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Isolation of Mutants of *Saccharomyces cerevisiae* with a Changed Cell Wall Composition by Screening on Resistance to Tannic Acid

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Summary

To isolate cell wall mutants, we started with the isolation of tannic acid resistant mutants. Tannic acid is a polyphenolic substance known for its inhibiting effect on yeast growth and for its good capacity to precipitate proteins and polysaccharides. We considered the cell wall as a good target for tannic acid since the cell wall of Saccharomyces cerevisiae consists of chitin, β-glucan and mannoproteins. By screening on an increased resistance to tannic acid after mutagenesis, 25 mutants were isolated. Differences in cell wall composition were checked by determining the sensitivity to zymolyase, a cell wall hydrolysing enzyme and the sensitivity to calcofluor white, a molecule interfering with cell wall structure. All the tannic acid resistant mutants were more resistant to zymolyase. This is a strong evidence that the cell wall of the tannic acid resistant mutants is different in composition and/or structure. This was confirmed by determining the sensitivity to calcofluor white showing that most of the mutants had a changed resistance towards calcofluor white. However, five mutants showed no difference in sensitivity to calcofluor white, although they had an increased resistance to zymolyase. The resistance to zymolyase and to calcofluor white varied among the mutants. This is a strong indication that there may be differences in cell wall composition among the tannic acid resistant mutants.

Key words: Saccharomyces cerevisiae, tannic acid, cell wall mutants

Introduction

Properties of the yeast cell such as adhesion, flocculation and mechanical strength are ascribed to the cell wall (*1*). Apart from yeast-yeast interaction, adhesion of yeast cells to each other results in flocculation. This interaction is, for example, an important step in beer or wine fermentation. Adhesion between yeasts and bacteria can also occur and may have practical significance in the fermentation industry. Next to these adhesion properties, the cell wall of yeast is determining its characteristic morphology and is giving physical and osmotic protection to the cell (*2*).

The cell wall of *Saccharomyces cerevisiae* is made of three components which form a layered structure (*2*): mannoproteins, β-glucans and chitin. Mannoproteins are mainly found in the exterior layers of the cell wall, while β-glucans, which consist of β-1,3- and β-1,6-glucan, are mainly situated at the inside of the cell wall. Chitin is mainly present in the bud scars, but also in minor amounts in the lateral wall (*3*). The mannoprotein layer is responsible for the cell surface characteristics and determines cell wall permeability (*4*). The β-glucan layer is responsible for the rigidity of the cell wall. These layers are structurally connected to each other by various link-

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ages: β-1,3-glucan is covalently linked with chitin (*5*). β-1,6-glucan is both connected to β-1,3-glucan and mannoproteins (*6*).

To study cell wall composition and its influence on cell wall properties, we have isolated a new class of cell wall mutants, tannic acid resistant mutants. Tannic acid consists of a central glucose molecule, with its hydroxylgroups esterified with gallic acid. Gallic acid residues in turn can be esterified with another gallic acid (*7*). Tannic acid was chosen because of its growth inhibiting effect on yeast (*8*) and for its capacity to precipitate proteins and polysaccharides (*9*). We assumed that the cell wall of yeast is a good target for tannic acid, since it consists of hypermannosylated proteins, β-glucan and chitin. This idea was supported by the observation that tannic acid probably does not enter the cell (*10*).

Here, we report the isolation of tannic acid resistant mutants and we show that these mutants have an altered cell wall composition.

Materials and Methods

Strains and media: *Saccharomyces cerevisiae ATCC 44771 E4: Mata steVC9 ura3-52 trp1-289 leu2-3 leu2-112 his3Δ1 ade1-101 can1-100. Saccharomyces cerevisiae WE372* and *Saccharomyces cerevisiae 831B* are strains commercially used in wine fermentation.

YPD [1 % yeast extract (Oxoid), 2 % bacteriological peptone (Oxoid), 2 % glucose] medium was used. If necessary, filter sterilised tannic acid (UCB) was added to the medium after sterilisation. Synthetic defined, SD, medium consisted of 0.67 % yeast nitrogen base (Difco) with 2 % glucose. To solidify, 2 % agar was added to the media.

Isolation of tannic acid resistant mutants: A diluted stationary phase culture was plated out on YPD agar with 50 ppm tannic acid and irradiated for 16 seconds with UV light at 312 nm. After 48 hours incubation at 28 °C, mutants were transferred to fresh YPD medium with 50 ppm tannic acid. The wild type strain did not form colonies on this medium within 48 hours of incubation. The resistance of the mutants was confirmed on the same media.

Quantitative determination of sensitivity to tannic acid was carried out in microtiter plates. Wells were filled with 200 μL YPD to which tannic acid was added to obtain 0, 60, 80, 100, 140 or 160 ppm. After inoculation with a 50 times diluted stationary phase culture, microtiter plates were incubated at 28 °C. Growth was followed by measuring the absorption at 595 nm in function of time using the BIORAD Microplate Reader Mode 450. For every strain, growth of three independent cultures was followed and the mean value calculated.

Tannic acid sensitivity is expressed as the delay in growth in the presence of tannic acid compared to growth in the absence of tannic acid. Because the difference in growth rate between strains could affect the result, absorption values in the presence of tannic acid were calculated from the growth curves at the same time when the absorption was 0.4 in the absence of tannic acid.

Determination of sensitivity to calcofluor white (*11*). Wild type and mutant colonies were picked up with a toothpick from YPD agar and inoculated on SD agar buffered at pH = 6 with 50 mM morpholinoethanesulfonic acid to which calcofluor white was added in concentrations varying from 0 to 4 mg/mL. Growth was checked after 4 days of incubation at 28 °C.

Determination of the sensitivity to zymolyase (*12,13*). Cells were grown in YPD medium up to early stationary phase, washed three times with water and suspended in 10 mM Tris HCl pH = 7. This suspension was diluted to an absorption of 0.07 at 800 nm. To one milliliter of this diluted culture 1 U of zymolyase (ICN) was added and the decrease of the absorption at 800 nm was followed in function of time. For each strain at least three cultures were independently grown and tested twice. The average values are presented as percent variation.

The zymolyase sensitivity is defined as the time at which the absorption of the solution was reduced by 50 % of the value at the start of the experiment.

Results

Isolation and tannic acid resistance of isolated mutants

After mutagenisation 25 mutants which had an increased resistance towards tannic acid were isolated. We searched for mutants at a subinhibitory concentration of tannic acid: the wild type strain could form colonies after 72 hours incubation on YPD agar containing 50 ppm tannic acid, but not in 48 hours. Because we isolated mutants at subinhibitory concentrations of tannic acid, and to confirm the resistance, we scored sensitivity to tannic acid quantitatively by following the growth of wild and mutant strains in the absence or the presence of different concentrations of tannic acid.

To test the reliability of the method used, we compared the growth of the wild type strain, *Sacch. cerevisiae 44771*, with the growth of the yeast strains used in wine production, *Sacch. cerevisiae WE372* and *Sacch. cerevisiae 831B*. It is expected that these strains are more resistant to tannic acid, due to the presence of tannins during wine fermentation. Fig. 1 shows that the growth of the wine yeasts was less inhibited by the concentrations of tannic acid used than that of *Sacch. cerevisiae 44771*. Since the difference detected between these strains was larger than the standard deviation, which was always 1 to 10 % between cultures of the same strain, we can conclude that our method is suitable to compare differences in sensitivity to tannic acid between different strains.

The resistance of some of the isolated mutants compared with the wild type is shown in Fig. 2. The other isolated mutants gave a similar profile (results not shown). Sensitivity to tannic acid did not disappear completely: high concentrations of tannic acid still inhibited growth. A possible explanation could be that the mutants were screened at a subinhibitory concentration of tannic acid.

All the isolated mutants were, as expected, more resistant to tannic acid than the wild type. This is shown by the inhibitory effect of tannic acid on the growth rate of wild type and mutant TAR18 (Fig. 3). The growth rate

Table 1. Zymolyase sensitivity of tannic acid resistant mutants

Strain	Zymolyase sensitivity min	Strain	Zymolyase sensitivity min	Strain	Zymolyase sensitivity min
44771	26.4	TAR10	41.8	TAR20	36.4
TAR1	33.9	TAR11	37.7	TAR21	38.8
TAR2	35.6	TAR12	42.5	TAR22	40.6
TAR3	35.5	TAR13	39.9	TAR23	41.8
TAR4	34.6	TAR14	41.7	TAR24	50.3
TAR5	53.1	TAR15	39.0	TAR25	38.5
TAR6	44.6	TAR16	30.8		
TAR7	38.3	TAR17	41.9		
TAR8	38.1	TAR18	31.8		
TAR9	40.3	TAR19	35.8		

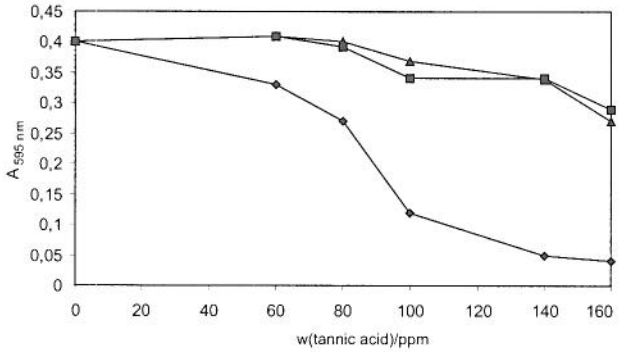

Fig. 1. Growth of *Sacch. cerevisiae 44771* (◆), and of the commercial wine strains *Sacch. cerevisiae WE372* (■) and *Sacch. cerevisiae 831B* (▲) in the presence of tannic acid

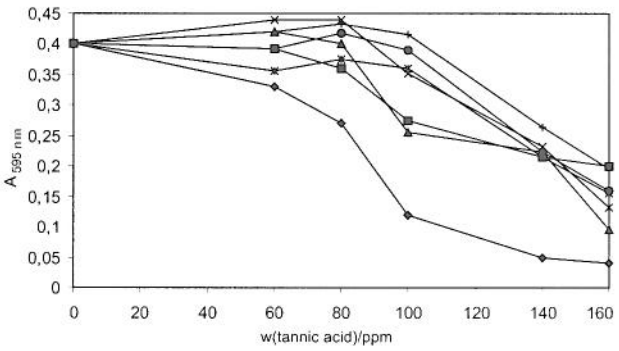

Fig. 2. Growth of wild type, *Sacch. cerevisiae 44771* (◆) and tannic acid resistant mutants (TAR) in the presence of tannic acid. TAR18 (■); TAR9 (▲); TAR22 (×); TAR4 (✳); TAR16 (●); TAR5 (+).

of the mutants was less affected by the presence of tannic acid than the growth rate of the wild type.

Cell wall characteristics of tannic acid resistant mutants

To check whether the mutants showed differences in their cell wall composition, the sensitivity to zymolyase and to calcofluor white was studied.

It is expected that a difference in cell wall composition or structure will be reflected in a difference in sensitivity to zymolyase (*12*), a cell wall hydrolysing enzyme with β-glucanase activity as main activity (*13*). Addition of zymolyase to a cell suspension resulted in a decrease in absorption due to the hydrolysis of the cell wall of the cells. The decrease in absorption was completely due to enzyme activity and not the result of spontaneous cell lysis in the buffer (Fig. 4). The level of variability shown in the same figure was always between 5 and 10 % for different cultures of the same strain.

All the tannic acid resistant mutants were more resistant to zymolyase than the wild type strain (Table 1). The sensitivity to zymolyase between the mutants varied between 30.8 and 53.1 min. This is an indication that the cell wall composition between the mutants is different.

The change in cell wall composition was confirmed by the sensitivity to calcofluor white. With respect to this sensitivity, the tannic acid resistant mutants can be divided in three groups (Table 2). One mutant is more resistant to calcofluor, 5 mutants have the same sensitivity, all the other mutants are hypersensitive to it. The differences in sensitivity among the mutants are implying also that the call wall composition varies among the tannic acid resistant mutants.

Discussion

It is clear from the results that by screening on an increased resistance to tannic acid, mutants can be isolated with a modified cell wall composition, as shown by both the analysis of sensitivity to zymolyase and to calcofluor white.

Remarkable is the great diversity found in sensitivity to zymolyase and calcofluor white between the tannic acid resistant mutants. Since it is to be expected that these differences reflect differences in cell wall composition, it is possible that different classes of cell wall mutants were isolated. This idea is corroborated by the fact that some tannic acid resistant mutants with an increased zymolyase resistance, do not show a difference in calcofluor white sensitivity. This implies that certain cell wall mutants cannot be isolated by a screening based on

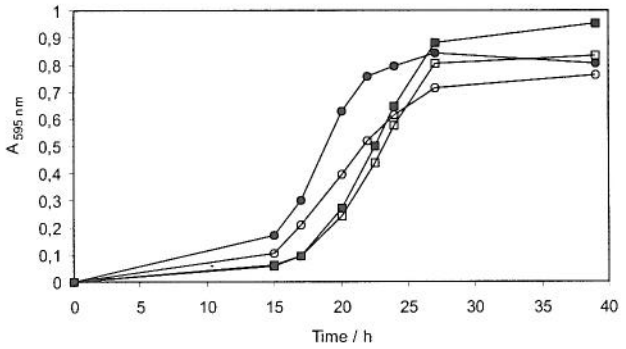

Fig. 3. Effect of 80 ppm tannic acid on the growth of wild type, *Sacch. cerevisiae 44771* (●, ○), and of a tannic acid resistant mutant, *TAR18* (■, □). Closed symbols represent growth in the absence of tannic acid; open symbols growth in the presence of 80 ppm tannic acid.

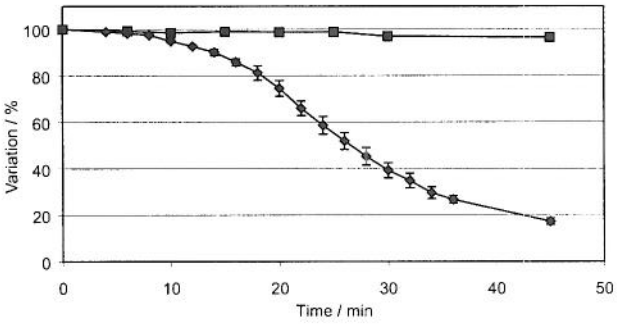

Fig. 4. Effect of addition of zymolyase to a cell suspension of *Sacch. cerevisiae 44771* (◆). As a control, the change in absorption of the cell suspension without addition of zymolyase was also followed (■).

Table 2. Calcofluor white sensitivity of tannic acid resistant mutants

Tannic acid resistant mutants hypersensitive to calcofluor white	Tannic acid resistant mutants with a comparable sensitivity as the wild type towards calcofluor white	Tannic acid resistant mutants resistant to calcofluor white
TAR2, TAR3, TAR5, TAR6, TAR7, TAR10, TAR12, TAR13, TAR14, TAR15, TAR16, TAR17, TAR18, TAR19, TAR20, TAR21, TAR24, TAR25	TAR1, TAR4, TAR8, TAR22, TAR23	TAR9

an altered calcofluor white sensitivity, although this is a routine procedure for isolation of mutants with a changed cell wall composition (*14,15*).

It is impossible to correlate the increased resistance to zymolyase directly with a change in a specific cell wall fraction, due to the following reasons. First, zymolyase has, apart from β-glucanase activity, also protease and mannanase activity. Secondly, a changed susceptibility of living yeast cells towards zymolyase depends on the structure as well as on the composition of the cell wall. Since the outer protein layer is mainly responsible for the cell wall porosity (*4*), a change in protein composition in the mutants or a change in *N*- or *O*-glycosylation can restrict the accessibility of the enzyme to the β-1,3-glucan. Structural changes in the β-glucan layer, such as the number of branches, could be responsible for the decreased susceptibility to zymolyase in the tannic acid resistant mutants. The change in cell wall composition of the mutants was confirmed by analysis of the calcofluor white sensitivity. Screening of a change in resistance to calcofluor white is based on the principle that cells with a weakened cell wall will not be able to withstand the extra disturbing effect of calcofluor white. Isolation of mutants with an altered calcofluor white sensitivity was yielding cell wall mutants who bore mutations in the β-glucan fraction as well as in the mannoprotein fraction (*14,15*). But there is also no direct information about the cell wall fraction(s) that is (are) affected.

The reason for the inhibitory effect on growth of tannic acid and its effect on the cell wall is not known. According to Scalbert (*16*), there are different mechanisms by which the toxicity of tannic acid can be explained. A first mechanism is enzyme inhibition or substrate deprivation caused by their ability to precipitate proteins. A second mechanism of toxicity can be the complexation of metal ions. This mechanism is suggested for the growth inhibition of *E. coli* by tannic acid since addition of iron could restore its growth in a tannic acid containing medium (*17*).

Which of these mechanisms is valid for yeast is not known yet. Since mutants with an altered cell wall composition were isolated, it seems that there is a direct effect of tannic acid on the cell wall. However, none of the described mechanisms of toxicity can be excluded at this stage. Lussier *et al.* (*15*) who isolated cell wall mutants by sensitivity to calcofluor white, classified the mutant genes in different classes related to the function of these genes. They found not only genes directly involved in cell wall synthesis, but also genes involved in nitrogen metabolism, genes related to mitochondrial function, and genes related to nucleic acid function. To explain these results they proposed regulatory associations between these cellular pathways and the cell wall. So, it is still possible that deprivation of nutrients is the main cause of tannic acid toxicity and that mutants were isolated in a pathway not directly related to the cell wall, but with a change in cell wall composition as a consequence.

Conclusions

After UV mutagenesis and by screening on resistance to tannic acid, mutants were isolated with a changed

cell wall composition as confirmed by an increased resistance to zymolyase and a changed sensitivity to calcofluor white. Because the resistance to zymolyase and calcofluor white varied among the mutants, it is expected that different classes of mutants were isolated with respect to their cell wall composition.

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Izolacija mutanata *Saccharomyces cerevisiae* s izmijenjenim sastavom staničnog zida dobivenih selekcijom na otpornost prema taninskoj kiselini

Sažetak

Mutanti stanica izolirani su prema njihovoj otpornosti na taninsku kiselinu. Taninska je kiselina polifenolni spoj poznat po tome što inhibira rast kvasca, a ujedno može taložiti proteine i polisaharide. Prema mišljenju autora stanični je zid pogodan za reakciju s taninskom kiselinom jer je u Sacch. cerevisiae izgrađen od hitina, β-glukana i manoproteina. Selekcijom s obzirom na povećanu otpornost prema taninskoj kiselini nakon mutageneze izolirano je 25 mutanata. Razlike u sastavu staničnog zida ispitane su određujući osjetljivost na zimoliazu, enzim koji hidrolizira stanični zid, te na osjetljivost prema »calcofluor white«, molekulom koja interferira sa strukturom staničnog zida. Svi mutanti otporni na taninsku kiselinu bili su otporniji i prema zimoliazi. To je očit dokaz da stanična stijenka mutanata, otpornih na taninsku kiselinu, ima drukčiji sastav i/ili strukturu. Također je to potvrđeno određivanjem osjetljivosti na »calcofluor white« gdje se pokazalo da najveći broj mutanata ima različitu otpornost prema tom spoju. Međutim, pet mutanata nije pokazalo različitu osjetljivost prema »calcofluor white«, iako su imali povećanu otpornost prema zimoliazi. Između mutanata postojala je različita otpornost prema zimoliazi i »calcofluor white«. To uvelike pokazuje da moraju postojati razlike u sastavu staničnog zida između mutanata otpornih na taninsku kiselinu.