microbial cell

Research Article

www.microbialcell.com

Sorbic acid triggers rapid formation of proteasome storage granules in *Saccharomyces cerevisiae*

Shoei Tanaka[1], Mitsuki Imajo[1], Mieko Hayashi[2], Akira Matsuura[2] and Shingo Izawa[1,*]

[1] Department of Applied Biology, Graduate School of Science and Technology, Kyoto Institute of Technology, Sakyo-ku, Kyoto 606-8585, Japan.
[2] Department of Biology, Graduate School of Science, Chiba University, Chiba 263-8522, Japan.

* Corresponding author:
Shingo Izawa Ph.D., Tel.: +81-75-724-7764; E-mail: thioredoxin@kit.ac.jp

ABSTRACT Proteasomes are primarily located within the nuclei of proliferating cells; however, their localization changes dynamically in response to environmental conditions. *Saccharomyces cerevisiae* forms proteasome storage granules (PSGs) in the cytoplasm upon glucose depletion, mitochondrial stress, transition to quiescence, or acetic acid stress, with intracellular acidification acting as a key trigger. Despite possessing similar dissociation constants, sorbic acid and acetic acid are noted to exert different physiological effects on yeast cells. In this study, we demonstrate that sorbic acid induces the fastest PSG formation ever reported (within 30 min) at lower concentrations than acetic acid, accompanied by the inhibition of proteasomal proteolysis. Conversely, our analysis of the required factors revealed that, similar to acetic acid, the proteasome subunits Sem1 and Rpn13 are essential for sorbic acid-induced PSG formation. In contrast, the shuttle factors Dsk2 and Rad23, along with the E3 ubiquitin ligase Hul5—which are required for PSG formation under mitochondrial stress or quiescence—are dispensable for sorbic acid-induced PSG formation. These findings not only identify a novel condition for rapid PSG formation but also provide new insights into the physiological effects of sorbic acid on the proteasome, highlighting both its similarities to and differences from acetic acid.

doi: 10.15698/mic2026.08.886
Received originally: 01.05.2026;
in revised form: 21.07.2026,
Accepted: 27.07.2026,
Published: 12.08.2026.

Keywords: sorbic acid, acidification, proteasome, proteasome storage granules, proteolysis, UBA-UBL shuttle factors, *Saccharomyces cerevisiae*.

Abbreviations:
pHi - intracellular pH,
PSG - proteasome storage granule,
SD medium - synthetic defined medium.

INTRODUCTION

The 26S proteasome is a highly conserved proteolytic machinery present in all eukaryotes, playing a pivotal role in maintaining proteostasis (protein homeostasis) and regulating diverse cellular functions [1, 2]. It mediates the degradation of damaged or misfolded proteins and regulatory proteins (e.g., cyclins, transcription factors) mainly via ubiquitination; this pathway is known as the ubiquitin-proteasome system (UPS) [2–4]. The 26S proteasome is a massive ATP-dependent protease complex (approximately 2.5 MDa), comprising a 20S core particle (20S CP) and one or two 19S regulatory particles (19S RPs) [5–7]. The 20S CP is a cylindrical structure composed of four stacked heptameric rings (two outer α-rings and two inner β-rings), where substrates are degraded within the internal lumen by trypsin-like, chymotrypsin-like, and caspase-like activities [3, 4, 8, 9]. The 19S RP, comprising 19 distinct subunits, caps one or both ends of the 20S CP. It utilizes ATP to mediate the recognition of ubiquitin chains, substrate deubiquitination, protein unfolding, and the subsequent translocation of substrates into the 20S CP [3, 4, 8, 9]. Beyond the ATP-dependent pathway, the free 20S CP can degrade proteins in a ubiquitin- and ATP-independent manner. Specifically, proteins with unstructured regions due to oxidative stress are highly susceptible to degradation by the free 20S CP [10, 11].

The 26S proteasome is primarily located in the nucleus of proliferating cells; however, its localization is dynamically regulated depending on stress and growth conditions [12–15]. Stress conditions, including amino acid starvation, hyperosmotic shock, and cellular senescence, induce the

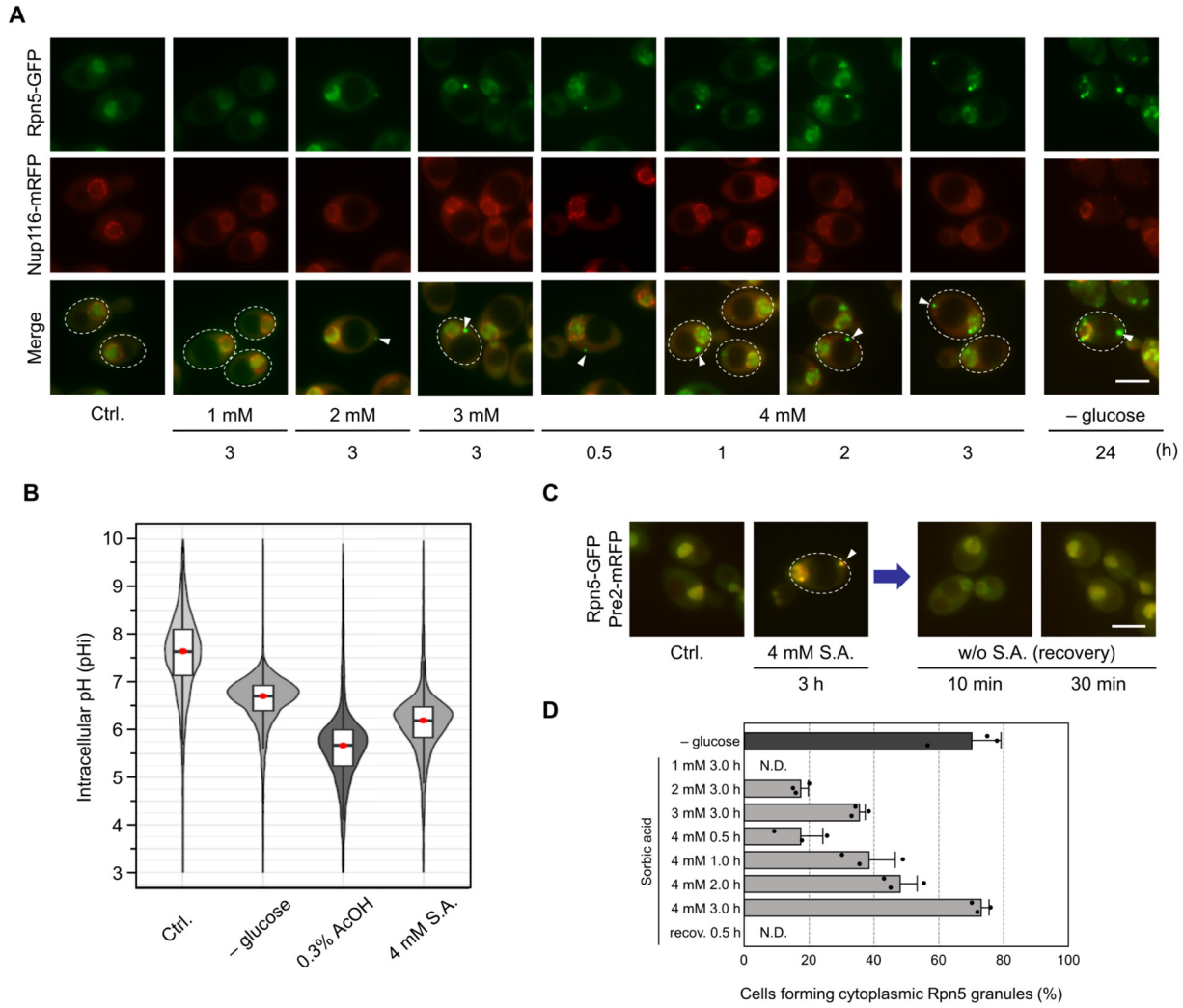

FIGURE 1 ● Sorbic acid stress causes the formation of cytoplasmic proteasome granules in yeast cells. **(A)** Yeast cells expressing Rpn5-GFP (a 19S RP lid subunit) and Nup116-mRFP (a nuclear envelope marker) were treated with sorbic acid stress or glucose depletion for the indicated times. White arrowheads denote cytoplasmic Rpn5 granules. **(B)** Cells were subjected to stress conditions: glucose depletion, 0.3% acetic acid, or 4 mM sorbic acid for 1 h. Subsequently, pHi was determined by measuring pHluorin2 via flow cytometry. **(C)** Reversibility of sorbic acid-induced proteasome granules. Yeast cells treated with 4 mM sorbic acid for 3 h were transferred to fresh SD medium without sorbic acid. **(D)** Quantitative analysis of cells containing cytoplasmic proteasome granules. Data are based on three independent experiments, with a total of over 300 living cells observed per condition. No granule formation was observed under non-stressed conditions. Scale bar, 5 μm. S.A., sorbic acid.

formation of nuclear proteasome condensates in mammalian cells [14–18]. These condensates sequester ubiquitinated proteins and the proteasome shuttle factor RAD23B, which contains ubiquitin-associated (UBA) and ubiquitin-like (UBL) domains [14, 17, 19–21]. Yeast proteasomes also localize to the perinuclear region in quiescent cells and in cells treated with the proteasome inhibitor MG132 [22, 23]. In the cytoplasm, the formation of proteasome storage granules (PSGs) is well known as a yeast-specific phenotype [18]. PSGs are also known to contain the UBA-UBL shuttle factors Dsk2 and Rad23 (a yeast homolog of mammalian RAD23B), as well as ubiquitinated proteins [24, 25]. A model has been proposed in which the formation of nuclear proteasome condensates and PSGs is driven by liquid–liquid phase separation (LLPS), mediated by multivalent interactions between ubiquitinated proteins, shuttle factors, and proteasomes [12, 14, 24, 26]. In contrast, a recent study has shown that PSGs are paracrystalline arrays composed of bundled fibers formed by the stacking of proteasome trimers [27]. While the mechanisms underlying PSG formation are thus becoming increasingly elucidated, their physiological significance remains largely elusive.

PSG formation is induced by acute glucose depletion, quiescence, and mitochondrial stress, all of which reduce intracellular pH (pHi) and ATP levels, alongside altering metabolism [12, 18, 24, 26–30]. Furthermore, it has been demonstrated that intracellular acidification alone is sufficient to induce PSG formation [26]. We recently reported that sublethal acetic acid stress (52.5 mM), which also reduces pHi [31–33], induces PSG formation with faster kinetics than conventional methods such as acute glucose depletion [25]. We also confirmed that the factors required for PSG formation differ among these induction conditions.

Sorbic acid is a lipophilic weak acid that has a dissociation constant similar to that of acetic acid (p*Ka* = 4.76 at 25 °C). Interestingly, while sorbic acid exhibits fungistatic effects on *S. cerevisiae* at significantly lower concentrations than acetic

acid, it exerts a more modest impact on pHi reduction [34–38]. Although it has been suggested that sorbic acid and acetic acid differ in their effects on yeast and bacterial cells, as well as in the corresponding yeast responses [39–42], the precise effects of sorbic acid on yeast cells are not yet fully understood. Similarly, the specific responses of yeast to this stressor remain insufficiently characterized.

Based on this background, we investigated whether sorbic acid possesses the capacity to induce PSG formation and compared its efficacy with that of acetic acid. Our results show that 4 mM sorbic acid rapidly induces PSG formation, exceeding the efficacy of acetic acid and other known PSG induction conditions. Additionally, we found that sorbic acid treatment inhibits proteasomal proteolysis. To elucidate the underlying mechanisms of PSG formation, we compared the factors required for PSG formation between sorbic acid and other established induction conditions. Our results offer novel insights into the physiological response of yeast to sorbic acid and the regulatory pathways of PSG formation, identifying similarities and differences in the regulatory pathways of PSG formation induced by acetic acid.

RESULTS

Sorbic acid causes the formation of PSGs

To investigate whether sorbic acid induces PSG formation, we used Rpn5-GFP as a canonical PSG marker [24, 26, 43]. Following a previous report that sublethal concentrations of acetic acid trigger PSG formation [25], we evaluated sorbic acid at concentrations up to 4 mM, a range established as a sublethal in prior studies [44, 45]. While 1 mM sorbic acid failed to induce cytoplasmic Rpn5 granules, concentrations of 2 mM or higher successfully triggered their formation (**Figure 1A**). Specifically, 4 mM sorbic acid induced the formation of cytoplasmic Rpn5 granules in approximately 20% of the cell population within 30 min, increasing to over 70% after 3 h (**Figures 1A** and **1D**). In contrast, concentrations of 3 mM or lower resulted in Rpn5 granule formation in less than half of the cells after 3 h. Based on these results, 4 mM sorbic acid was used for all subsequent experiments. Treatment with 4 mM sorbic acid was confirmed to reduce pHi (**Figure 1B**).

Upon removal of sorbic acid by medium exchange, the cytoplasmic Rpn5-GFP granules induced by 4 mM sorbic acid treatment rapidly dissociated, and the signal relocalized to the nucleus (**Figure 1C**). This reversibility supports the notion that sorbic acid-induced Rpn5 granules are characteristically similar to PSGs [24, 25].

Next, we examined the localization changes of other proteasome subunits. Similar to Rpn5, other subunits formed cytoplasmic granules with nearly identical granule formation rates under 4 mM sorbic acid stress (**Figure 2**). Given that all examined proteasome subunits formed cytoplasmic granules and colocalized with Pre2 or Pre4 (20S CP subunits), we conclude that sorbic acid induces the formation of PSGs, mirroring the effects of acetic acid.

Sorbic acid inhibits proteasomal proteolysis and causes the accumulation of ubiquitinated proteins

Next, we examined the relationship between PSG formation and proteasome activity under sorbic acid stress. *In vivo* proteasomal proteolysis was monitored via cycloheximide (CHX)-chase analysis using an auxin-inducible degron (AID) system [46]. Although the addition of indole-3-acetic acid (IAA) rapidly induced the proteasomal degradation of Paf1–AID*–6FLAG under non-stressed conditions [47, 48], this effect was clearly suppressed by 4 mM sorbic acid (**Figures 3A** and **3C**).

We also examined the effect of sorbic acid on ubiquitin-independent proteasomal proteolysis by monitoring the degradation of ornithine decarboxylase Spe1, which is known to be degraded in a ubiquitin-independent manner in the presence of polyamines such as spermidine [48–51]. CHX-chase analysis revealed that spermidine-induced proteasomal proteolysis of Spe1-3HA was also inhibited in the presence of 4 mM sorbic acid (**Figures 3B** and **3C**). The extent of this inhibition was dependent on the sorbic acid concentration (1–4 mM) and showed a trend toward correlation with the degree of PSG formation (Figure S1).

Furthermore, increased levels of ubiquitinated proteins were observed in yeast cells under sorbic acid stress (**Figure 3D**). These results clearly indicate that proteasomal proteolysis was inhibited under sorbic acid stress, similar to the effect of acetic acid stress [25].

Shuttle factors are dispensable for PSG formation under sorbic acid stress

The UBA–UBL shuttle factors, Dsk2 and Rad23, are crucial for PSG formation during mitochondrial stress or quiescence, but are dispensable for that induced by acute glucose depletion or acetic acid [24, 25]. To determine their role in response to sorbic acid stress, we monitored PSG formation in *dsk2Δrad23Δ* cells. Exposure to 4 mM sorbic acid induced PSG formation in over 50% of the double-mutant cells (**Figure 4A**), indicating that Dsk2 and Rad23 are not strictly required for PSG formation under sorbic acid stress.

Notably, however, both Dsk2-GFP and Rad23-GFP colocalized with the proteasome subunit Pre2-mRFP upon sorbic acid stress (**Figure 4B**). These results suggest that while these shuttle factors are sequestered into PSGs under this condition—consistent with other induction conditions [24, 25]—they are not the primary drivers of PSG formation in this context.

Another yeast UBA–UBL shuttle factor, Ddi1, possesses a viral protease domain that cleaves substrates with long polyubiquitin chains [24, 52]. Consistent with this, *ddi1Δ* cells exhibit accumulation of polyubiquitinated substrates and spontaneous PSG formation even under non-stressed conditions [24, 51]. While Ddi1 is known to form cytoplasmic condensates in quiescent cells and under glucose depletion or acetic acid stress, these condensates localize independently of PSGs [24, 25]. We found that sorbic acid also induced the formation of Ddi1 condensates, which localized independently of PSGs (**Figure 5**).

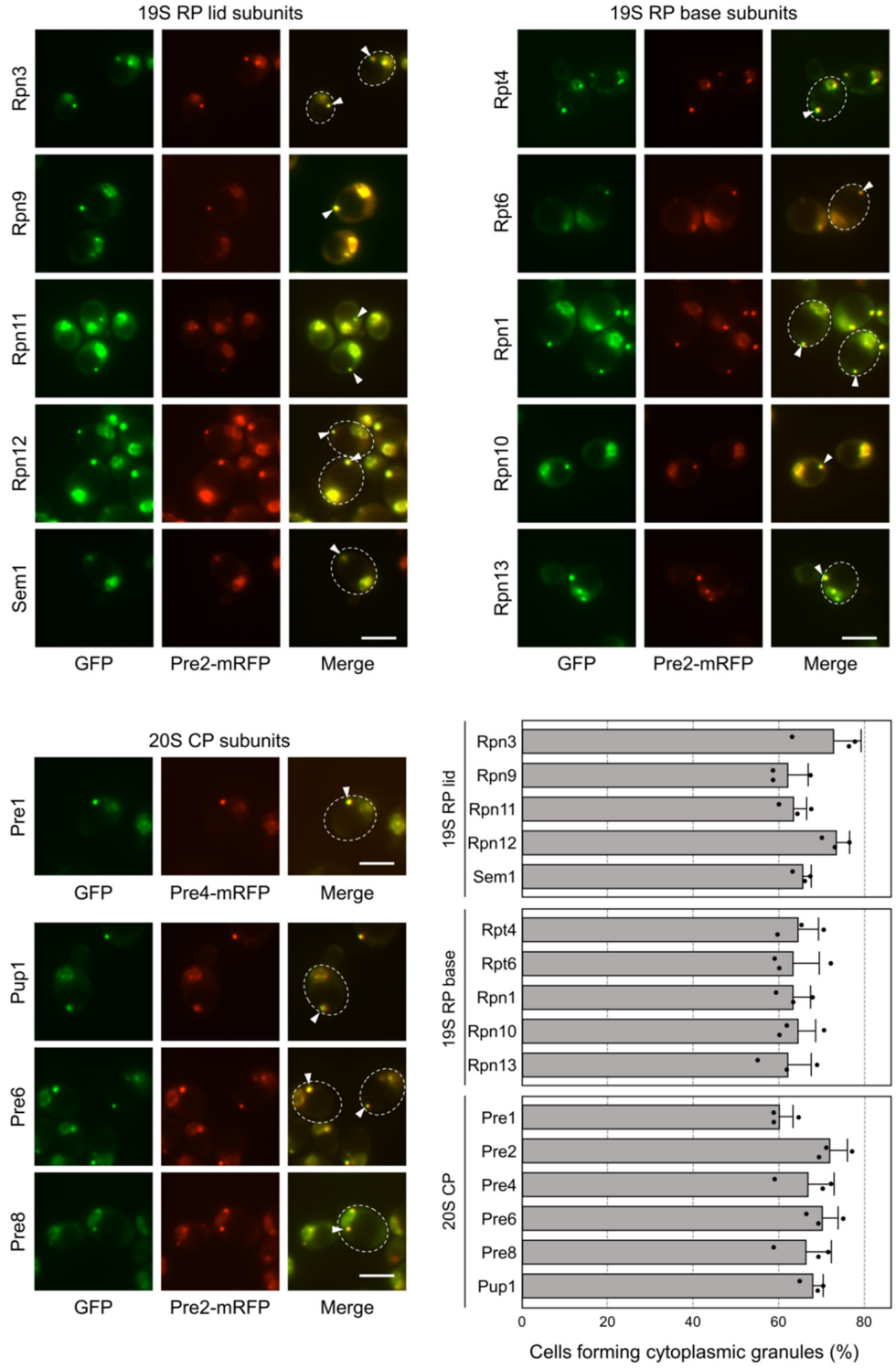

FIGURE 2 ● **19S RP subunits form granules with 20S CP subunits under sorbic acid stress.** Cells expressing one of the GFP-tagged proteasome subunits and either mRFP-tagged Pre2 (β5 of 20S CP) or Pre4 (β7 of 20S CP) were treated with 4 mM sorbic acid for 3 h. White arrowheads indicate the cytoplasmic proteasome granules. Scale bar, 5 μm. The right bottom panels indicate the granule formation rate of each proteasome subunit examined in this study. No granule formation was observed in any subunit under non-stressed conditions.

Sem1 and Rpn13 are indispensable for sorbic acid-induced PSG formation

In addition to UBA-UBL shuttle factors, the factors required for PSG formation vary depending on the induction conditions [23–25, 53]. In this study, we examined sorbic acid-induced PSG formation in strains deficient in the *HUL5* gene or non-essential proteasome subunit genes (*PRE9*, *RPN10*, *RPN13*, and *SEM1*).

Sem1, which is indispensable across all previously reported induction conditions [25, 54], was also essential for sorbic acid-induced PSG formation; accordingly, *sem1Δ* cells failed to

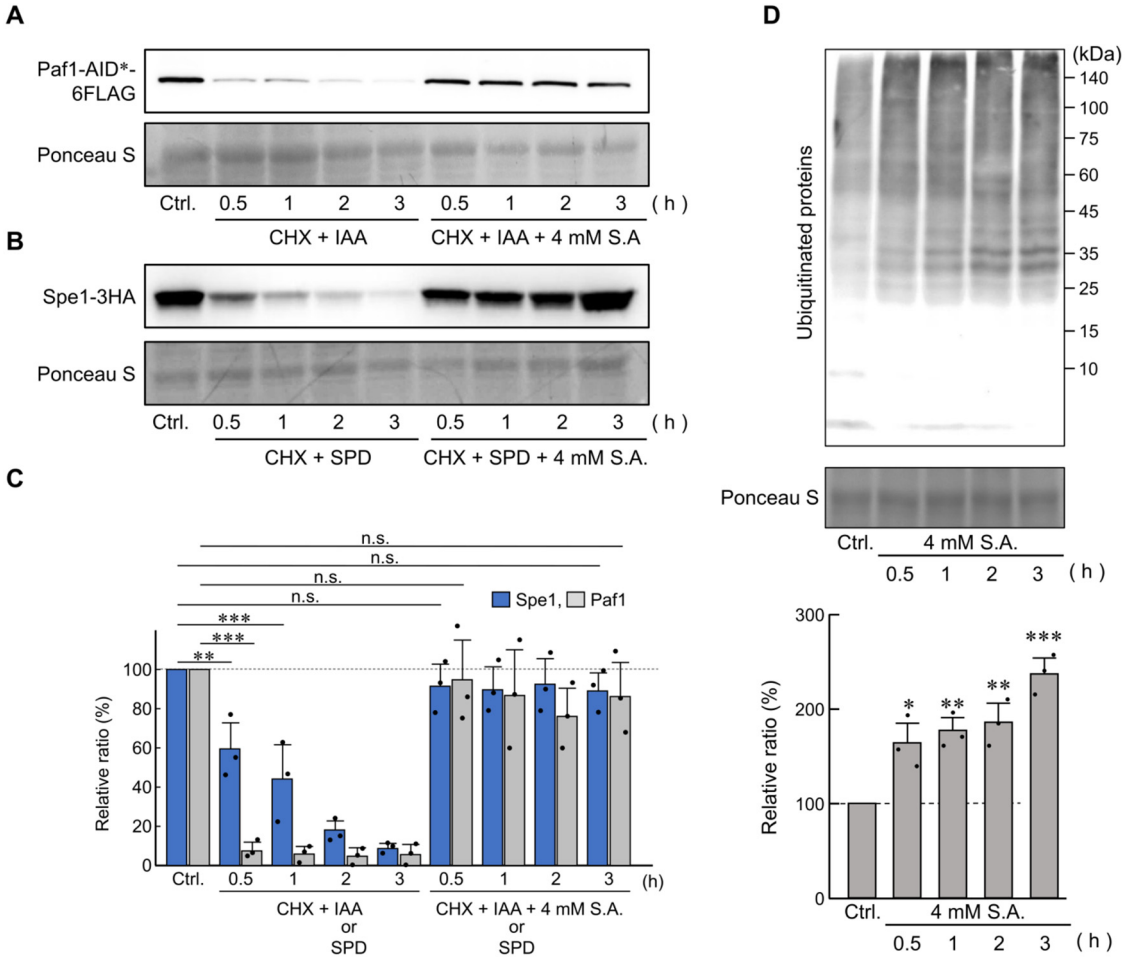

FIGURE 3 ● Sorbic acid stress inhibits proteasomal proteolysis. **(A)** Assessment of proteasomal proteolysis efficiency using the auxin-inducible degron (AID) system. Cells expressing Paf1–AID*–6FLAG were treated with 0.75 mM indole-3-acetic acid (IAA) and 200 μg/mL cycloheximide (CHX), with or without 4 mM sorbic acid, for the indicated durations. **(B)** Assessment of ubiquitin-independent proteasomal degradation using Spe1-3HA. Cells were treated with 1 mM spermidine (SPD) and 200 μg/mL CHX to induce Spe1 degradation, in the presence or absence of 4 mM sorbic acid. **(C)** Quantitative analysis of Paf1–AID*–6FLAG and Spe1-3HA levels normalized to Ponceau S staining. n.s., statistically not significant; $^{**}p < 0.01$, $^{***}p < 0.005$ (Dunnett's test, $n = 3$). **(D)** The levels of ubiquitinated proteins were detected by western blotting using an anti-ubiquitin antibody. Ubiquitinated protein levels were normalized to Ponceau S staining (*right bottom panel*). Statistical significance was assessed using Dunnett's test ($n = 3$); $^{*}p < 0.05$,$^{**}p < 0.01$, $^{***}p < 0.005$. S.A., sorbic acid.

form PSGs in response to sorbic acid stress (**Figure 6**). Similarly, Rpn13—required for PSG formation under acetic acid stress but not during glucose depletion [24, 25]—was necessary for sorbic acid-induced PSG formation. The essentiality of Sem1 and Rpn13 was further verified through complementation assays employing pRS-*SEM1* and pRS-*RPN13* (**Figure 6**). In contrast, the deletion of *PRE9* and *RPN10* had no discernible effect on PSG formation under sorbic acid stress.

Hul5, a proteasome-interacting E3 ubiquitin ligase, is required for PSG formation during mitochondrial stress or quiescence, but is dispensable for PSG formation triggered by acute glucose depletion or acetic acid stress [23, 25]. Similar to observations under glucose depletion and acetic acid stress, *hul5Δ* cells induced PSG formation under sorbic acid stress (**Figure 6**). These results indicate that there is no difference between sorbic acid and acetic acid regarding the factors required for PSG formation, as far as we investigated in this study. The survival rates of the null mutants used in this study remained high after treatment with 4 mM sorbic acid for 3 h (**Figure 7**).

DISCUSSION

This study revealed a novel physiological effect of sorbic acid on yeast proteasomes. Treatment with 4 mM sorbic acid induced the formation of PSGs, accompanied by the inhibition of proteasomal proteolysis. The most noteworthy finding is that sorbic acid triggers PSG formation with remarkably accelerated kinetics. Compared to conventional induction conditions—such as glucose deprivation, transition to quiescence, or mitochondrial inhibition, which require 12 to 24 h for sufficient PSG formation [12, 24, 29] or acetic acid stress, which necessitates 9 h [25]—4 mM sorbic acid induced detectable PSG formation within approximately 30 min. Furthermore, 4 mM sorbic acid stress induced PSGs in the majority of the cell population within 3 h. This rapid induction provides a significant methodological advantage for enhancing the throughput and efficiency of PSG research.

Consistent with its established antimicrobial properties [37, 39, 41], sorbic acid induced PSG formation at lower concentrations than acetic acid [25]. In contrast, previous

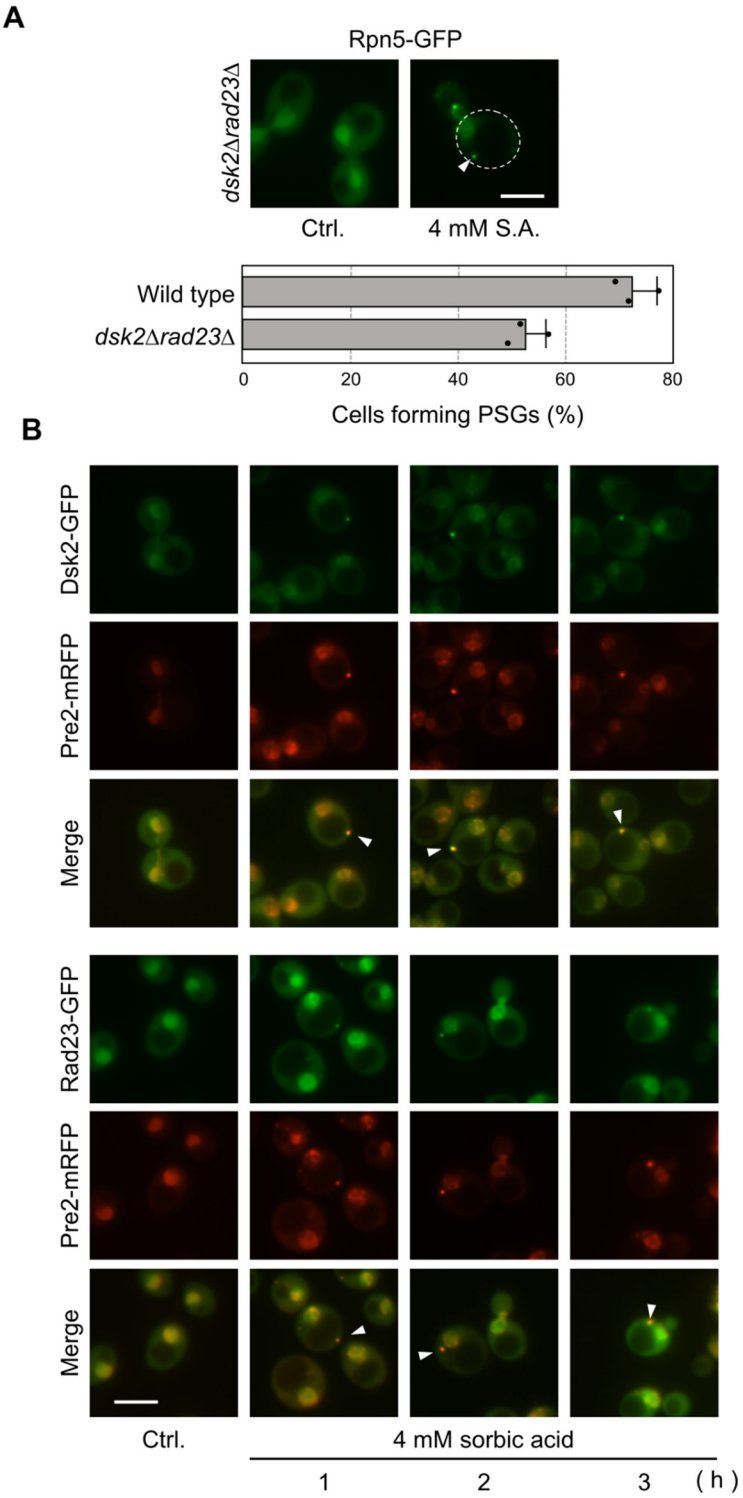

FIGURE 4 ● **Dsk2 and Rad23 colocalize with PSGs but are not essential for PSG formation under sorbic acid stress.** (**A**) PSG formation was examined using Rpn5-GFP in *dsk2Δrad23Δ* cells under 4 mM sorbic acid stress for 3 h. White arrowheads indicate PSGs. The lower panel shows the PSG formation rates of wild-type and *dsk2Δrad23Δ* cells exposed to 4 mM sorbic acid for 3 h. (**B**) Intracellular localization of Dsk2-GFP and Rad23-GFP was examined in wild-type cells expressing Pre2-mRFP. Cells were exposed to 4 mM sorbic acid stress for the indicated periods. White arrowheads indicate granules where Dsk2-GFP or Rad23-GFP colocalizes with Pre2-mRFP. Scale bar, 5 μm.

studies have reported that sorbic acid exerts a weaker effect on pHi reduction than acetic acid [36, 37]. Our data corroborate these reports, demonstrating that sorbic acid-induced pHi reduction was more modest than that caused by acetic acid, even at PSG-inducing concentrations. Nevertheless, the degree of acidification under sorbic acid stress exceeded that triggered by glucose depletion. These results suggest that, unlike lactic acid and 10% ethanol [25], sorbic acid is capable of reducing pHi below the critical threshold required for PSG formation [26]. Notably, the kinetics of PSG formation did not correlate

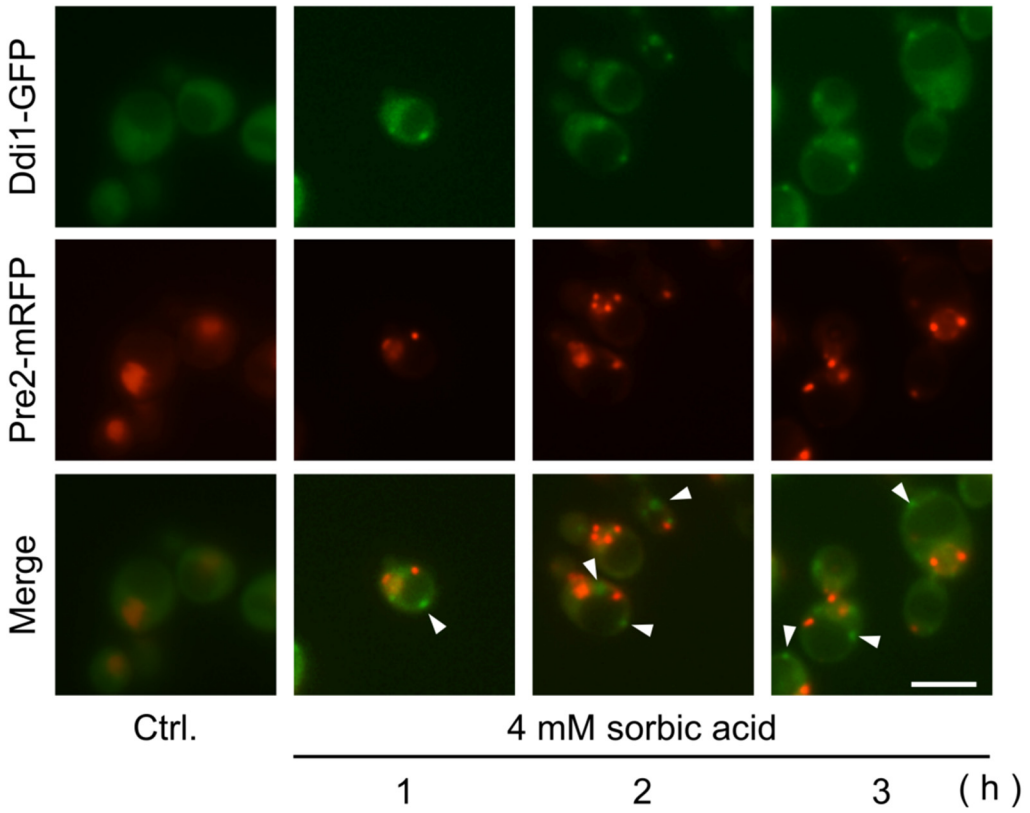

FIGURE 5 ● **Sorbic acid induces Ddi1 condensate formation.** Ddi1-GFP localization was examined in wild-type yeast cells expressing Pre2-mRFP. Cells were subjected to sorbic acid stress for the indicated periods. White arrowheads indicate Ddi1-GFP condensates that did not colocalize with Pre2-mRFP. Scale bar, 5 μm.

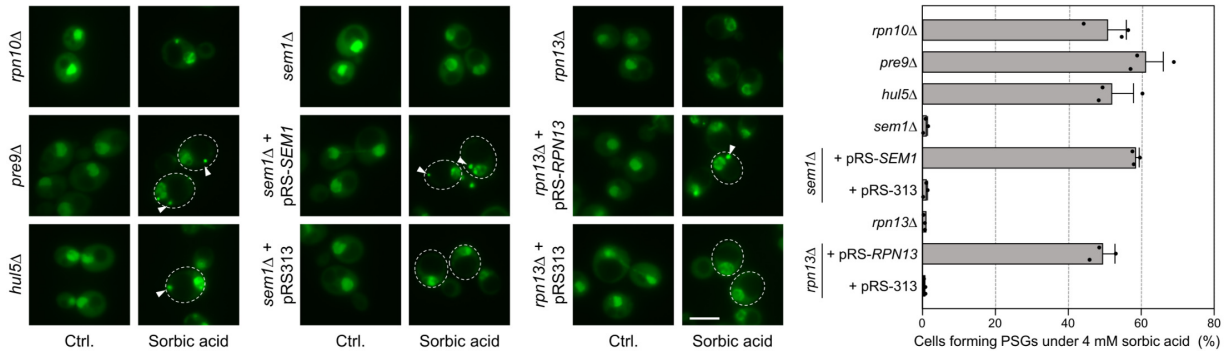

FIGURE 6 ● **PSG formation is mitigated in *rpn13Δ* and *sem1Δ* cells under sorbic acid stress.** PSG formation in *rpn10Δ*, *pre9Δ*, *hul5Δ*, *sem1Δ*, and *rpn13Δ* cells was examined using Rpn5-GFP. Cells were treated with 4 mM sorbic acid for 3 h. *sem1Δ* and *rpn13Δ* cells were transformed with pRS313-*SEM1* and pRS313-*RPN13*, respectively, while the empty vector (pRS313) served as a control. White arrowheads indicate PSGs and the PSG formation rates after 3 h of treatment with 4 mM sorbic acid are shown in the right panel. No granule formation was observed under non-stressed conditions. Scale bar, 5 μm.

with the magnitude of the pHi decline. Although the degree of acidification varied depending on the stressors [26, 30, 33, 55], these differences were not reflected in the timeline of PSG formation. This suggests that the extent of acidification is not the rate-limiting factor governing the kinetics of PSG assembly. Furthermore, the distinct kinetics of PSG formation between acetic and sorbic acids may be influenced by modes of action independent of their pHi-lowering effects [41, 45, 56–58].

PSG formation induced by the transition to quiescence or mitochondrial stress is significantly impaired in *dsk2Δrad23Δ* and *hul5Δ* cells [23–25, 53]. In contrast, these genetic deficiencies have a negligible impact on PSG formation triggered by sorbic acid (Figures 4A and 6) or acetic acid stress [25]. While the requirements for Rpn10 and Rpn13 in PSG formation vary depending on the induction conditions [24, 25], Rpn10 was dispensable for sorbic acid-induced PSG formation, consistent with its role in the response to acetic acid stress [25]. Conversely, Rpn13 was essential for PSG formation under both sorbic and acetic acid stress conditions. Additionally, we confirmed that

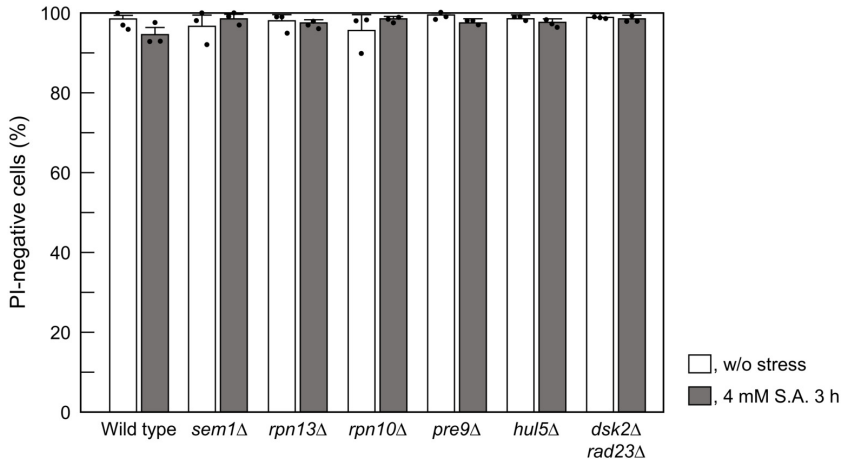

FIGURE 7 ● **Cell viability following sorbic acid stress treatment.** Cells were exposed to 4 mM sorbic acid in SD medium for 3 h. Cell viability was then assessed by propidium iodide (PI) staining, with PI-negative cells considered viable. Data are based on three independent experiments, with a total of over 300 living cells observed per condition. S.A., sorbic acid.

Sem1 is essential for PSG formation under all conditions examined [25, 54]. Despite differences in formation kinetics and effective concentrations, the shared necessity of these factors suggests a highly similar underlying mechanism for PSG formation induced by sorbic and acetic acids. Elucidating the effects of various weak acids may provide critical insights into the physicochemical triggers and the diversity of induction pathways of PSG formation.

Although both Rpn10 and Rpn13 serve as ubiquitin receptors for the proteasome, only Rpn13 is essential for sorbic acid-induced PSG formation. Although further studies are warranted, as discussed previously [25], this difference may tentatively be explained by distinct post-translational modifications of these two subunits. In contrast, Sem1 is required for PSG formation under all tested conditions, acting as a chaperone for 19S RP assembly [59]. Previous studies have also demonstrated that *sem1* deletion reduces 26S proteasome stability and impairs its activity [60, 61]. Together, these findings suggest that the inefficient 19S RP assembly in *sem1Δ* cells limits the cellular abundance of fully assembled 26S proteasomes, consequently impairing PSG formation.

The present study also demonstrates that sorbic acid inhibits proteasomal proteolysis and causes the accumulation of ubiquitinated proteins, paralleling the effects of acetic acid stress and other PSG induction conditions [25, 62–66]. Combined with our recent finding that sorbic acid suppresses translation activity [45], it is clear that sorbic acid inhibits both protein synthesis and degradation within the yeast proteostasis network. Given that all known PSG induction conditions—including sorbic acid stress—lead to decreased intracellular ATP levels and reduced ATP consumption [24, 27, 42, 67–70], this suppression likely represents an energy-saving response necessitated by limited ATP availability. Under glucose starvation, yeast cells enter an energy-saving and protective survival state [71]; a comparable state may be induced by sorbic acid stress. Furthermore, since ATP depletion destabilizes the proteasome [15, 62, 65], our results suggest that the inhibition of proteasomal proteolysis is both a consequence and a driver of the metabolic stress response, forming a feedback loop that regulates energy status and proteostasis.

It is also of considerable interest to compare sorbic acid-induced PSGs with mammalian LLPS-driven proteasome condensates [14]. While mammalian nuclear proteasome condensates facilitate the degradation of unassembled ribosomal proteins [14], specific substrates for proteasomes that co-localize with yeast PSGs remain to be identified. Furthermore, yeast proteasomal activity is significantly impaired under stress conditions [25], including sorbic acid treatment. Collectively, these observations suggest that yeast PSGs likely serve a distinct physiological role rather than acting as proteolytic hubs.

A recent cryo-electron tomography study demonstrated that quiescent phase-induced PSGs form paracrystalline arrays in an inactive S1-like conformation [27]. Because the biophysical properties and ultrastructure of sorbic acid-induced PSGs were not investigated in the present study, whether these granules also adopt a paracrystalline structure or represent a distinct type of proteasome condensate remains to be elucidated. Further structural analyses utilizing cryo-electron tomography will be indispensable to define their precise organization. In line with this, the nuclear localization pattern of proteasomes under sorbic acid stress appears to differ from that observed during the quiescent phase [25]. This finding may reflect distinct underlying mechanisms or kinetics of PSG formation associated with these two stressors.

In conclusion, this study demonstrates that sorbic acid induces rapid PSG formation and suppresses proteasomal proteolysis. This proteostatic disruption likely contributes to its fungistatic effects by interfering with the precise regulation of key proteins, such as cyclins. Furthermore, sorbic acid represents a valuable tool for promptly inducing PSG formation. This rapid induction—occurring within a few hours—significantly enhances downstream experimental efficiency, particularly when screening for factors required for PSG formation, while minimizing the confounding effects typically associated with prolonged stress treatments.

MATERIALS AND METHODS

Yeast strains and stress treatment

The wild-type strain BY4742 (*MATα ura3Δ0 his3Δ1 leu2Δ0 lys2Δ0*) and its isogenic knockout mutants (*hul5Δ::kanMX, pre9Δ::kanMX, rpn10Δ::kanMX, rpn13Δ::kanMX,* and *sem1Δ ::kanMX*) were purchased from Open Biosystems (Huntsville). Yeast strains expressing GFP- or mRFP-tagged proteasome subunits and *dsk2Δrad23Δ* used in this study are as described in our recent publication [25]. Rpn5-GFP is integrated into the *RPN5* locus on the genome (present at a single copy per cell) and is under the expression control of the endogenous *RPN5* promoter. All other proteasome subunits tagged with GFP or mRFP were similarly integrated into the genome. Synthetic defined (SD) medium (2% glucose, 0.67% yeast nitrogen base without amino acids, 20 mg/L uracil, 30 mg/L L-lysine HCl, 100 mg/L L-leucine, and 20 mg/L L-histidine HCl, pH 5.6) was used for cultivation of yeast cells with reciprocal shaking (120 rpm) at 28 °C, and exponentially growing cells were harvested at an optical density at 600 nm (OD_{600}) of 0.5. The sorbic acid stress treatment was conducted by resuspending the harvested cells in fresh SD medium containing sorbic acid. The cell death rate was assessed using propidium iodide (PI) staining [72].

CHX-chase analysis and western blotting

The efficiency of proteasomal degradation was monitored by CHX-chase analysis using 200 μg/mL CHX (Nacalai Tesque, Kyoto, Japan) [48]. Ubiquitinated proteins were detected using an anti-ubiquitin antibody (P4D1; Santa Cruz Biotechnology, Dallas, TX, USA). Other antibodies used for western blotting included anti-FLAG antibody (F1804; Sigma-Aldrich, St Louis, MO, USA), anti-HA monoclonal antibody (M180–3; Medical and Biological Laboratories Co., Ltd., Tokyo, Japan), and anti-mouse IgG, HRP-linked antibody (7076S; Cell Signaling Technology, Danvers, MA, USA). Signals were detected using Chemi-Lumi One L (Nacalai Tesque) and a Multi Imager II Chemi Box (BioTools Inc., Gunma, Japan). Equal protein loading and transfer were confirmed by Ponceau S staining. Protein levels were quantified using ImageJ and normalized to the Ponceau S staining intensity.

Fluorescent microscopic analysis

Proteasome granule formation was analyzed using an Olympus IX83 microscope (Tokyo, Japan). Ten consecutive images were acquired per field of view by stepping the focal plane along the z-axis, and proteasome condensates were counted using the entire z-stack. Proteasome condensates were then quantified using the entire z-stack. Images were processed and visually re-confirmed using ImageJ to obtain quantitative data. To ensure statistical significance, more than 100 live cells were observed per condition across three independent experiments (totaling over 300 cells).

Measurement of intracellular pH

Intracellular pH (pHi) was measured using the genetically encoded pH sensor pHluorin2 as previously described [25, 33, 73].

Statistical analysis

Statistical significance was evaluated using one-way ANOVA with Dunnett's post-hoc test using RStudio (https://posit.co/products/open-source/rstudio/). Data are expressed as the mean ± standard deviation (n = 3).

DATA AVAILABILITY

All data discussed in this article is available upon request

AUTHOR CONTRIBUTIONS

S.T.: conceptualization; investigation; writing-original draft preparation. M.I.: investigation; writing-reviewing. M.H.: investigation; writing-reviewing. A.M.: investigation; writing-reviewing and editing. S.I.: conceptualization; project administration; funding acquisition; writing-reviewing and editing.

ACKNOWLEDGMENTS

This study was supported by the Japan Society for the Promotion of Science (Grant number 24K01679) and by the Institute of Fermentation, Osaka to S. I.

We thank Dr. Nguyet for her helpful suggestions regarding this study. The AID system strain was provided by the National BioResource Project (Yeast), Japan.

SUPPLEMENTAL MATERIAL

All supplemental data for this article are available online at www.microbialcell.com.

CONFLICT OF INTEREST

No potential conflicts of interest were reported by the authors.

COPYRIGHT

Please cite this article as: Shoei Tanaka, Mitsuki Imajo, Mieko Hayashi, Akira Matsuura, Shingo Izawa (**2026**). Sorbic acid triggers rapid formation of proteasome storage granules in *Saccharomyces cerevisiae*. **Microbial Cell** 13: 329-339. doi: 10.15698/mic2026.08.886

REFERENCES

1. Pohl C, Dikic I (**2019**). Cellular quality control by the ubiquitin-proteasome system and autophagy. **Science** 366 (6467): 818–822. doi:10.1126/science.aax3769
2. Jayaraj GG, Hipp MS, Hartl FU (**2020**). Functional modules of the proteostasis network. **Cold Spring Harb Perspect Biol** 12 (1): a033951. doi:10.1101/cshperspect.a033951
3. Finley D, Ulrich HD, Somme T, Kaiser P (**2012**). The ubiquitin-proteasome system of *Saccharomyces cerevisiae*. **Genetics** 192 (2): 319–360. doi:10.1534/genetics.112.140467
4. Saeki Y (**2017**). Ubiquitin recognition by the proteasome. **J Biochem** 161 (2): 113–124. doi:10.1093/jb/mvw091

5. Tanaka K (**2009**). The proteasome: Overview of structure and functions. **Proc Jpn Acad Ser B** 85 (1): 12–36. doi:10.2183/pjab.85.12

6. Bard JAM, Goodall EA, Greene ER, Jonsson E, Dong KC, Martin A (**2018**). Structure and function of the 26S proteasome. **Annu Rev Biochem** 87: 697–724. doi:10.1146/annurev-biochem-062917-011931

7. Vimer S, Ben-Nissan G, Morgenstern D, Kumar-Deshmukh F, Polkinghorn C, Quintyn RS, Vasil'ev YV, Beckman JS, Elad N, Wysocki VH, Sharon M (**2020**). Comparative structural analysis of 20s proteasome ortholog protein complexes by native mass spectrometry. **ACS Cent Sci** 6 (4): 573–588. doi:10.1021/acscentsci.0c0008

8. Finley D, Chen X, Walters KJ (**2016**). Gates, channels, and switches: Elements of the proteasome machine. **Trends Biochem Sci** 41 (1): 77–93. doi:10.1016/j.tibs.2015.10.009

9. Collins GA, Goldberg AL (**2017**). The logic of the 26S proteasome. **Cell** 169 (5): 792–806. doi:10.1016/j.cell.2017.04.023

10. Raynes R, Pomatto LCD, Davies KJA (**2016**). Degradation of oxidized proteins by the proteasome: Distinguishing between the 20S, 26S, and immunoproteasome proteolytic pathways. **Mol Aspects Med** 50: 41–55. doi:10.1016/j.mam.2016.05.001

11. Pepelnjak M, Rogawski R, Arkind G, Leushkin Y, Fainer , Ben-Nissan G, Picotti P, Sharon M (**2024**). Systematic identification of 20S proteasome substrates. **Mol Syst Biol** 20 (4): 403–427. doi:10.1038/s44320-024-00015-y

12. Laporte D, Salin B, Daignan-Fornier B, Sagot I (**2008**). Reversible cytoplasmic localization of the proteasome in quiescent yeast cells. **J Cell Biol** 181 (5): 737–745. doi:10.1083/jcb.200711154

13. Pack CG, Yukii H, Toh-e A, Kudo T, Tsuchiya H, Kaiho A, Sakata E, Murata S, Yokosawa H, Sako Y, Baumeister W, Tanaka K, Saeki Y (**2014**). Quantitative live-cell imaging reveals spatio-temporal dynamics and cytoplasmic assembly of the 26S proteasome. **Nat Commun** 5 (3396). doi:10.1038/ncomms4396

14. Yasuda S, Tsuchiya H, Kaiho A, Guo Q, Ikeuchi K, Endo A, Arai N, Ohtake F, Murata S, Inada T, Baumeister W, Fernández-Busnadiego R, Tanaka K, Saeki Y (**2020**). Stress- and ubiquitylation-dependent phase separation of the proteasome. **Nature** 578 (7794): 296–300. doi:10.1038/s41586-020-1982-9

15. Enenkel C, Ernst OP (**2025**). Proteasome dynamics in response to metabolic changes. **Front Cell Dev Biol** 13: 1523382. doi:10.3389/fcell.2025.1523382

16. Fu A, Cohen-Kaplan V, Avni N, Livneh I, Ciechanover A (**2021**). p62-containing, proteolytically active nuclear condensates, increase the efficiency of the ubiquitin-proteasome system. **Proc Natl Acad Sci USA** 118 (33): e2107321118. doi:10.1073/pnas.2107321118

17. Uriarte M, Nkwe NS, Tremblay R, Ahmed O, Messmer C, Mashtalir N, Barbour H, Masclef L, Voide M, Viallard C, Daou S, Abdelhadi D, Ronato D, Paydar M, Darracq A, Boulay K, Desjardins-Lecavalier N, Sapieha P, Masson JY, Sergeev M, Kwok BH, Hulea L, Mallette FA, Milot E, Larrivée B, Wurtele H, Affar EB (**2021**). Starvation-induced proteasome assemblies in the nucleus link amino acid supply to apoptosis. **Nat Commun** 12 (1): 6984. doi:10.1038/s41467-021-27306-4

18. Enenkel C, Kang RW, Wilfling F, Ernst OP (**2022**). Intracellular localization of the proteasome in response to stress conditions. **J Biol Chem** 298 (7): 102083. doi:10.1016/j.jbc.2022.102083

19. Kang Y, Vossler RA, Diaz-Martinez LA, Winter NS, Clarke DJ, Walters KJ (**2006**). UBL/UBA ubiquitin receptor proteins bind a common tetraubiquitin chain. **J Mol Biol** 356 (4): 1027–1035. doi:10.1016/j.jmb.2005.12.001

20. Tsuchiya H, Ohtake F, Arai N, Kaiho A, Yasuda S, Tanaka K, Saeki Y (**2017**). In vivo ubiquitin linkage-type analysis reveals that the Cdc48-Rad23/Dsk2 axis contributes to K48-linked chain specificity of the proteasome. **Mol Cell** 66 (4): 488–502. doi:10.1016/j.molcel.2017.04.024.e7

21. Iriki T, Iio H, Yasuda S, Masuta S, Kato M, Kosako H, Hirayama S, Endo A, Ohtake F, Kamiya M, Urano Y, Saeki Y, Hamazaki J, Murata S (**2023**). Senescent cells form nuclear foci that contain the 26S proteasome. **Cell Rep** 42 (8): 112880. doi:10.1016/j.celrep.2023.112880

22. Kaganovich D, Kopito R, Frydman J (**2008**). Misfolded proteins partition between two distinct quality control compartments. **Nature** 454 (7208): 1088–1095. doi:10.1038/nature07195

23. Ohigashi N, Hirayama S, Yashiroda H, Murata S (**2025**). Vacuolar Sts1 degradation-induced cytoplasmic proteasome translocation restores cell proliferation. **Genes Cells** 30 (2): e70004. doi:10.1111/gtc.70004

24. Waite KA, Vontz G, Lee SY, Roelofs J (**2024**). Proteasome condensate formation is driven by multivalent interactions with shuttle factors and ubiquitin chains. **Proc Natl Acad Sci USA** 121 (10): e2310756121. doi:10.1073/pnas.2310756121

25. Imajo M, Tanaka S, Anh Nguyet VT, Hayashi M, Matsuura A, Izawa S (**2026**). Acetic acid induces proteasome storage granule formation and inhibits proteasomal proteolysis: Comparison with other induction conditions. **J Biol Chem** 302 (4): 111297. doi:10.1016/j.jbc.2026.111297

26. Peters LZ, Hazan R, Breker M, Schuldiner M, Ben-Aroya S (**2013**). Formation and dissociation of proteasome storage granules are regulated by cytosolic pH. **J Cell Biol** 201 (5): 663–671. doi:10.1083/jcb.201211146

27. Tang X, Qu L, Wilfling F, Beck F, Ernst OP, Schulman BA, Baumeister W, Enenkel C (**2026**). Metabolically regulated proteasome supramolecular organization *in situ*. **Cell** 189 (4): 1153–1169.e16. doi:10.1016/j.cell.2025.12.035

28. Yedidi RS, Fatehi AK, Enenkel C (**2016**). Proteasome dynamics between proliferation and quiescence stages of *Saccharomyces cerevisiae*. **Crit Rev Biochem Mol Biol** 51 (6): 497–512. doi:10.1080/10409238.2016.1230087

29. Waite KA, Roelofs J (**2022**). Proteasome granule formation is regulated through mitochondrial respiration and kinase signaling. **J Cell Sci** 135 (17): jcs259778. doi:10.1242/jcs.259778

30. Dechant R, Binda M, Le SS, Pelet S, Winderickx J, Peter M (**2010**). Cytosolic pH is a second messenger for glucose and regulates the PKA pathway through V-ATPase. **EMBO J** 29 (15): 2515–2526. doi:10.1038/emboj.2010.138

31. Guldfeldt LU, Arneborg N (**1998**). Measurement of the effects of acetic acid and extracellular pH on intracellular pH of nonfermenting, individual *Saccharomyces cerevisiae* cells by fluorescence microscopy. **Appl Environ Microbiol** 64 (2): 530–534. doi:10.1128/AEM.64.2.530-534.1998

32. Arneborg N, Jespersen L, Jakobsen M (**2000**). Individual cells of *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* exhibit different short-term intracellular pH response to acetic acid. **Arch Microbiol** 174 (1–2): 125–128. doi:10.1007/s002030000185

33. Fujii R, Katsukawa R, Takeda E, Itakura E, Matsuura A (**2025**). Regulatory dynamics of Sch9 in response to cytosolic acidification: From spatial reconfiguration to cellular adaptation to stresses. **iScience** 28 (1): 111573. doi:10.1016/j.isci.2024.111573

34. Stratford M, Anslow PA (**1998**). Evidence that sorbic acid does not inhibit yeast as a classic 'weak acid preservative'. **Lett Appl Microbiol** 27 (4): 203–206. doi:10.1046/j.1472-765x.1998.00424.x

35. Pampulha ME, Loureiro-Dias MC (**2000**). Energetics of the effect of acetic acid on growth of *Saccharomyces cerevisiae*. **FEMS Microbiol Lett** 184 (1): 69–72. doi:10.1111/j.1574-6968.2000.tb08992.x

36. Ullah A, Orij R, Brul S, Smits GJ (**2012**). Quantitative analysis of the modes of growth inhibition by weak organic acids in *Saccharomyces cerevisiae*. **Appl Environ Microbiol** 78 (23): 8377–8387. doi:10.1128/AEM.02126-12

37. Stratford M, Nebe-von-Caron G, Steels H, Novodvorska M, Ueckert J, Archer DB (**2013**). Weak-acid preservatives: pH and proton movements in the yeast *Saccharomyces cerevisiae*. **Int J Food Microbiol** 161 (3): 164–171. doi:10.1016/j.ijfoodmicro.2012.12.013

38. Guaragnella N, Bettiga M (**2021**). Acetic acid stress in budding yeast: From molecular mechanisms to applications. **Yeast** 38 (7): 391–400. doi:10.1002/yea.3651

39. Stratford M, Plumridge A, Nebe-von-Caron G, Archer DB (**2009**). Inhibition of spoilage mould conidia by acetic acid and sorbic acid involves different modes of action, requiring modification of the classical weak-acid theory. **Int J Food Microbiol** 136 (1): 37–43. doi:10.1016/j.ijfoodmicro.2009.09.025

40. Giannattasio S, Guaragnella N, Zdralević M, Marra E (**2013**). Molecular mechanisms of *Saccharomyces cerevisiae* stress adaptation and programmed cell death in response to acetic acid. **Front Microbiol** 4 (33). doi:10.3389/fmicb.2013.00033

41. van Beilen JWA, Teixeira de Mattos MJ, Hellingwerf KJ, Brul S (**2014**). Distinct effects of sorbic acid and acetic acid on the electrophysiology and metabolism of *Bacillus subtilis*. **Appl Environ Microbiol** 80 (19): 5918–5926. doi:10.1128/AEM.01391-14

42. Stratford M, Vallières C, Geoghegan IA, Archer DB, Avery SV (**2020**). The preservative sorbic acid targets respiration, explaining the resistance of fermentative spoilage yeast species. **mSphere** 5 (3): e00273-20. doi:10.1128/msphere.00273-20

43. Peters LZ, Karmon O, Miodownik S, Ben-Aroya S (**2016**). Proteasome storage granules are transiently associated with the insoluble protein deposit in *Saccharomyces cerevisiae*. **J Cell Sci** 129 (6): 1190–1197. doi:10.1242/jcs.179648

44. Warth AD (**1985**). Resistance of yeast species to benzoic and sorbic acids and to sulfur dioxide. **J Food Prot** 48 (7): 564–569. doi:10.4315/0362-028X-48.7.564

45. Yoshiyama H, Nomura W, Izawa S (**2026**). Fungistatic effect of sorbic acid on yeast cells via translational repression involving eIF2α phosphorylation and formation of Ded1- and eIF2B-granules. **Microbial Cell** 13: 250–260. doi:10.15698/mic2026.06.880

46. Nishimura K, Fukagawa T, Takisawa H, Kakimoto T, Kanemaki M (**2009**). An auxin-based degron system for the rapid depletion of proteins in non-plant cells. **Nat Methods** 6 (12): 917–922. doi:10.1038/nmeth.1401

47. Maudlin IE, Beggs JD (**2019**). Spt5 modulates cotranscriptional spliceosome assembly in *Saccharomyces cerevisiae*. **RNA** 25 (10): 1298–1310. doi:10.1261/rna.070425.119

48. Nguyet VTA, Furutani N, Ando R, Izawa S (**2022**). Acquired resistance to severe ethanol stress-induced inhibition of proteasomal proteolysis in *Saccharomyces cerevisiae*. **Biochim Biophys Acta Gen Subj** 1866 (12): 130241. doi:10.1016/j.bbagen.2022.130241

49. Gödderz D, Schäfer E, Palanimurugan R, Dohmen RJ (**2011**). The N-terminal unstructured domain of yeast ODC functions as a transplantable and replaceable ubiquitin-independent degron. **J Mol Biol** 407 (3): 354–367. doi:10.1016/j.jmb.2011.01.051

50. Erales J, Coffino P (**2014**). Ubiquitin-independent proteasomal degradation. **Biochim Biophys Acta** 1843 (1): 216–221. doi:10.1016/j.bbamcr.2013.05.008

51. Kandasamy G, Andréasson C (**2018**). Hsp70-Hsp110 chaperones deliver ubiquitin-dependent and -independent substrates to the 26S proteasome for proteolysis in yeast. **J Cell Sci** 131 (6): jcs210948. doi:10.1242/jcs.210948

52. Yip MCJ, Bodnar NO, Rapoport TA (**2020**). Ddi1 is a ubiquitin-dependent protease. **Proc Natl Acad Sci USA** 117 (14): 7776–7781. doi:10.1073/pnas.1902298117

53. van Deventer S, Menendez-Benito V, van Leeuwen F, Neefjes J (**2015**). N-terminal acetylation and replicative age affect proteasome localization and cell fitness during aging. **J Cell Sci** 128 (1): 109–117. doi:10.1242/jcs.157354

54. Li J, Breker M, Graham M, Schuldiner M, Hochstrasser M (**2019**). AMPK regulates ESCRT-dependent microautophagy of proteasomes concomitant with proteasome storage granule assembly during glucose starvation. **PLoS Genet** 15 (11): e1008387. doi:10.1371/journal.pgen.1008387

55. Imai T, Ohno T (**1995**). Measurement of yeast intracellular pH by image processing and the change it undergoes during growth phase. **J Biotechnol** 38 (2): 165–172. doi:10.1016/0168-1656(94)00130-5

56. Kim MS, Cho KH, Park KH, Jang J, Hahn JS (**2019**). Activation of Haa1 and War1 transcription factors by differential binding of weak acid anions in *Saccharomyces cerevisiae*. **Nucleic Acids Res** 47 (3): 1211–1224. doi:10.1093/nar/gky1188

57. Terashima M, Yoshiyama H, Nomura W, Izawa S (**2026**). Acetic acid-induced translational repression involves eIF2B body formation and Ded1 sequestration into stress granules in yeast. **J Biosci Bioeng** (*in press*)

58. Stratford M, Plumridge A, Archer DB (**2007**). Decarboxylation of sorbic acid by spoilage yeasts is associated with the *PAD1* gene. **Appl Environ Microbiol** 73 (20): 6534–6542. doi:10.1128/AEM.01246-07

59. Tomko RJ Jr, Hochstrasser M (**2014**). The intrinsically disordered Sem1 protein functions as a molecular tether during proteasome lid biogenesis. **Mol Cell** 53 (3): 433–443. doi:10.1016/j.molcel.2013.12.009

60. Sone T, Saeki Y, Toh-e A, Yokosawa H (**2004**). Sem1p is a novel subunit of the 26 S proteasome from *Saccharomyces cerevisiae*. **J Biol Chem** 279 (27): 28807–28816. doi:10.1074/jbc.M403165200

61. Funakoshi M, Li X, Velichutina I, Hochstrasser M, Kobayashi H (**2004**). Sem1, the yeast ortholog of a human BRCA2-binding protein, is a component of the proteasome regulatory particle that enhances proteasome stability. **J Cell Sci** 117 (Pt 26): 6447–6454. doi:10.1242/jcs.01575

62. Bajorek M, Finley D, Glickman MH (**2003**). Proteasome disassembly and downregulation is correlated with viability during stationary phase. **Curr Biol** 13 (13): 1140–1144. doi:10.1016/s0960-9822(03)00417-2

63. Chen Q, Thorpe J, Ding Q, El-Amouri IS, Keller JN (**2004**). Proteasome synthesis and assembly are required for survival during stationary phase. **Free Radic Biol Med** 37 (6): 859–868. doi:10.1016/j.freeradbiomed.2004.05.025

64. Livnat-Levanon N, Kevei É, Kleifeld O, Krutauz D, Segref A, Rinaldi T, Erpapazoglou Z, Cohen M, Reis N, Hoppe T, Glickman MH (**2014**). Reversible 26S proteasome disassembly upon mitochondrial stress. **Cell Rep** 7 (5): 1371–1380. doi:10.1016/j.celrep.2014.04.030

65. Gu ZC, Wu E, Sailer C, Jando J, Styles E, Eisenkolb I, Kuschel M, Bitschar K, Wang X, Huang L, Vissa A, Yip CM, Yedidi RS, Friesen H, Enenkel C (**2017**). Ubiquitin orchestrates proteasome dynamics between proliferation and quiescence in yeast. **Mol Biol Cell** 28 (19): 2479–2491. doi:10.1091/mbc.E17-03-0162

66. Choi WH, Yun Y, Byun I, Kim S, Lee S, Sim J, Levi S, Park SH, Jun J, Kleifeld O, Kim KP, Han D, Chiba T, Seok C, Kwon YT, Glickman MH, Lee MJ (**2023**). ECPAS/Ecm29-mediated 26S proteasome disassembly is an adaptive response to glucose starvation. **Cell Rep** 42 (7): 112701. doi:10.1016/j.celrep.2023.112701

67. Piper P, Mahé Y, Thompson S, Pandjaitan R, Holyoak C, Egner R, Mühlbauer M, Coote P, Kuchler K (**1998**). The pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. **EMBO J** 17 (15): 4257–4265. doi:10.1093/emboj/17.15.4257

68. Ullah A, Chandrasekaran G, Brul S, Smits GJ (**2013**). Yeast adaptation to weak acids prevents futile energy expenditure. **Front Microbiol** 4 (142). doi:10.3389/fmicb.2013.00142

69. Joyner RP, Tang JH, Helenius J, Dultz E, Brune C, Holt LJ, Huet S, Müller DJ, Weis K (**2016**). A glucose-starvation response regulates the diffusion of macromolecules. **Elife** 5: e09376. doi:10.7554/eLife.09376

70. Lahtvee PJ, Kumar R, Hallström BM, Nielsen J (**2016**). Adaptation to different types of stress converge on mitochondrial metabolism. **Mol Biol Cell** 27 (15): 2505–2214. doi:10.1091/mbc.E16-03-0187

71. Marini G, Nüske E, Leng W, Alberti S, Pigino G (**2020**). Reorganization of budding yeast cytoplasm upon energy depletion. **Mol Biol Cell** 31 (12): 1232–1245. doi:10.1091/mbc.E20-02-0125

72. Davey HM, Hexley P (**2011**). Red but not dead? Membranes of stressed *Saccharomyces cerevisiae* are permeable to propidium iodide. **Environ Microbiol** 13 (1): 163–171. doi:10.1111/j.1462-2920.2010.02317.x

73. Mahon MJ (**2011**). pHluorin2: an enhanced, ratiometric, pH-sensitive green florescent protein. **Adv Biosci Biotechnol** 2 (3): 132–137. doi:10.4236/abb.2011.23021