine (AdoMet) to methylate the L-isoaspartyl residue side chain and form a methyl ester. This methyl ester is non-enzymatically readily hydrolyzed to reform the succinimidyl intermediate, which once again can convert either into the L-isoaspartyl or L-aspartyl residues. The PCMT repair cycle continues until the L-isoaspartyl residue is fully converted to the normal L-aspartyl residue. Additionally, the succinimide is racemization prone and can also form D-isoaspartyl and D-aspartyl products, the latter of which is also a substrate for PCMT methylation (9,11).

**Figure 2. PCMT is highly conserved in many organisms, except for some Gram-positive bacteria and a few fungal species including *S. cerevisiae*.** A protein-protein BLAST search was performed comparing various organisms to human PCMT (UniProt P22061). The UniProt ID of the top ranking alignment for each organism is indicated, along with their respective E-values. To construct a phylogenetic tree, a blastp protein-protein search was performed using the human PCMT protein (UniProt P22061) and the tree was constructed in MEGA5. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. All positions containing gaps and missing data were eliminated. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. ‡, PCMT has been previously detected in the organism; \*, the enzyme has been inferred by BLAST analysis; X, no PCMT homolog is found in the organism by BLAST analysis or by experimental evidence (49-51).

**Figure 3. *S. cerevisiae* does not have PCMT activity.** Strain BY4742 was cultured in YPD for 1, 3, or 7 d at 30 °C, and extracts were prepared as described in “Experimental Procedures”. (A) *In vitro* vapor diffusion assays to measure the formation of methyl esters were performed as described in “Experimental Procedures.” Briefly, 25 µg of extract protein with or without 5 µg of recombinant human PCMT was incubated with or without 50 µM of the isoaspartyl-containing peptide KASA(isoD)LAKY in the presence of 10 µM [<sup>14</sup>C]AdoMet for 2 h at 37 °C. Technical duplicates for each condition were analyzed and each point represents the average of three radioactivity measurements. The horizontal bar indicates the average and the error bars represent the standard deviation. (B) *In vitro* SDS-PAGE/fluorography to visualize isoaspartyl-containing species in reaction mixtures prepared as in panel A. 25 µg of bovine serum albumin incubated with or without 5 µg recombinant human PCMT served as the positive and negative controls, respectively. The reactions were quenched by the addition of SDS-PAGE loading buffer and analyzed as described in “Experimental Procedures”. Polypeptide molecular weight markers (Bio-Rad broad range, catalog # 161-0317) were electrophoresed in a parallel lane and include myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), lysozyme (14 kDa), and aprotinin (6 kDa). Fluorography was performed by treating the gel with EN<sup>3</sup>HANCE and exposing the dried gel to film for 7 d at -80 °C. The arrow denotes the position of recombinant human PCMT and its automethylation band at approximately 30 kDa.

**Figure 4. *S. cerevisiae* has low isoaspartyl damage.** (A) Strain BY4742 or (B) strain LWY7235 parent cells were cultured in 30 ml of YPD or CSM media for 1, 3, or 7 d at 30 °C and 250 rpm, and extracts were prepared as described in “Experimental Procedures.” Isoaspartyl damage was quantified by vapor diffusion assay using 25 µg of extract, 5 µg of recombinant human PCMT, and 10 µM [<sup>14</sup>C]AdoMet as described in “Experimental Procedures.” The individual extracts were measured in duplicate; each data point is the average of three radioactivity measurements. The horizontal lines represent the average and the error bars are standard deviations.

**Figure 5. Low levels of isoaspartyl residue damage in *S. cerevisiae* are not due to the low frequency of susceptible aspartic acid and asparagine residues.** (A) The frequency of amino acids in proteins (fragments excluded) in *S. cerevisiae* are compared to *H. sapiens*, *M. musculus*, and *E. coli* using the Integr8 server (<http://www.ebi.ac.uk/integr8/>), which was recently closed and archived. (B) Particularly susceptible asparagine-glycine, asparagine-serine, asparagine-cysteine, and asparagine-alanine sequences are compared in the proteins of in *H. sapiens*, *M. musculus*, *E. coli*, and *S. cerevisiae*. The number of proteins containing at least one of the above sequences as determined by the Motif Search at GenomeNet (<http://www.genome.jp/tools/motif/>) is compared to the total number of protein coding genes from the KEGG genome statistics.

**Figure 6. The proteasome and autophagy pathways are not significantly involved in limiting isoaspartyl residue damage in *S. cerevisiae*.** Isoaspartyl residue quantification by vapor diffusion assays of *S. cerevisiae* strains cultured with inhibitors or in yeast mutated in the proteasome and autophagy pathways were performed as described in “Experimental Procedures.” (A) Overnights of *Δpdr5* cells were used to inoculate 30 ml of YPD. After 8 h or 24 h at 30 °C and 250 rpm, the media was removed and the cells were washed. The cells were resuspended in 30 ml of water with or without 20 µM MG132 and/or 5 mM 3-methyladenine. Control cultures were not incubated with the inhibitors. After 24 h of incubation, extracts were prepared as described in “Experimental Procedures.” Isoaspartyl quantification was determined by incubating 15-75 µg of extract, 5 µg of recombinant human PCMT, and 10 µM [<sup>14</sup>C]AdoMet. Three independent experiments were performed for each condition, with each data point representing a technical duplicate of the sample. The horizontal line denotes the average and the error bars represent standard deviations. (B) To determine if combining proteasome mutations with chemical inhibition would increase isoaspartyl damage in *S. cerevisiae*, overnights of congenic strains *PUP1PRE3pdr5Δ* and *pup1pre3pdr5Δ* cells in YPAD media were used to inoculate 30 ml of YPAD media with or without 50 µM MG132. The cells were cultured for 8 h or 24 h at 30 °C and 250 rpm, and lysed as described in “Experimental Procedures.” Isoaspartyl residues were quantified by incubating 4-89 µg of extract with 5 µg of recombinant human PCMT and 10 µM [<sup>14</sup>C]AdoMet. The data represents two individual experiments, with each data point as an average of two technical duplicates. The horizontal line denotes the average and the error bars are standard deviations. (C) To determine if other autophagy inhibitors could increase isoaspartyl damage, BY4741 or BY4742 overnight cultures were used to inoculate 5 ml YPD cultures supplemented with 10 nM or 1 µM concanamycin A, or 10 mM, 100 mM, and 200 mM ammonium chloride. BY4741 or BY4742 cells not incubated with inhibitors served as the control. After culturing for 7 d at 30 °C and 250 rpm, the cells were spun down and lysed according to the “Experimental Procedures.” Isoaspartyl residues were quantified by incubating 35-120 µg of extract with 5 µg of recombinant human PCMT and 10 µM [<sup>14</sup>C]AdoMet for 2 h at 37 °C. The data represent two individual experiments with each data point as the average of technical duplicates. The horizontal line denotes the average and the error bars represent standard deviations. (D) Other proteasome and autophagy components were tested by incubating various strains for 7 d in 30 ml of YPD media at 30 °C and 250 rpm. The cells were spun down and lysed as described in “Experimental Procedures.” Isoaspartyl residues were quantified by incubating 25-130 µg of extract with 5 µg of recombinant human PCMT and 10 µM [<sup>14</sup>C]AdoMet as described in “Experimental Procedures.” The data represents two individual experiments with each data point representing the average of two technical replicates. The horizontal line denotes the average and the error bars represent standard deviations.

**Figure 7. Comparison of *in vitro* aged *S. cerevisiae* cytosolic extracts to other organisms.** To determine if isoaspartyl residues accumulate over time in *S. cerevisiae* extracts, we incubated 25  $\mu\text{g}$  of extract in Tris, pH 7.5, buffer as described in “Experimental Procedures.” 25  $\mu\text{g}$  of *M. musculus* PCMT-/- brain cytosolic extract, 25  $\mu\text{g}$  of cytosolic extract from a mixed population of N2 *C. elegans*, and 25  $\mu\text{g}$  of OP50 *E. coli* cytosolic extract were prepared as described in the “Experimental Procedures” and aged identically to *S. cerevisiae* to serve as a comparison. Isoaspartyl residues were quantified via vapor diffusion assaying by incubating 12.5  $\mu\text{g}$  of *in vitro* aged extract with 5  $\mu\text{g}$  of recombinant human PCMT, and 10  $\mu\text{M}$  [ $^{14}\text{C}$ ]AdoMet as described in “Experimental Procedures.” Results are given for two independent experiments, with each data point representing the average of three radioactivity measurements.

**Figure 8. *S. cerevisiae* cytosolic extracts incubated with EDTA accumulate isoaspartyl residues over time.** Cytosolic protein extracts of BY4742 *S. cerevisiae* cultured in YPD for 1 d or 7 d were prepared as described in “Experimental Procedures.” 25  $\mu\text{g}$  of protein was incubated in either Tris-HCl, pH 7.5, buffer alone, or Tris-HCl with a final concentration of 2 mM EDTA, 0.7 mM PMSF, 2  $\mu\text{g}/\text{ml}$  aprotinin, 10  $\mu\text{g}/\text{ml}$  leupeptin, 100  $\mu\text{g}/\text{ml}$  soybean trypsin inhibitor, Roche inhibitor solution, or Roche inhibitor solution and 0.7 mM PMSF for 0, 2, 4, or 6 d at 37  $^{\circ}\text{C}$ . (A, B) Isoaspartyl residues were quantified via vapor diffusion assay by incubating 12.5  $\mu\text{g}$  of *in vitro* aged extract with 5  $\mu\text{g}$  of recombinant human PCMT, and 10  $\mu\text{M}$  [ $^{14}\text{C}$ ]AdoMet as described in “Experimental Procedures. The *in vitro* aging was performed in duplicate, with each data point representing a single technical measurement. (C, D) To determine the molecular weights of the isoaspartyl-damaged species, SDS-PAGE was performed on the aged extracts. 5  $\mu\text{g}$  of *in vitro* aged extract was incubated with 1  $\mu\text{g}$  of recombinant human PCMT, and 0.3  $\mu\text{M}$  [ $^3\text{H}$ ]AdoMet for 2 h at 37  $^{\circ}\text{C}$ . SDS-PAGE and fluorography (E, F; 5 d exposure) were performed as in Fig. 3B. The red asterisks denote the positions of recombinant human PCMT and its automethylation at approximately 30 kDa, and the black arrows indicate the region where an increase in isoaspartyl residues is observed with samples incubated in EDTA.

**Figure 9. *In vitro* aged extracts incubated with EDTA have an increased amount of isoaspartyl-containing peptides.** To determine the amount of isoaspartyl-damage in the peptides and proteins of *in vitro* aged *S. cerevisiae* samples, we aged 15  $\mu\text{g}$  of BY4742 cytosolic extract derived from 1 or 7 d cultures with or without EDTA as described in “Experimental Procedures.” After aging for 5 d, trifluoroacetic acid precipitation was used to separate a protein and peptide fraction as described in “Experimental Procedures.” Isoaspartyl residues were quantified for isoaspartyl residues via vapor diffusion assay by incubating 25  $\mu\text{l}$  of sample with 5  $\mu\text{g}$  of recombinant human PCMT and 10  $\mu\text{M}$  [ $^{14}\text{C}$ ]AdoMet as described in “Experimental Procedures.” Each sample was analyzed once, with each bar representing the average of three radioactivity measurements.

**Figure 10. HPLC analysis confirms an increase in isoaspartyl-containing peptides in *in vitro* aged *S. cerevisiae* extract incubated with EDTA.** To confirm an increase in isoaspartyl-containing peptides in *S. cerevisiae* extracts treated with EDTA, 100  $\mu\text{g}$  of *S. cerevisiae* extracts cultured for 1 d (A, C) or 7 d (B, D) were *in vitro* aged for without (A, B) or with (C, D) EDTA. After approximately 5 d, the samples were precipitated with 5% trifluoroacetic acid and an equivalent of 47  $\mu\text{g}$  of extract was separated by reverse-phase HPLC as described in “Experimental Procedures.” The absorbance (mAu) is represented on the left y-axis by the black line. 1 ml fractions were collected, taken to dryness under vacuum centrifugation, and resuspended in 25  $\mu\text{l}$  of 0.2 M Bis-Tris-HCl, pH 6.4 buffer. Isoaspartyl residues were quantified by vapor diffusion assay using 5  $\mu\text{g}$  of recombinant human PCMT and 10  $\mu\text{M}$  [ $^{14}\text{C}$ ]AdoMet as described in “Experimental Procedures” and the radioactivity is indicated on the right y-axis by the red trace. The brackets at the bottom indicate fractions 17, 18, and 19 of each experiment that were taken for mass spectrometry analysis.

**Figure 11. Isoaspartyl-containing peptides have a distinctive N-terminus.** The four amino acids present at the N- and C-termini of the peptides identified by mass spectrometry were analyzed. The amino acid frequency of both non-isoaspartyl (A, B) and isoaspartyl-containing (C, D) peptides from 1 (A, C) and 7 (B, D) day cultures *in vitro* aged for 5 days were inputted into WebLogo. We analyzed only peptides with an ion score greater than 20 and ppm error less than 13.

**Figure 12. Model of isoaspartyl-damage removal in *S. cerevisiae*.** Even though *S. cerevisiae* lacks the PCMT enzyme, isoaspartyl damage is low compared to other organisms with PCMT, suggesting an active mechanism to remove isoaspartyl-damage. The proteasome and autophagy pathways do not appear to be significantly involved in isoaspartyl control. However, one or more metallopeptidases may be involved in preventing isoaspartyl residue accumulation. These enzymes may be a part of the normal protein turnover or may be specific in recognizing isoaspartyl residues.

Table 1. *S. cerevisiae* strains used in this study.

Strain	Genotype	Biological function	Source
BY4741	MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Wild type	Open Biosystems
BY4742	MATalpha <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	Wild type	Open Biosystems
LWY7235	MATa <i>leu2-3,112 ura3-52 his3Δ200 trp1Δ901 lys2-801 suc2Δ9</i>	Wild type	Lois S. Weisman (92)
<i>pdr5Δ</i>	BY4742 background	Drug transport	Open Biosystems
<i>atg8Δ</i>	BY4742 background	Autophagy and cytoplasm-to-vacuole targeting pathway	Open Biosystems
<i>atg18Δ</i>	BY4742 background	Autophagy and cytoplasm-to-vacuole targeting pathway	Open Biosystems
<i>mon1Δ</i>	BY4742 background	Autophagy and cytoplasm-to-vacuole targeting pathway	Open Biosystems
<i>doa4Δ</i>	BY4742 background	Recycling ubiquitin from proteasome-bound ubiquitinated intermediates	Open Biosystems
<i>rpn4Δ</i>	BY4742 background	Transcription factor that stimulates expression of proteasome genes	Open Biosystems
<i>ubr2Δ</i>	BY4742 background	Cytoplasmic ubiquitin-protein ligase (E3); required for ubiquitylation of Rpn4p	Open Biosystems
<i>ubc4Δ</i>	BY4742 background	Ubiquitin-conjugating enzyme (E2); mediates degradation of abnormal or excess proteins	Open Biosystems
GAC201	MATalpha <i>his3-11 leu2-3,112; ura3-52 lys2-801 trp1-1 pdr5Δ::KanMX6 pre3Δ2::HIS3 pup1Δ::leu2-HIS3 [pRS317-PUP1][YCplac22-PRE3] gal<sup>+</sup></i>	Wild type	William Tansey (48)
GAC202	MATalpha <i>his3-11 leu2-3,112 ura3-52 lys2-801 trp1-1 pdr5Δ::KanMX6; pre3Δ2::HIS3 pup1Δ::leu2-HIS3 [pRS317-pup1-T30A][YCplac22-pre3-T20A] gal<sup>+</sup></i>	<i>Pre3</i> : Beta 1 subunit of the 20S proteasome; cleavage after acidic residues in peptides <i>Pup1</i> : Beta 2 subunit of the 20S proteasome; endopeptidase with trypsin-like activity that cleaves after basic residues	William Tansey (48)

Table 2. Deamidation sites in *in vitro* aged 1-day extracts incubated with EDTA.

		Rank	Abundance (ppm)	t <sub>1/2</sub> (min)	Deamidation sites
<b>Glycolysis, glyoxylate cycle, gluconeogenesis, TCA cycle, and electron transport</b>					
Adh1/Adh2	Alcohol dehydrogenase	11/247	8179/1010	nd/41	
Cdc19	Pyruvate kinase	9	12228	nd	<u>NGVHMV</u> <u>NFSHGSYIEY</u>
Eno1/Eno2	Enolase	16/1	7491/24563	nd/27	<u>NVL</u> <u>NGGSHAGGALA</u> <u>NGGSHAGGALALQE</u>
Fba1	Fructose 1,6-bisphosphate aldolase	3	21368	104	
Gpm1	Phosphoglycerate mutase	18	6668	stable	
Hxk1/Hxk2	Hexokinase	410/32	437/3413	49/nd	<u>NLRVVLVKLGGN</u> <u>H</u>
Pgi1	Phosphoglucose isomerase	26	4112	540	
Pgk1	3-phosphoglycerate kinase	5	16232	199	
Qcr2	Subunit 2 of ubiquinol cytochrome-c reductase	591	259	352	
Tdh2/Tdh3	Glyceraldehyde-3-phosphate dehydrogenase	15/2	7519/22369	nd/nd	<u>INSTGVFKEL</u> <u>AINSTGVFKELDTA</u>
<b>Alcoholic fermentation</b>					
Pdc1	Pyruvate decarboxylase	4	18671	29	<u>NATFPGVQMK</u> <u>HHTLGNDFTV</u> <u>NGIAGSYAEHVGVL</u> <u>FVLNNNGYTIE</u> <u>PVFDAPQNLVKQAKLT</u>
<b>Ribosomal proteins and translation</b>					
Egd1/Egd2	Subunit of the nascent polypeptide-associated complex	111/161	1925/1552	stable/263	
Efb1	Translation elongation factor 1 beta	61	2542	nd	
Eft1/Eft2	Elongation factor 2	128/40	1767/3015	155/stable	
Rpl13B	Ribosomal 60S subunit protein L13B	76	2327	67	
Rpp0	Ribosomal protein P0 of the ribosomal stalk	117	1872	nd	
Rps7A	Ribosomal 40S subunit protein S7A	85	2184	nd	
Rps12	Ribosomal 40S subunit protein S12	142	1673	nd	
Rps19A	Ribosomal 40S subunit protein S19A	176	1430	stable	
Ssb1/Ssb2	Ribosome-associated molecular chaperone	77/49	2320/2791	149/712	
Tef2	Translation elongation factor EF-1 alpha	8	12435	30	
Tif2	Translation initiation factor eIF4A	153	1590	nd	
Yef3	Subunit of translational elongation factor eEF1B	17	6951	191	
<b>Cofactor, purine, pyrimidine, amino acid, and glycerol biosynthesis</b>					
Gln1	Glutamine synthetase	71	2389	73	
Hom6	Homoserine dehydrogenase	270	885	56	
Shm2	Serine hydroxymethyltransferase	187	1306	nd	
Ura1	Dihydroorotate dehydrogenase	118	1871	203	
Rhr2	DL-glycerol-3-phosphate phosphatase	38	3125	113	
<b>Protein folding, sorting, and translocation</b>					
Hsp82	Hsp90 chaperone	90	2117	nd	
Ssa1/Ssa2/Ssa3	ATPase involved in protein folding; HSP70 family	12/13/547	8178/7986/290	77/nd/59	
Ssc1	Constituent of the TIM23 complex; HSP70 family	306	709	nd	
Sse1	ATPase component of heat shock protein Hsp90 chaperone complex	106	1959	101	
<b>Vacuolar acidification and proteases</b>					
Vma1	Subunit of vacuolar H <sup>+</sup> -ATPase	180	1406	109	
<b>Stress adaptation and viability</b>					
Ahp1	Thiol-specific peroxiredoxin	10	9384	stable	
Ipp1	Cytoplasmic inorganic pyrophosphatase	221	1125	883	
Sod1	Cytosolic copper-zinc superoxide dismutase	73	2358	stable	
Trx1	Cytoplasmic thioredoxin	305	715	nd	
<b>Transcription factors, DNA replication, nuclear localization, cell cycle, spore development</b>					
Gna1	Glucosamine-6-phosphate acetyltransferase	3007	11.9	25	
Gsp1	Ran GTPase	403	446	nd	SAPAANGEVPTFKL
Mbf1	Transcriptional coactivator and suppressor of frameshift mutations	206	1206	106	
Rnr4	Ribonucleotide-diphosphate reductase small subunit	140	1681	372	

PaxDB integrated dataset for protein abundance, rank out of 6153 proteins (77); Protein half-life data from Belle *et al.* (78); nd, no data; isoaspartyl sites are underlined

Table 3. Deamidation sites in *in vitro* aged 7-day extracts incubated with EDTA.

		Rank	Abundance (ppm)	t <sub>1/2</sub> (min)	Deamidation sites
<b>Glycolysis, glyoxylate cycle, gluconeogenesis, TCA cycle, and electron transport</b>					
Aco1	Aconitase	340	595	72	
Acs1	Acetyl-coA synthetase	2944	12.8	29	
Adh1/Adh2	Alcohol dehydrogenase	11/247	8179/1010	nd/41	
Atp3	Subunit of the F1 sector of mitochondrial F1F0 ATP synthase	443	384	stable	
Cdc19	Pyruvate kinase	9	12228	nd	<u>N</u> VVAGSDLRRTSIIGTIGPK
Eno1/Eno2	Enolase	16/1	7491/24563	nd/27	<u>N</u> IDVKDQKAVDDFL <u>V</u> LNGGSHAGGALALKEF
Fba1	Fructose 1,6-bisphosphate aldolase	3	21368	104	
Gpm1	Phosphoglycerate mutase	18	6668	stable	
Hxk1/Hxk2	Hexokinase	410/32	437/3413	49/nd	
Icl1	Isocitrate lyase	3353	7.55	nd	
Idh1	Isocitrate dehydrogenase	499	320	29	
Mls1	Malate synthase	1675	46.2	nd	
Pck1	Phosphoenolpyruvate carboxykinase	2059	31.6	nd	
Pgk1	3-phosphoglycerate kinase	5	16232	199	
Qcr2	Subunit 2 of ubiquinol cytochrome-c reductase	591	259	352	
Tdh1/Tdh2/Tdh3	Glyceraldehyde-3-phosphate dehydrogenase	48/15/2	2792/7519/22369	85/nd/nd	<u>N</u> KETTYDEIKKVVKA <u>N</u> GFGRIGRL
<b>Alcoholic fermentation</b>					
Pdc1	Pyruvate decarboxylase	4	18671	29	<u>H</u> HTLNGNGDFT <u>F</u> VLNNNGYTIE <u>P</u> VFDAPQNLVKQAKLT
<b>Ribosomal proteins and translation</b>					
Efb1	Translation elongation factor 1 beta	61	2542	nd	
Grs1	Cytoplasmic and mitochondrial glycyl-tRNA synthase	273	878	44	
Hyp2	Translation elongation factor eIF-5A	167	1484	nd	
Rpp0	Ribosomal protein P0 of the ribosomal stalk	117	1872	nd	
Rps5	Ribosomal 40S subunit protein S5	30	3476	nd	
Ssb1/Ssb2	Ribosome-associated molecular chaperone	77/49	2320/2791	149/712	
Tef2	Translation elongation factor EF-1 alpha	8	12435	30	
Tma19	Associates with ribosomes	98	2078	166	
Yef3	Subunit of translational elongation factor eEF1B	17	6951	191	
<b>Cofactor, purine, pyrimidine, and amino acid biosynthesis</b>					
Adk1	Adenylate kinase	97	2078	217	
Gln1	Glutamine synthetase	71	2389	73	
Hom6	Homoserine dehydrogenase	270	885	56	
Rnr4	Ribonucleotide-diphosphate reductase small subunit	140	1681	372	
Ura1	Dihydroorotate dehydrogenase	118	1871	203	
Ura2	Carbamoylphosphate synthetase-aspartate transcarbamylase	383	494	22	
<b>Protein folding, sorting, and translocation</b>					
Hsp26	Small heat shock protein with chaperone activity	366	530	stable	
Hsp82	Hsp90 chaperone	90	2117	nd	
Hsp104	Disaggregase	304	721	61	
Ssa1/Ssa2/Ssa3	ATPase involved in protein folding; HSP70 family	12/13/547	8178/7986/290	77/nd/59	
Ssc1	Constituent of the TIM23 complex; HSP70 family	306	709	nd	
Sse1	ATPase component of heat shock protein Hsp90 chaperone complex	106	1959	101	
<b>Vacuolar acidification and proteases</b>					
Ape3	Vacuolar aminopeptidase Y	512	309	9	
Pep4	Vacuolar aspartyl protease	652	225	nd	
Vma2	Subunit of vacuolar H <sup>+</sup> -ATPase	238	1045	29	
<b>Stress adaptation and viability</b>					
Cpr1	Cytoplasmic peptidyl-prolyl cis-trans isomerase	55	2661	stable	
Ctt1	Cytosolic catalase T	2401	22.8	33	
Dak1	Dihydroxyacetone kinase	508	312	42	
Gnd1	6-phosphogluconate dehydrogenase	27	3865	nd	
Pst2	Similar to flavodoxin-like proteins	427	411	40	
Sub2	Component of the TREX complex required for nuclear mRNA export	462	361	17	
Trx1	Cytoplasmic thioredoxin	305	715	nd	
Uga1	Gamma-aminobutyrate transaminase	1006	110	67	
<b>Transcription factors, DNA replication, nuclear localization, cell cycle, spore development</b>					
Gsp1	Ran GTPase	403	446	nd	<u>S</u> APA <u>N</u> GEVPTFKL
Po11	Subunit of the DNA polymerase I alpha-primase complex	3102	10.3	stable	<u>N</u> VLQGTLLANPP
Vik1	Forms a kinesin-14 heterodimeric motor with Kar3	5569	0.54	nd	

PaxDB integrated dataset for protein abundance, rank out of 6153 proteins (77); Protein half-life data from Belle *et al.* (78); nd, no data; isoaspartyl sites are underlined

Figure 1

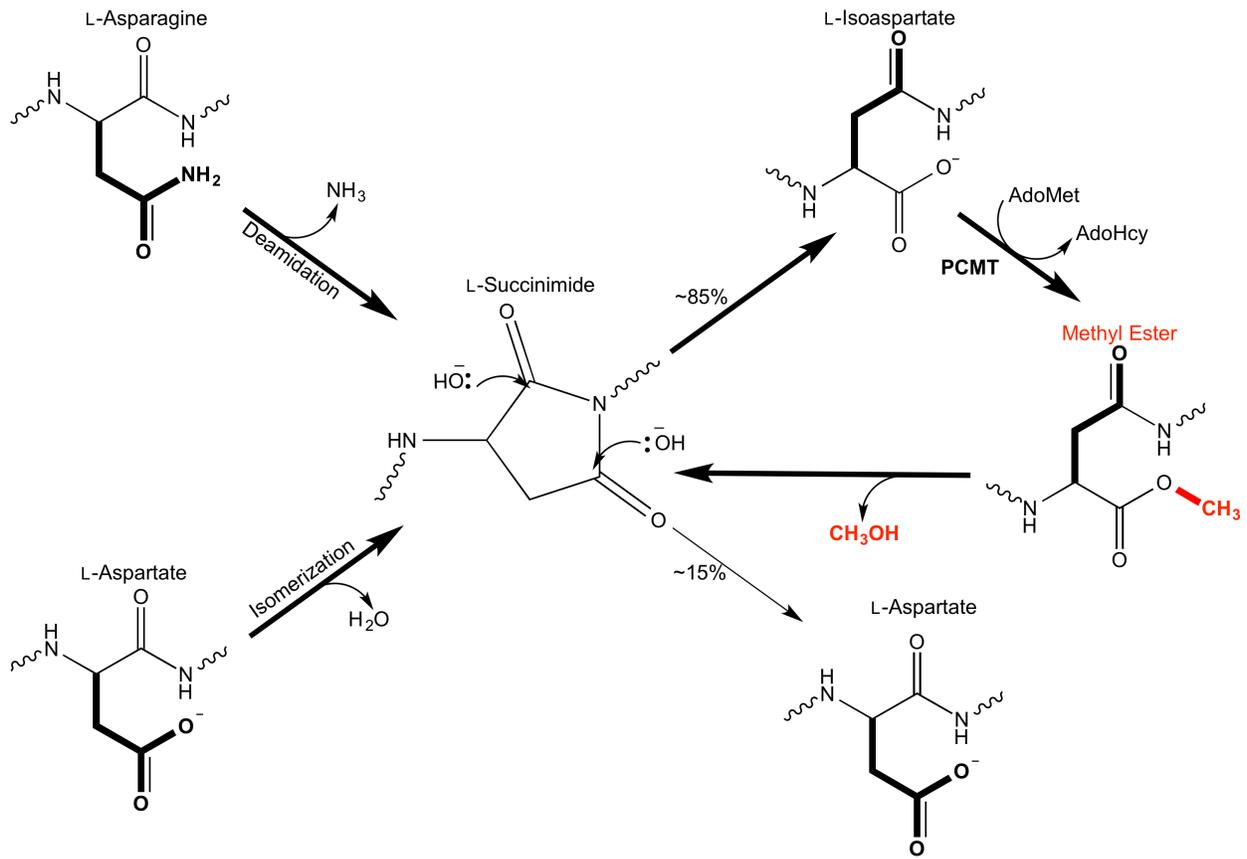


Figure 2

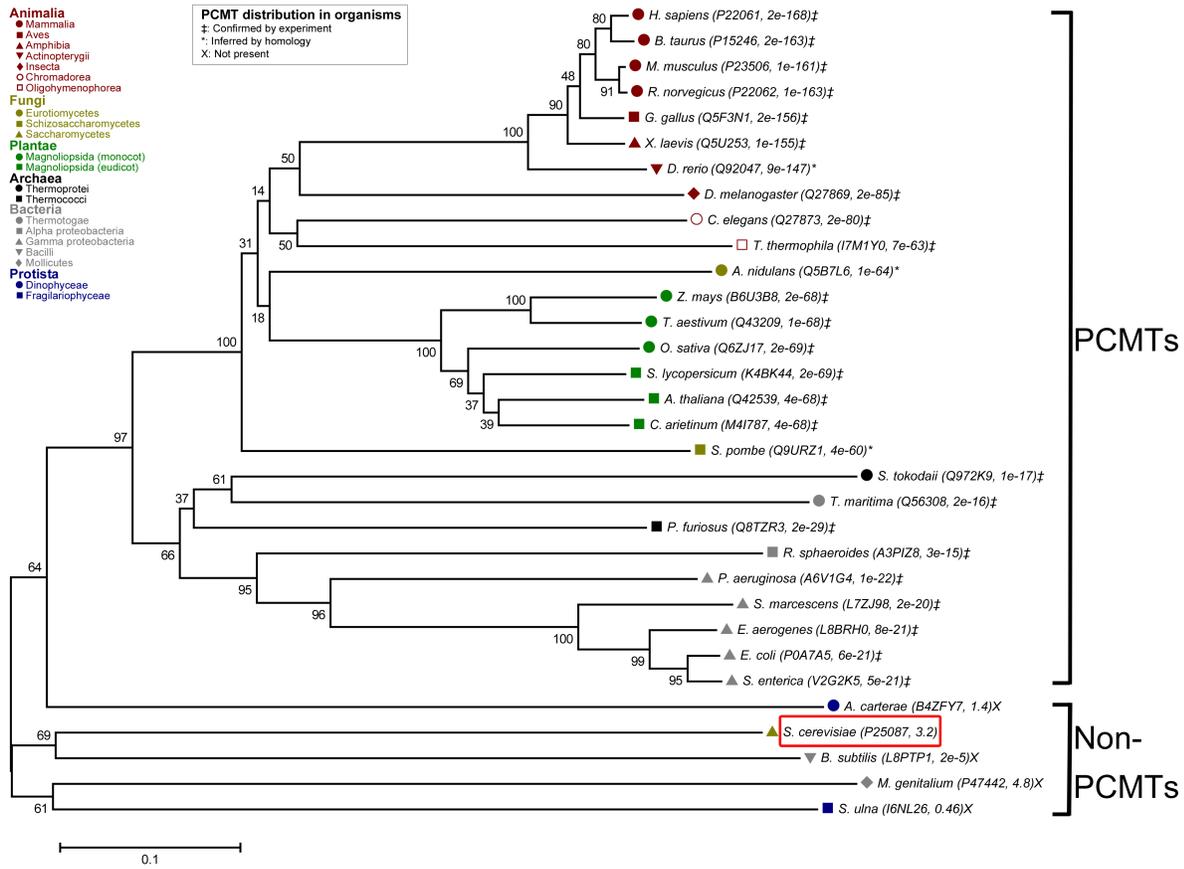


Figure 3

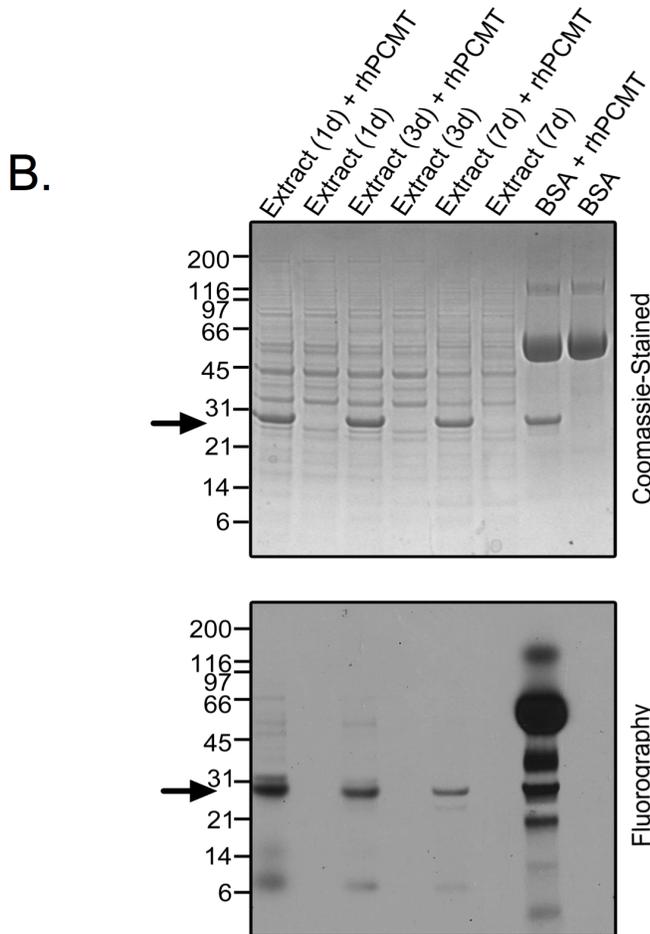
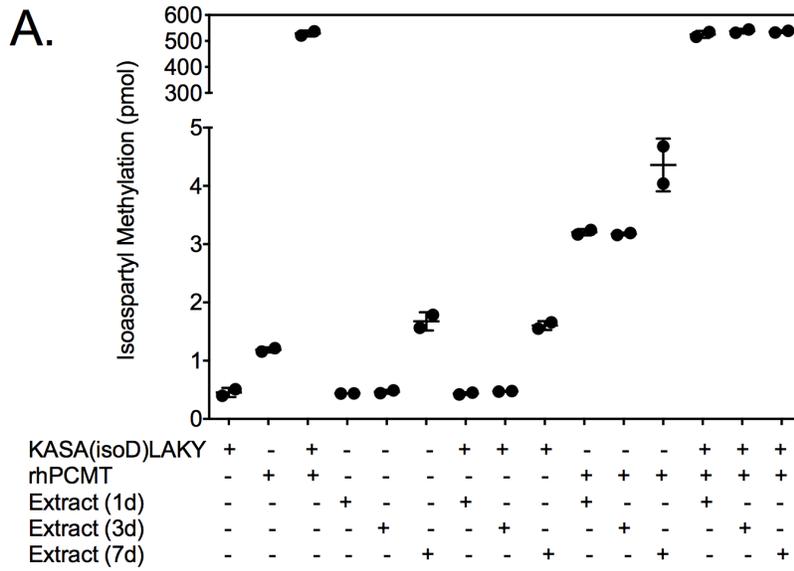


Figure 4

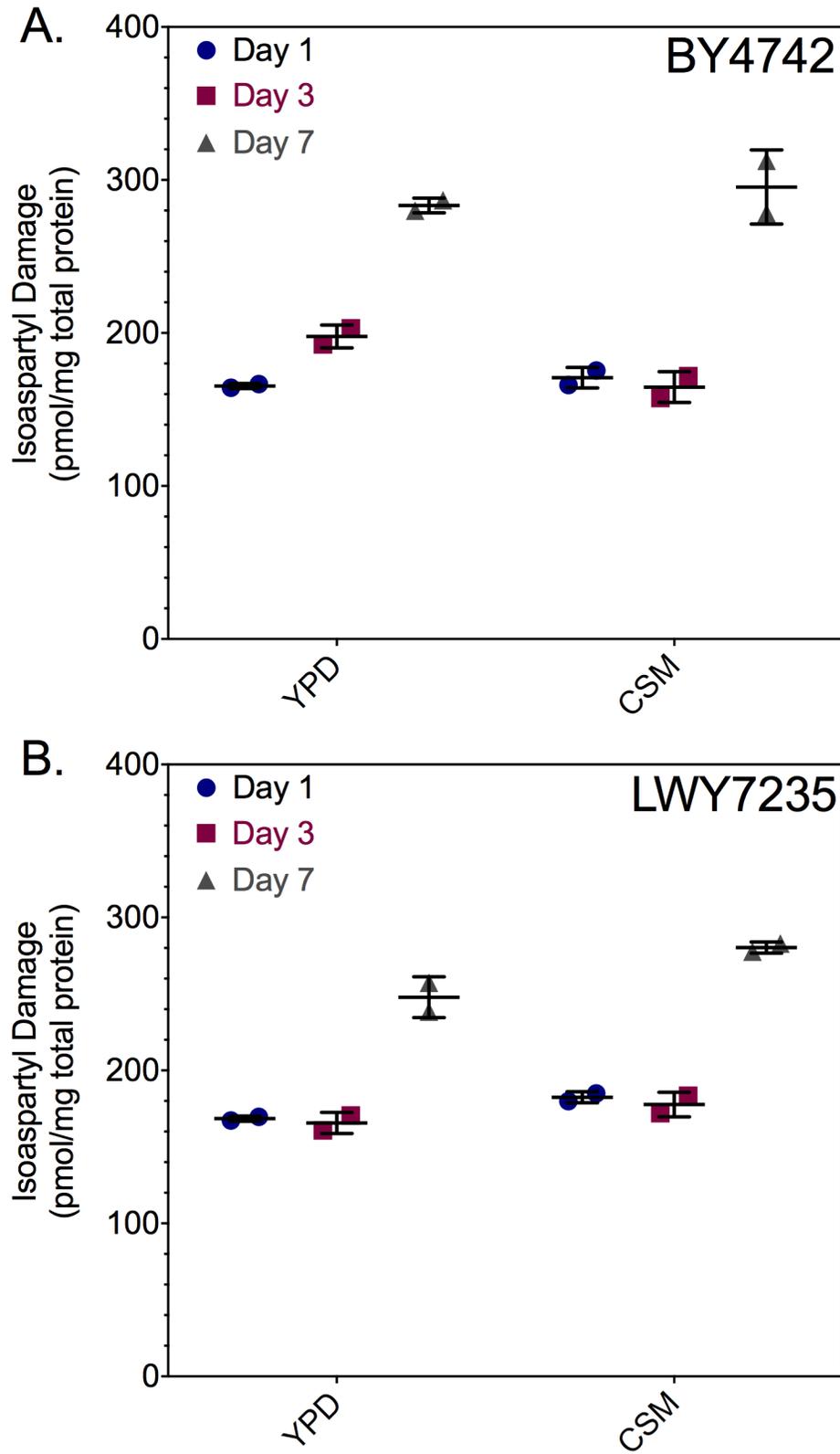


Figure 5

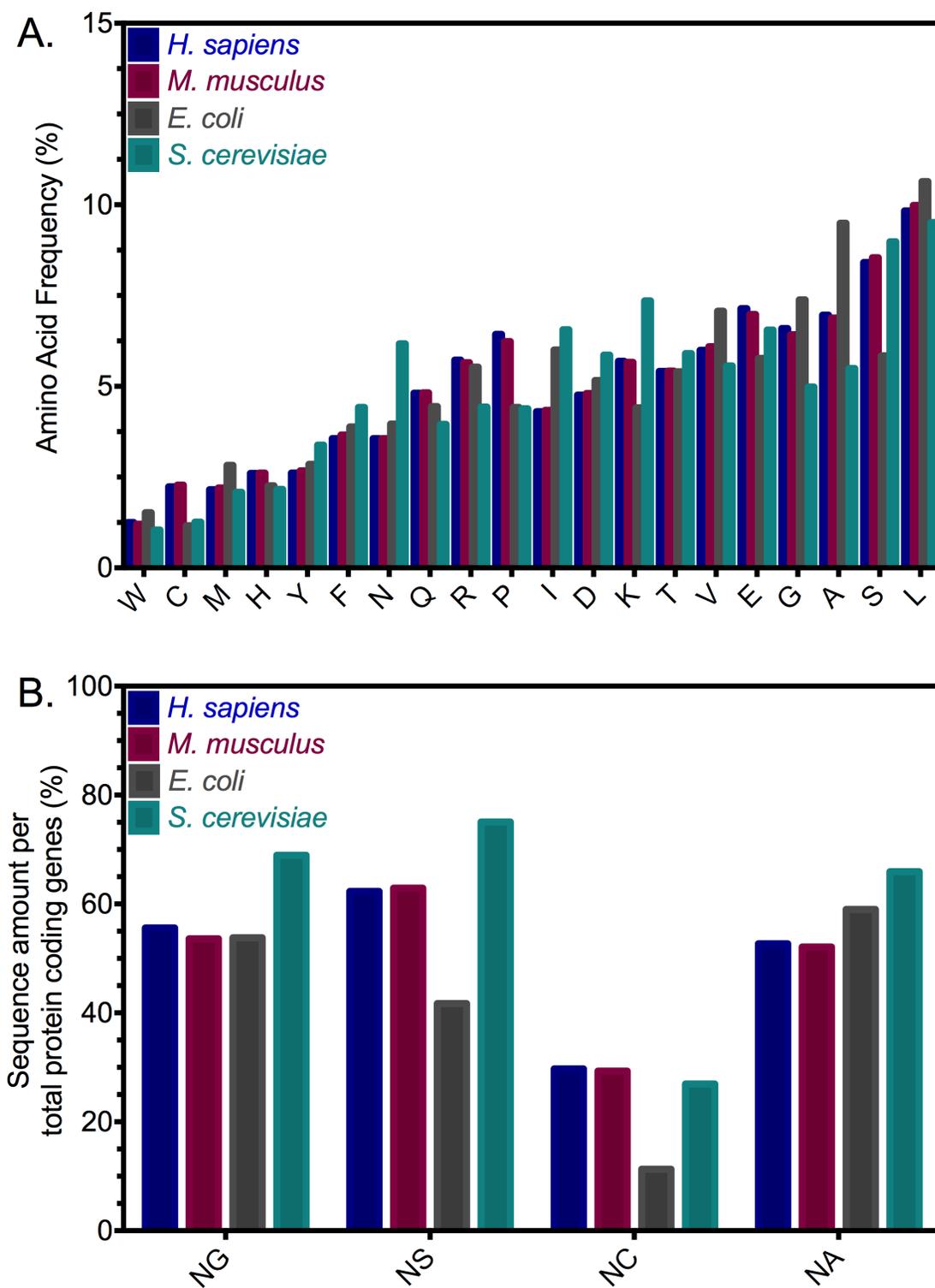


Figure 6

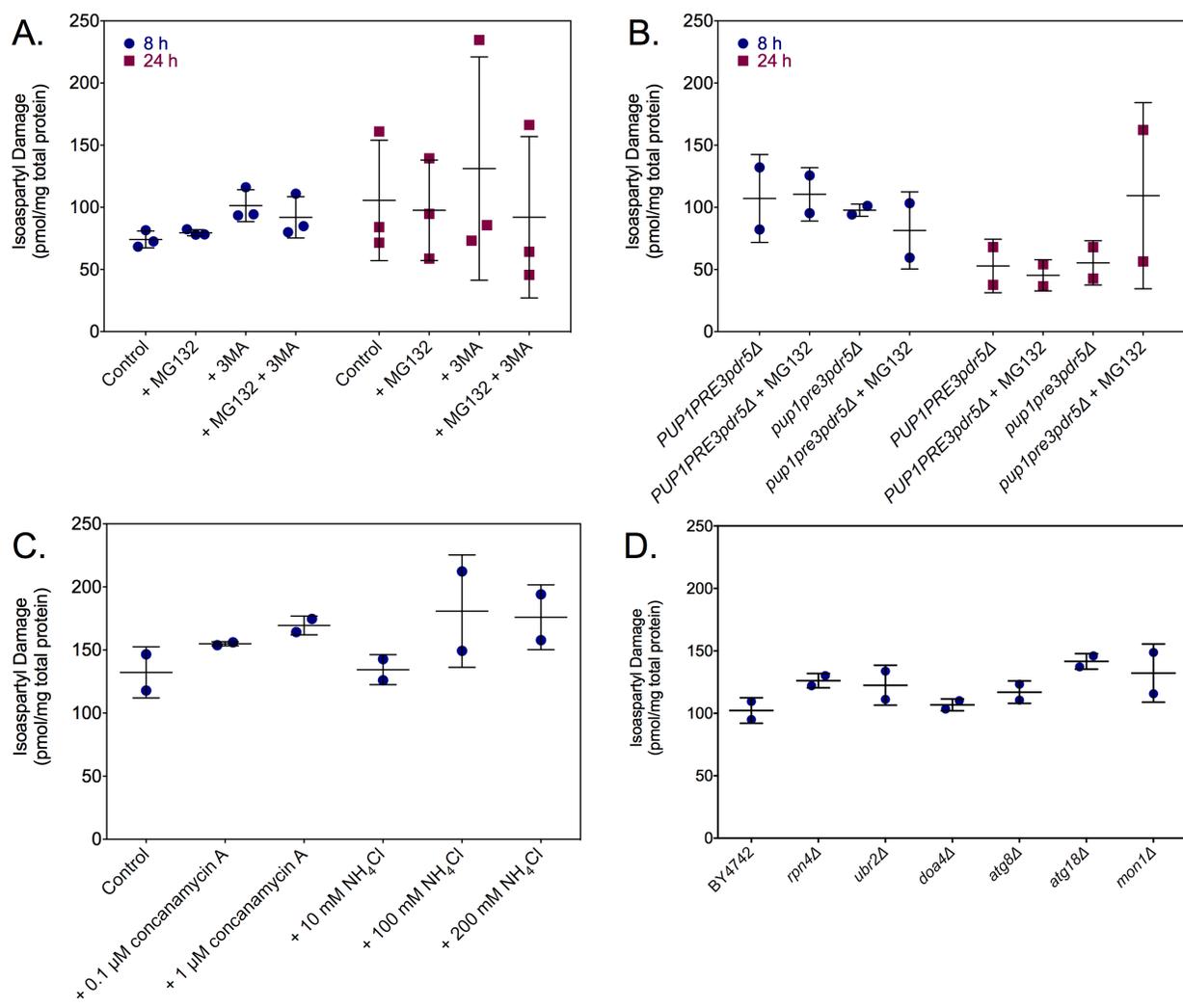


Figure 7

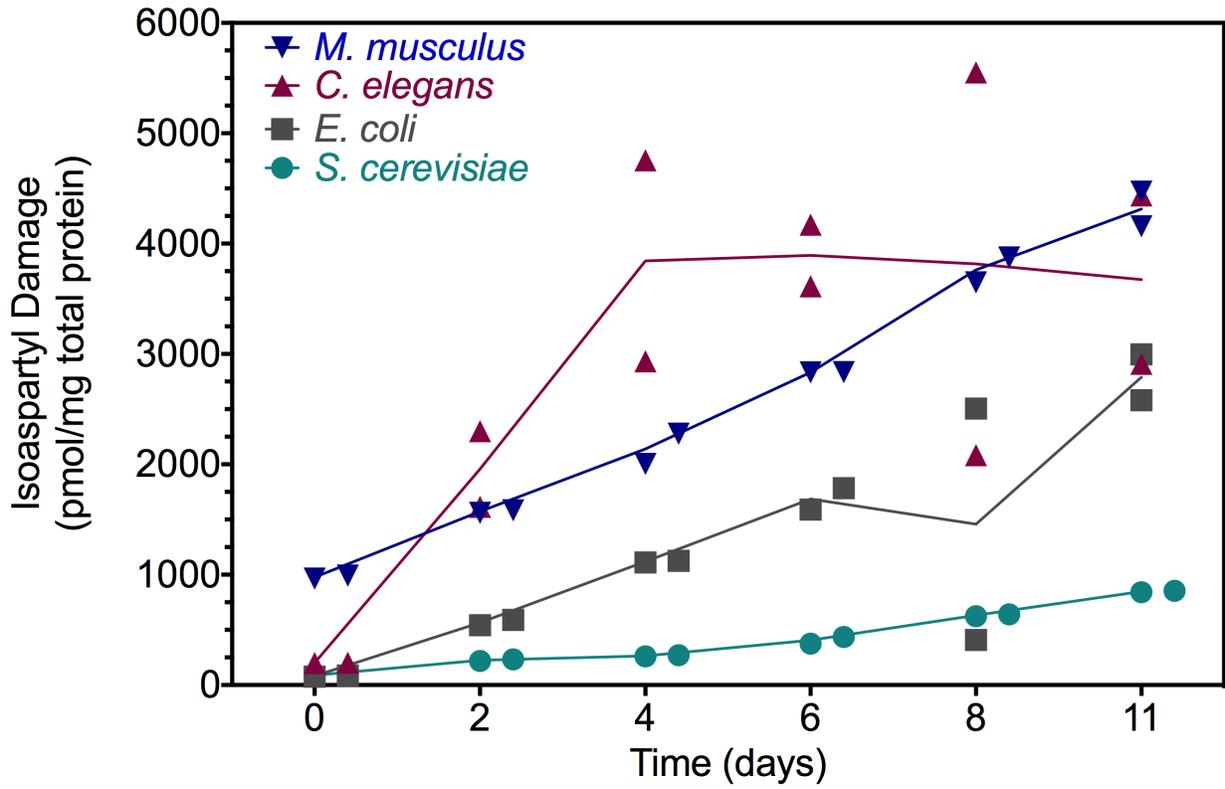


Figure 8

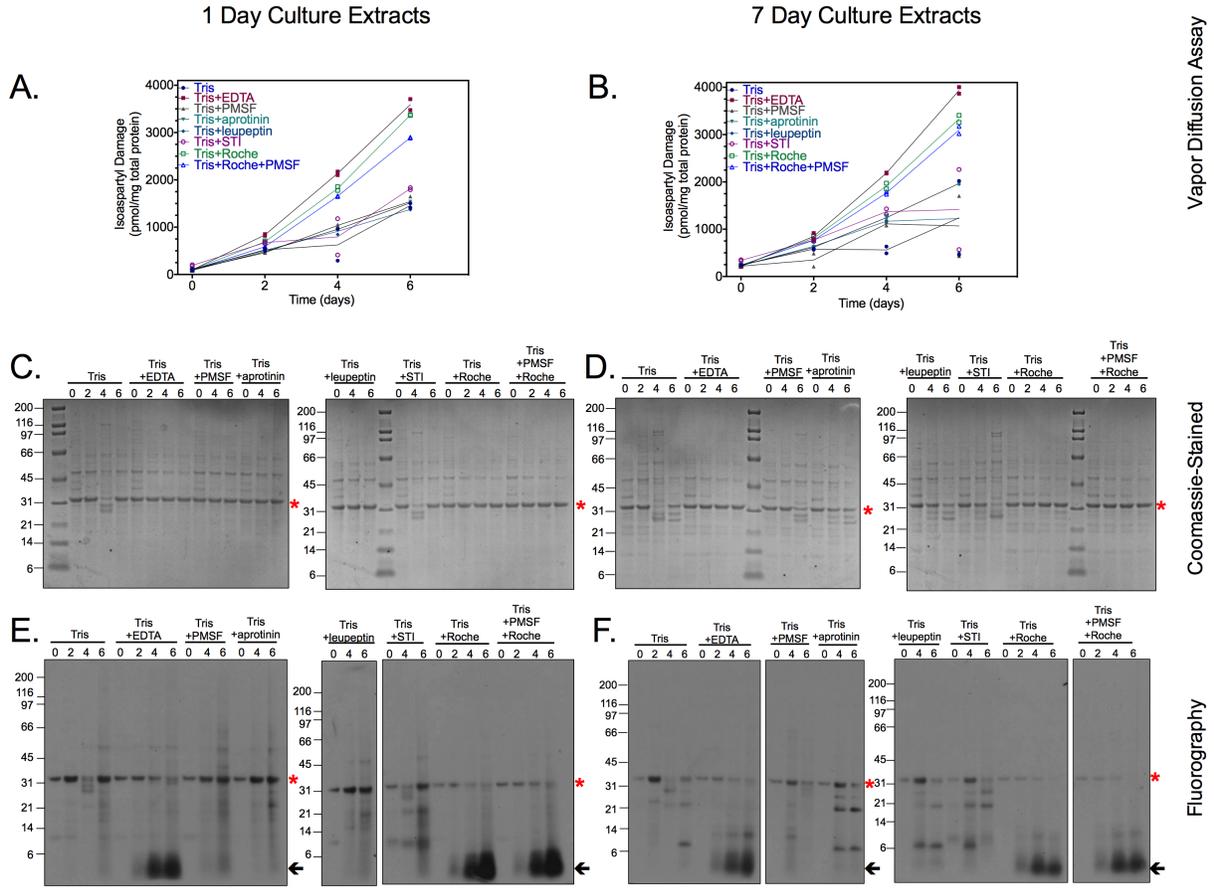


Figure 9

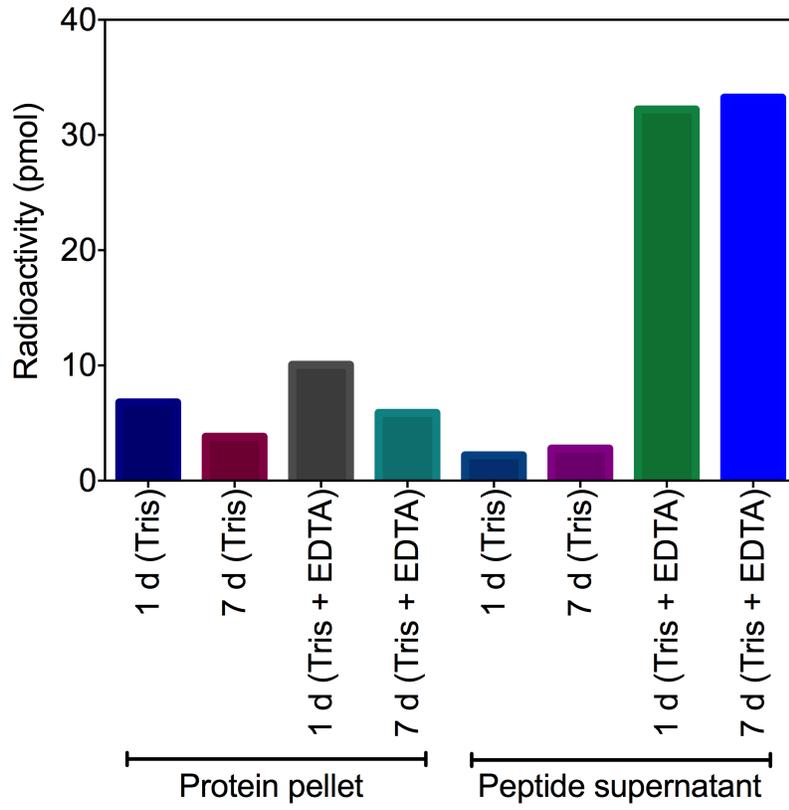


Figure 10

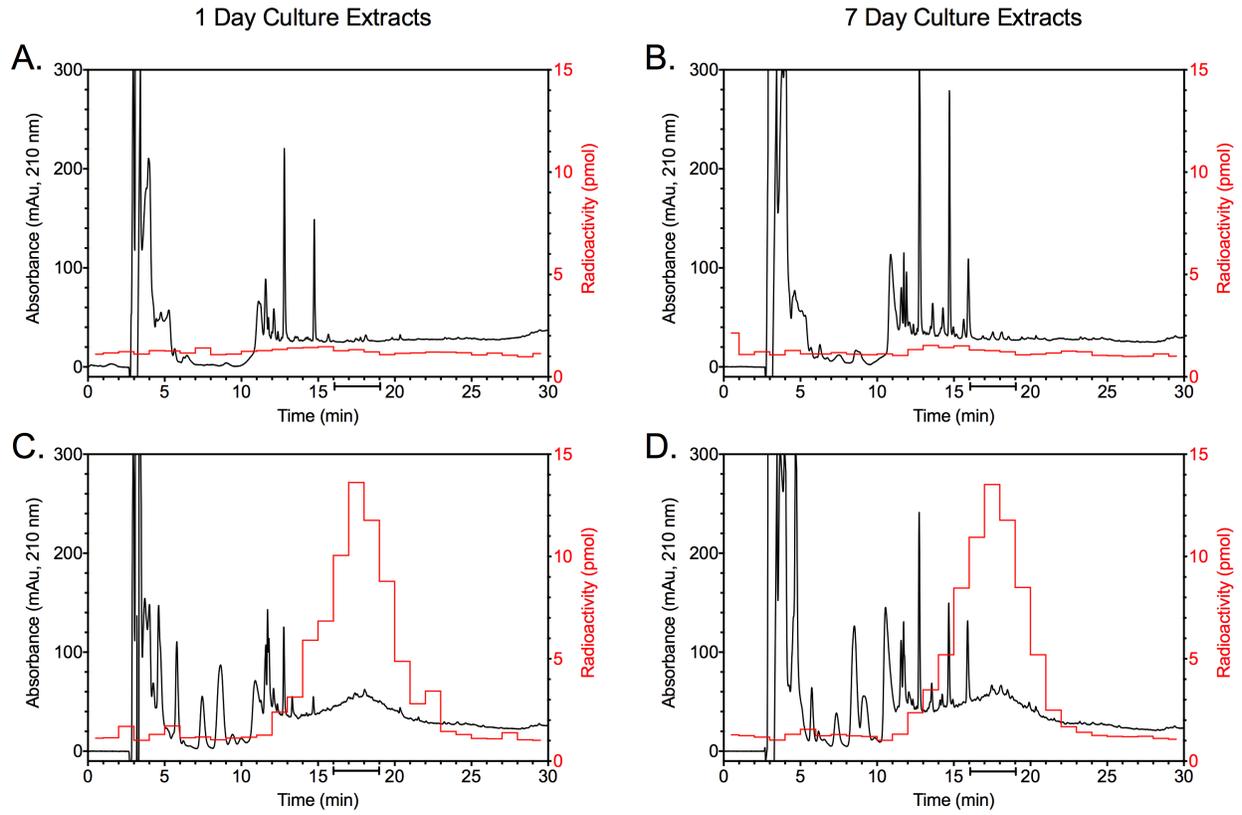




Figure 12

