Disruption of protein synthesis as antifungal mode of action by chitosan

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A B S T R A C T

The antimicrobial activity of chitosan has been acknowledged for more than 30 years and yet its mode-of-action remains ambiguous. We analyzed chemical–genetic interactions of low-molecular weight chitosan using a collection of ≈4600 S. cerevisiae deletion mutants and found that 31% of the 107 mutants most sensitive to chitosan had deletions of genes related primarily to functions involving protein synthesis. Disruption of protein synthesis by chitosan was substantiated by an in vivo β-galactosidase expression assay suggesting that this is a primary mode of antifungal action. Analysis of the yeast gene deletion array and secondary assays also indicate that chitosan has a minor membrane disruption effect – a leading model of chitosan antifungal activity.

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1. Introduction

Chitosan has gained attention as an abundant and inexpensive bioactive substance with potential applications in agricultural, food, pharmaceutical and textile industries (Raafat et al., 2008). It is produced by alkaline N-deacetylation of chitin, which is estimated to be the second most abundant biopolymer on earth after cellulose, owing to its prevalence in the exoskeletons of arthropods and the cell wall of most fungi (Cohen, 1987). Chitosan is a highly basic, linear polycationic heteropolysaccharide (β-1,4 linked N-acetylglucosamine units) comprised of about 6.9% nitrogen that has a molecular weight range of 50 to 2000 kDa. It has a slightly variable pKa of ≈6.0 ± 0.3 depending on the degree of deacetylation, which ranges from about 75 to 95% (Costa et al., 2012; Goy et al., 2009). Native chitosan is water insoluble but can be dissolved in slightly acidic solutions at a pH lower than its pKa, when the amino groups are in their protonated form.

Chitosan exhibits a diversity of biological properties including antimicrobial, antibacterial and antiviral activities (Allan and Hadwiger, 1979; Sudarshan et al., 1992; Chirkov, 2002). This wide-spectrum of antimicrobial activity is of particular interest for applications in the manufacture of coatings for perishable foods, in seed treatments, wound dressings, and for microbe resistant packaging materials (Raafat et al., 2008). This spectrum of antimicrobial activity seems to be influenced by various determinants such as the developmental stage of the target microorganism, and physico-chemical properties of the chitosan used, including molecular weight, degree of solubilization, acetylation, and charge density, and environmental growth conditions such as pH, temperature, exposure time, etc. (Kong et al., 2010). In spite of this well documented activity, the mechanism(s) of antimicrobial action of chitosan remains ambiguous (Rabea et al., 2003; Raafat et al., 2008). Three main mechanisms of antimicrobial activity have been proposed: 1) Metal-chelation, which might destabilize the outer membrane in Gram negative bacteria (Helander et al., 2001) or microbial cell walls; 2) Electrostatic interactions between negative residues of cell surfaces and the amino protonated groups of chitosan that would lead to cell wall permeability (Raafat et al., 2008) and plasma membrane perturbation (Zakrzewska et al., 2007; Palma-Guerrero et al., 2010); and 3) Interaction between microbial DNA and the internalized chitosan that might interfere with gene expression (Goy et al., 2009).

The present study investigates the antifungal mechanism of action of low molecular weight (LMW) chitosan using a gene deletion array (GDA) with the yeast Saccharomyces cerevisiae. The GDA system employs the exposure of about 4600 non-essential gene deletion mutants of yeast to a sub-lethal concentration of a compound (Mir-Rashed et al., 2010; Galván et al., 2008). Identification of the most susceptible (supersensitive) mutants gives an indication of the cellular pathway(s) perturbed by the compound, hence provides clues regarding its mode of action. Based on results of our GDA analysis, we used secondary assays to verify that chitosan negatively affects yeast protein synthesis and cell membranes.

2. Materials and methods

2.1. Strains, growth conditions and antifungal assays

LMW chitosan with a degree of deacetylation between 75 and 85%, viscosity of 20–200 cps and average molecular weight (Mw) of ≈150 × 103 (repeat units) was purchased from Sigma-Aldrich.
domly selected sensitive and nonsensitive mutant strains (based on the
150 rpm. Cell cultures were adjusted to
YPD-chitosan (1.5 mg/ml) or YPD-no chitosan, and incubated for 4
2 days at 30 °C, 150 rpm. A 10-fold serial dilution of each culture was performed
plate counts were performed to estimate growth inhibition due to chitosan
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YPD agar plates with and without chitosan (1.75 mg/ml) were inoculat-
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cultures were adjusted to an OD600nm
amaran analysis

The molecular activity of LMW chitosan was investigated through a
large-scale drug sensitivity screen by monitoring colony size reduction in a set of 4600 haploid deletion mutants of
S. cerevisiae using methods similar to those of Parsons et al. (2004). This gene deletion array (GDA) was developed by Winzeler et al. (1999) using the strain BY4741, a de-
was encapsulated in large unilamellar vesicles (LUV ≈ 100 nm diam-
eter) of dioleoylphosphatidylcholine (DOPC, Avanti Polar Lipids, Al-
ester, AL, USA). After having established the appropriate dilution of the LUV suspension, the membrane disruption assay was carried out in a 96-well microplate (black/clear OptiLux™ flat bottom; BD Biosci-
ence, San Jose, CA, USA). The threshold fluorescence intensity of the LUV suspension for each experimental well was measured (excitation 492 nm/emission 517 nm) with a FLUostar microplate reader (OPTI-
MA BMG LABTECH Inc., Durham NC, USA) to obtain the fluorescence at time zero (F0). Chitosan (30 mg/ml) was two-fold serially diluted in HEPES buffer before adding to the LUV suspension (1:10) to obtain final concentrations of between 6 and 3000 μg/ml. Fluorescence in-
tensities were also determined after the addition of the chitosan
lar solvent (1% acetic acid) and HEPES buffer. After adding the chitosan, the microplate was incubated in darkness for 1 h at room temperature and the fluorescent emission was measured to obtain F
(fluorescent intensity of the vesicles after chitosan addition). The F100 (Fluorescence intensity with 100% leakage of CF) was read 10 min after triton X-100 (10% v/v) was added. The % leakage (%L) was calculated by the equation: 
3. Results and discussion

3.1. Chitosan minimum inhibitory concentration
We initiated this study by determining that the chitosan MIC50 for
S. cerevisiae strain 288C was 1.5 mg/ml. This value differs from some previous studies in which the chitosan MIC50 has been reported to be
as low as 100 μg/ml for S. cerevisiae (Jaime et al., 2012). However, dif-
ferent MIC values for chitosan are noted, owing to variables such as mo-
lecular weight and degree of acetylation of the utilized chitosan and the identity of target microorganism and its cell surface properties (Mellegård et al., 2011). Our determination of MIC50 of LMW-chitosan for yeast strain 288C was necessary for subsequent GDA analyses.

3.2. Identification of S. cerevisiae deletion mutants most sensitive to chitosan
The antifungal activity of LMW-chitosan was investigated using a chemical-genomic screen by exposing the GDA of S. cerevisiae to 1.75 mg/ml of chitosan and detecting mutants with an increased sensitivity to the drug. Supersensitive mutants were identified by a significant colony size reduction (≥40%, Supplementary Table S1). As shown in Fig. 1A, among the top 2.5% most inhibited haploid deletion mutants, we distinguished 7 functional categories based on KOGs. Four of the categories represent the majority (> 71%) of the supersensitive mutants and will be discussed briefly below. A fifth large category represents deletions of genes with unknown function.

We found a significant enrichment (p-value 1.95 × 10−5) of super-
sensitive mutants had deletions of genes involved in protein biosynthesis. For example, 18 of the 33 mutant strains in this category have deletions of genes that encode ribosomal proteins (Table S1). The second largest group of mutants supersensitive to chitosan comprises 18 strains that

2.2. Gene deletion array (GDA) analysis

The molecular functions of genes deleted in the supersensitive mu-

2 days at 30 °C and MIC50 was determined as the chitosan concentration at which there was a ≈50% reduction in CFU compared to plates without chitosan. MIC50 determinations were done in triplicate.

2.3. β-Galactosidase expression assay

A standard β-galactosidase expression assay was performed to esti-
mate the effect of chitosan on the efficiency of protein translation (Alamgir et al., 2008). The assay employs the vector p416 (Munberg et al., 1994) which contains the yeast GAL1 promoter controlling lacZ (β-galactosidase expression; transcription of lacZ, and thus β-galactosidase biosynthesis, is repressed in the presence of glucose and induced with galactose). The vector p416 was transformed into the yeast strain W303. The transformed yeast cells were grown in synthetive medium lacking uracil (SC-URA with glucose) and incubated at 30 °C for 24 h, before resuspending in SC-URA with galactose. The assays were performed at time zero (F0) and incubated for 10 min after triton X-100 (10% v/v) was added. The % leakage (%L) was calculated by the equation:

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have deletions of genes involved in cell cycle and DNA processing (Table S1). The high sensitivity to LMW-chitosan of deletion mutants related to protein biosynthesis and processing, and cell cycle and DNA processing (first and second largest groups, respectively) is in agreement with the hypothesis that cationic chitosan can interact with DNA and/or RNA (Hardwiger et al., 1985) which may, in turn, inhibit protein synthesis.

The next largest groups represent deletions that effect non-vesicular ion trafficking and Golgi/endosomal transport (16 deletion mutants), and cell wall/cell membrane biogenesis (9 mutants). Interestingly, this last group includes deletions of genes that are involved in sphingolipid (e.g. ipt1Δ, skn1Δ, lcb3Δ) and ergosterol (e.g. erg3Δ, erg5Δ) biosynthesis, factors that affect the plasma membrane fluidity, which has previously been proposed to influence cell sensitivity to chitosan (Palma-Guerrero et al., 2010). The membrane topology and its dynamics affect the endosomal transport and trafficking. A drastic perturbation in the plasma membrane might produce defective invaginations impeding the proper trafficking and therefore inefficient endosomal transport (McMahon and Gallop, 2005). It was recently proposed that the plasma membrane in yeast is organized in domains (compartments) and networks which are involved in the regulation of processes such as cell polarity, signaling and membrane protein and lipid turnover (Ziółkowska et al., 2012). By extension, perturbation of the plasma membrane by chitosan may alter other cell processes as evident in our GDA analysis. Our observations that strains with deletions of genes effecting cell wall and cell membrane biogenesis are highly sensitive to chitosan support a leading hypothesis that the antifungal mechanism of action of chitosan is through perturbation of the plasma membrane (Zakrzewska et al., 2005; Park et al., 2008).

3.3. Effect of LMW-chitosan on β-galactosidase activity as an estimate of protein synthesis efficiency

To test our GDA results that indicate chitosan mainly affects protein biosynthesis, we investigated whether or not yeast that carry the inducible GAL1 promoter/lacZ fusion construct exhibit decreased

Fig. 1. Evidence that chitosan interferes with protein synthesis and membrane integrity. A) Functional distribution of the 107 most sensitive gene deletion strains to 1.75 mg/ml chitosan. Deleted genes in supersensitive strains were classified by function using KOG. B) β-Galactosidase expression assay suggests that chitosan inhibits protein biosynthesis in a dose-dependent manner. The efficiency of protein synthesis was indirectly evaluated using an inducible β-galactosidase reporter gene (p416 construct) and measuring enzyme activity spectrophotometrically based on ONPG conversion. Enzyme activity is plotted relative to no-chitosan controls. C) LMW-chitosan disrupted DOPC-LUVs based on carboxyfluorescein-loaded liposome leakage measurements. The effect of carrier solvent (no LMW-chitosan) on liposome leakage is also presented.
induction of β-galactosidase activity when exposed to chitosan. Yeast cells containing the fusion construct were transferred to media containing galactose with or without subinhibitory concentrations of chitosan (0.35–1.25 mg/ml, Fig. 1B). After 8 h, β-galactosidase conversion of substrate ONPG, as expressed in enzymatic units, can be indirectly considered an estimation of translation efficiency of the enzyme (Firoozan et al., 1991; Alamgriz et al., 2008). As shown in Fig. 1B, when yeast cells were exposed to 0.35 mg/ml of chitosan the β-galactosidase activity was reduced to 32% of no-chitosan controls. β-Galactosidase activity was further reduced with increasing chitosan concentrations of up to 1.25 mg/ml, the highest concentration tested in this assay, which resulted in 13% β-galactosidase activity compared to no-chitosan controls. The inhibitory effect of 1.25 mg/ml chitosan on protein synthesis was less pronounced than with the positive control: 10 μg/ml of cyclohexamide resulted in 2% of control translation (data not shown). Nevertheless, the results clearly show that LMW-chitosan reduces β-galactosidase activity at concentrations that are well below the MIC50 (1.5 mg/ml), and in a dose-dependent manner. It should be noted that decreased β-galactosidase activity as measured in this assay could result from interference with transcription efficiency and other processes in addition to translation. However, the observed reduction in β-galactosidase activity in the presence of chitosan is consistent with GDA results that indicate chitosan mainly affects protein biosynthesis.

Previous studies showed that electrostatic interactions can occur between positively charged amino groups from the N-glucosamine, forming-monomers of chitosan and negatively charged phosphate groups on DNA and RNA, or carboxyl groups on proteins (Ma et al., 2009; Souza et al., 2009). Such interactions between chitosan and DNA, RNA and protein could partly explain the effects of chitosan on translation efficiency and could also help explain our GDA results that found increased sensitivity of strains deleted for genes involved in protein biosynthesis, and DNA replication, recombination and repair (Fig. 1A).

3.4. Membrane disruption assay

A membrane disruption assay was performed, since this is considered a main mechanism of antifungal action of chitosan and our GDA results also show high chitosan sensitivity by mutants with deletions in genes involved in membrane transport and cell wall/membrane functions. The membrane-disruptive properties of LMW chitosan were investigated using carboxyfluorescein (CF) loaded liposomes. Similar to what was reported by Park et al. (2008), we found that LMW-chitosan at 0.75 μg/μl caused moderate (∼7%) leakage of carboxyfluorescein contained in large unilamellar vesicles (Fig. 1C). This finding is in accord with our GDA results that showed increased chitosan sensitivity of strains lacking genes involved in cell wall/membrane/envelope biogenesis and transport and secretion processes.

3.5. Conclusions on chitosan mode of action

Analysis of chemical–genetic interactions using the S. cerevisiae mutant collection provides insight into potential molecular targets of antifungals. This study shows that chitosan interferes primarily with protein synthesis in yeast. Disruption of such a fundamental process may account for some of the diverse additional effects of chitosan implicated by our GDA analysis, including cellular trafficking, secession, and cell cycle progression. Alternatively, chitosan may interfere directly with multiple cellular targets. Of significance, inhibitors that target multiple processes would be difficult to overcome by resistance mechanisms — a major concern in antimicrobial chemotherapy.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2013.03.025.

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References


