A combined chemical and enzymatic method to determine quantitatively the polysaccharide components in the cell wall of yeasts

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Abstract
A reliable method to determine cell wall polysaccharides composition in yeast is presented, which combines acid and enzymatic hydrolysis. Sulphuric acid treatment is used to determine mannans, whereas specific hydrolytic enzymes are employed in a two sequential steps to quantify chitin and the proportion of β-(1,3) and β-(1,6)-glucan in the total β-glucan of the cell wall. In the first step, chitin and β-(1,3)-glucan were hydrolysed into their corresponding monomers N-acetylglucosamine and glucose, respectively, by the combined action of a chitinase from Streptomyces griseus and a pure preparation of endo/exo-β-(1,3)-glucanase from Trichoderma species. This step was followed by addition of recombinant endo-β-(1,6)-glucanase from Trichoderma harzianum with β-glucosidase from Aspergillus niger to hydrolyse the remaining β-glucan. This latter component corresponded to a highly branched β-(1,6)-glucan that contained about 75–80% of linear β-(1,6)-glucose linked units as deduced from periodate oxidation. We validated this novel method by showing that the content of β-(1,3), β-(1,6)-glucan or chitin was dramatically decreased in yeast mutants defective in the biosynthesis of these cell wall components. Moreover, we found that heat shock at 42 °C in Saccharomyces cerevisiae and treatment of this yeast species and Candida albicans with the antifungal drug caspofungin resulted in 2- to 3-fold increase of chitin and in a reduction of β-(1,3)-glucan accompanied by an increase of β-(1,6)-glucan, whereas ethanol stress had apparently no effect on yeast cell wall composition.

Introduction
The cell wall is vital for growth, survival and morphogenesis of fungi. In the yeast Saccharomyces cerevisiae, this external structure is a complex interplay of four major polysaccharides: β-(1,3) glucan which is a linear chains of around 1500 glucose units linked in β-(1,3), β-(1,6) glucan composed of 140–350 residues of glucose linked by a β-(1,6) linkage, mannans which comprises mannose units linked in α-(1,2); α-(1,3); α-(1,6) and chitin, a polymer of 100–90 N-acetylglucosamine units linked by β-(1,4) linkages. Moreover, the cell wall also harbours several proteins that are implicated in molecular recognition and adhesion (Levin, 2005; Klis et al., 2006, 2009). Other polymers of glucose (α-(1,3) and α-(1,4)-glucans) as well as galactomannans are part of the cell wall structure of other fungal species such as Schizosaccharomyces pombe and Aspergillus species (Free, 2013). Over the last 15 years, the complexity of the cell wall architecture has emerged from detailed genetic, molecular and biochemical studies, which led to the discovery of several interconnections between the wall components to form macromolecular
complexes (see Free, 2013; Orlean, 2012; for a recent review on this topic). In addition, the molecular architecture of the cell wall is constantly remodelled according to growth conditions, morphological development or in response to cell surface stresses. This cell wall remodelling process is mainly under the control of the cell wall integrity (CWI) signalling pathway that transmits the signal from cell surface sensors to a MAP kinases cascade (Levin, 2011), with the main consequence to reorganize cell wall architecture through changes in cell wall carbohydrates content, in cross-links between chitin and β-glucan polymers, and in a transient redistribution of the cell wall repair machinery to the site of the cell wall injuries (Klis et al., 2006; Lesage & Bussey, 2006).

The yeast cell wall is endowed with remarkable biochemical properties, which are exploited in different biotechnological industrial sectors. In winemaking, cell wall mannoproteins are used to reduce astringency caused by tannins (Dupin et al., 2000) and to capture some wine aromatic contaminants that give a musty taste to wine. In animal nutrition, cell wall β-glucan serves as a valuable microbiological binder of mycotoxins (Yiannikouris et al., 2004). This polymer is also well known to have strong immunostimulating activities that could be beneficial for health and resistance to diseases and cancer (Brown & Gordon, 2003; Chen & Seviour, 2007). Taken together, these biological functions of the yeast cell wall appeal for a precise quantitative determination of its composition, owing to the fact that cell wall mass and the proportion of each of its component may dramatically vary according to growth conditions and process methods (Nguyen et al., 1998; Aguilar-Uscanga & Francois, 2003).

Several methods aiming at isolating cell wall and quantifying its composition have been already developed. Most of them make use of chemical treatments of the cell wall with strong acids such as hydrochloric, hydrofluoric, trifluoroacetic and sulphuric acids, which can break down the cell wall polymers into their monomers of glucose, mannose and glucosamine (De Ruiter et al., 1992; Ram et al., 1994; Dallies et al., 1998; Freidmund et al., 2005). However, these acidic methods suffer from at least three major problems. Firstly, they are unable to make distinction between β-(1,3) and β-(1,6)-glucan. Secondly, they are rather harsh and may destroy the monomers and hence underestimate the content of the corresponding polysaccharide in the wall if not well controlled in terms of duration and temperature. Thirdly, most of these methods are not efficient enough to hydrolyse all chitin, leading to an underestimation of this polymer. To estimate the proportion of β-(1,3) and β-(1,6)-glucan, Fleet and Manners (Fleet & Manners, 1976) developed a chemical fractionation method that consisted in the separation of the cell wall into an alkali-soluble and alkali-insoluble fraction, and the latter was again separated into an alkali-insoluble acid-insoluble and alkali-insoluble acid-soluble fractions that contain the bulk of β-(1,6)-glucan, whereas β-(1,3)-glucan is essentially present in the alkali-soluble and alkali-soluble acid-insoluble fraction. Aimanianda et al. (2009) applied an enzymatic digestion after this fractionation procedure to determine β-(1,3)- and β-(1,6)-glucan using recombinant endo-β-(1,3)-glucanase from Thermotoga neapolitana and endo-β-(1,6) glucanase from Trichoderma harzianum. However, this fractionation procedure is time-consuming and imperfect as it leaves some 10–20% of β-(1,6)-glucan in the alkali-insoluble acid-soluble fraction (Catley, 1988; Ha et al., 2002). To be more accurate on these polysaccharides quantification, Hong et al. (1994) and Magnelli et al. (2002) have proposed a method based on the chemical and enzymatic fractionation of the cell wall obtained from yeast cells cultivated with radiolabeled glucose. Although this method allowed to trace and quantify each component in the different fractions, they could not be applied for regular cell wall analysis because they require cultivating yeast cells with a radiolabeled sugar prior to hydrolysis.

With the objective to determine more precisely chitin levels and to distinguish β-(1,6) from β-(1,3)-glucan in cell wall β-glucan, we report a simple and reliable method that is based on the sequential treatment of cell walls with specific hydrolytic enzymes, namely chitinase, endo and exo-β-(1,3)-glucanases followed by a mixture of endo-β-(1,6)-glucanase and β-glucosidase. Combined with the chemical treatment that hydrolyses mannans into mannos units, this new method allowed faithfully quantifying each of the different polymers that compose the cell wall. This method has been applied to determine the cell wall composition of mutants defective in genes involved in cell wall synthesis and remodelling, as well as to assess effects of ethanol stress, heat shock and the antifungal drug caspofungin on the cell wall composition of yeasts.

**Material and methods**

**Chemicals and biochemicals**

Laminarin (from Laminaria digitata), chitin (from shrimp shells) and mannann (from S. cerevisiae) were purchased from Sigma-Aldrich. Pustulan isolated from Umbilicaria papulosa was from Calbiochem. All these polysaccharides were verified for their relative purity after acid hydrolysis as described in Dallies et al. (1998). Values around 65–78% of the dry powder were obtained based on the quantification of the sugars monomers released after this acid hydrolysis (see Table 1). Note that all commercial polysaccharides were kept in a jar under vacuum to maintain them dry. Except for β-D-gentiobiose, which was...
Table 1. Screening of commercial enzymatic preparation on β-(1,3) glucan, β-(1,6) glucan, chitin and mannans

<table>
<thead>
<tr>
<th>Commercial name (company)</th>
<th>Origin</th>
<th>Description of enzymes activities present in the commercial preparation</th>
<th>Laminarin (Laminaria digitata) µg glucose mg⁻¹ powder</th>
<th>Pustulan (Umbilicaria pustulata) µg glucose mg⁻¹ powder</th>
<th>Chitin (Shrimp shells) µGlcNac mg⁻¹ powder</th>
<th>Mannan (S. cerevisiae) µG mannose mg⁻¹ powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucanex</td>
<td></td>
<td>Enzyme preparation Endo-β-(1,3)-glucanase β-(1,6)glucanase Cellulase Protease Chitinase</td>
<td>570.9 ± 13.6</td>
<td>576.0 ± 20.2</td>
<td>556.9 ± 7.2</td>
<td>574.7 ± 20.2</td>
</tr>
<tr>
<td>Glucozyme</td>
<td></td>
<td>Enzyme preparation Endo &amp; Exo-β-(1,3)-glucanase Chitinase β-glucosidase</td>
<td>757.4 ± 8.9</td>
<td>799.9 ± 20.3</td>
<td>26.5 ± 2.5</td>
<td>23.8 ± 5.3</td>
</tr>
<tr>
<td>Zymolyase Arthobacter luteus</td>
<td></td>
<td>β-(1,3)-laminarin pentao-hydrolase Mannanase Protease</td>
<td>409.2 ± 35.7</td>
<td>39.9 ± 16.9</td>
<td>13.9 ± 2.8</td>
<td>0</td>
</tr>
<tr>
<td>Laminarinase Trichoderma species</td>
<td></td>
<td>Endo-β-(1,3) glucanase Cellulase α-amylase</td>
<td>752.3 ± 11.3</td>
<td>740.9 ± 33.7</td>
<td>6.0 ± 2.3</td>
<td>15.7 ± 9.2</td>
</tr>
<tr>
<td>Exo-β-(1,3) glucanase Trichoderma species</td>
<td></td>
<td>Endo-β-(1,3) glucanase</td>
<td>249.6 ± 19.2</td>
<td>48.5 ± 11.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Endo-β-(1,3) glucanase Trichoderma species</td>
<td></td>
<td>Endo-β-(1,3) glucanase</td>
<td>745.3 ± 33.4</td>
<td>46.2 ± 18.5</td>
<td>0</td>
<td>6.9 ± 1.6</td>
</tr>
<tr>
<td>Endo-β-(1,6) glucanase Trichoderma harzianum</td>
<td></td>
<td>Recombinant Endo-β-(1,6)glucanase</td>
<td>0</td>
<td>559.8 ± 42.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>β-glucosidase Aspergillus niger</td>
<td></td>
<td>β-(1,4)-glucanase</td>
<td>663.3 ± 62.0</td>
<td>311.5 ± 50.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chitinase Trichoderma viride</td>
<td></td>
<td>Chitinase</td>
<td>0</td>
<td>0</td>
<td>161.3 ± 19.5</td>
<td>0</td>
</tr>
<tr>
<td>Streptomyces griseus</td>
<td></td>
<td>Chitinase</td>
<td>0</td>
<td>0</td>
<td>975.0 ± 17.5</td>
<td>0</td>
</tr>
<tr>
<td>Sulphuric acid method</td>
<td></td>
<td></td>
<td>720.9 ± 25.4</td>
<td>695.6 ± 13.3</td>
<td>491.3 ± 25.0</td>
<td>651.3 ± 22.4</td>
</tr>
</tbody>
</table>
Strains, culture conditions and phenotypic tests

The BY4741 and its isogenic YKO strains [deletion mutants made with the kanMX6 cassette according to (Wach, 1996)] were obtained from Open Biosystem. Unless otherwise stated, the yeast cells were cultivated in YPD medium (1% w/v of yeast extract; 1% w/v of peptone, 2% w/v glucose) and were collected at the exponential growth phase (OD600 nm around 1.5 corresponding to 2 × 10⁷ cells mL⁻¹) by centrifugation at 4 °C for 15 min. The cell wall-containing pellets were frozen in nitrogen liquid and then lyophilised (E-EXBGL), endo-β-(1,3)-glucanase from Trichoderma harzianum expressed in Pichia pastoris (a kind gift from Dr J. Chapman, Unilever, the Netherlands) was prepared according to Bom et al. (1998).

Isolation of cell walls

Each pellet was resuspended in 0.5 mL cold water and transferred to lysis matrix tubes (MPBio, 6960-500) containing 0.5 mm glass beads. Cells were disrupted using a Fastprep system (MP Biomedicals) for 20 s with intervals of 1 min on ice. The disruption cycle was repeated until more than 95% of the cells were lysed as estimated by methylene blue colouration according to (Cot et al., 2007). Usually, about 10 cycles were needed to reach this yield. The cell suspension was collected, and the glass beads were extensively washed with cold deionized water. The supernatant and washings were pooled and centrifuged at 4000 g for 15 min. The cell wall-containing pellet was again washed two times with cold deionized water. Pellets were frozen in nitrogen liquid and then lyophilized until complete dryness.

Chemical treatment of cell walls

Acid sulphuric hydrolysis of the cell wall was carried out as described previously (Dallies et al., 1998). This acid hydrolysis released glucose from β-glucan, mannose from mannans and glucosamine from chitin (since the N-acetyl residue in N-acetyl-glucosamine is acid-labile).

Enzymatic treatment of commercial polysaccharides and yeast cell wall

Pustulan, laminarin, chitin, mannan and yeast β-glucan (alkali-soluble from Megazyme) were prepared as a 1–10 mg mL⁻¹ solution in 50 mM potassium acetate pH 5.0. Unless otherwise stated, commercial enzymes were added at 1 U and incubation was carried out at 37 °C for 8–16 h. One unit is defined as the amount of enzyme that releases 1 μmol of product which is either glucose or N-acetylglucosamine per min under the condition of the assay. An aliquot was taken for measurement of glucose residues and N-acetylglucosamine (see below).

Enzymatic treatment of yeast cell wall to determine chitin, β-(1,3) and β-(1,6)-glucan

The procedure for quantitative determination of chitin, β-(1,3)- and β-(1,6)-glucan from purified yeast cell wall is based on a two steps process as depicted in Fig. 1.

Step 1: enzymatic treatment for chitin and β-(1,3)-glucan determination

The lyophilised cell wall (about 10 mg of dry mass) was resuspended in 200 μL of potassium acetate 50 mM pH 5.0. After heating at 65 °C for 5 min, 1 U chitinase from Streptomyces griseus, 5 U of exo-β-(1,3)-glucanase and 5 U endo-β-(1,3)-glucanase from Trichoderma species were added. Hydrolysis was carried out at 37 °C for 6–12 h.

Step 2: enzymatic hydrolysis for β-(1,6)-glucan estimation

To the remaining hydrolysate in step 1, 2 U of purified endo-β-(1,6)-glucanase prepared as described above and 1 U of β-glucosidase from A. niger (Megazyme, E-BGLUC) were added. Hydrolysis was carried out at 37 °C for 6–12 h.

Quantification of sugars by HPAEC-PAD

After each enzymatic digestion, a sample was withdrawn and centrifuged in a microfuge (10 min at 10 000 g at

obtained from Carbosynth, other biochemicals were purchased from Sigma-Aldrich (France) and were of the purest quality. The following enzyme preparation known to act on β-glucans were purchased from Megazyme (Ireland): Glucanex (K-EBHLG), exo-β-(1,3)-glucanase from Trichoderma species (E-EXBGL), endo-β-(1,3)-glucanase from Trichoderma species (E-LAMSE), recombinant endo-β-(1,3)-glucanase from Hordeum vulgare (E-LAMHV) and β-glucosidase from Aspergillus niger (E-BGLUC). Glucanex was from Novozymes (L1412), Zymolyase 20T (L5263), laminarinase from Trichoderma species (L5272), chitinase from Streptomyces griseus (C6137) and from Trichoderma viride (C8241) were obtained from Sigma-Aldrich. Recombinant endo-β-(1,6)-glucanase from Trichoderma harzianum expressed in Pichia pastoris (a kind gift from Dr J. Chapman, Unilever, the Netherlands) was prepared according to Bom et al. (1998).
4 °C). The supernatant was analysed by HPAEC-PAD on an ICS 5000 system (Thermofisher Scientific, Courtaboeuf, France). Separation of the released monosaccharides (glucose, mannose and glucosamine) and oligosaccharides were performed on a CarboPac PA100 analytical column (250 × 4 mm) with a guard column CarboPac PA100 using NaOH (100 mM) and NaOAc/NaOH (500 mM/100 mM) as solvent A and B, respectively. The chromatographic conditions set up by Aimanianda et al. (2009) were followed. The column was pre-equilibrated by 98% A and 2% B. Following sample injection, a gradient run (flow rate 1 mL min\(^{-1}\)) was performed as follows: 0–2 min, isocratic step (98% A-2% B), 2–15 min 98% A-2% B to 65% A-35% B, 15–22 min 65% A 35% B to 57% A-43% B, 22–23 min 57% A-43% B to 100% B, and 23–25 min 100% B. Sugar residues were detected on a pulsed amperometric system equipped with a gold electrode.

**Colorimetric method to determine chitin levels**

Chitin was determined by the colorimetric method as described by Reissig et al. (1955) and adapted for a microplate reader method, using N-acetylglucosamine as a standard. Briefly, a 125 μL of the digestion mixture was heated with 25 μL of 0.8 M potassium tetraborate pH 9.0 at 100 °C for 8 min. After cooling down the samples to room temperature, 750 μL of Reissig reagent diluted 10 times in glacial acetic acid was added, and the tubes were incubated 40 min at 37 °C. The absorbance was read at 585 nm. The 10× Reissig solution was prepared by dissolving 10 g of 4-dimethylamino- benzaldehyde in 12.5 mL of HCl 10 N and 87.5 mL of glacial acetic acid.

**Other miscellaneous methods**

Preparation of alkali-soluble and insoluble fractions from purified yeast cell wall was carried out as described in Magnelli et al. (2002). The nitrogen content of the cell walls was determined by a micro-Kjeldahl method in a Leco nitrogen auto analyser FP428 (Leco Corporation) according to the manufacturer procedure with EDTA as internal standard (9.56% N). The protein content was calculated as the N content × 6.25, based on the estimation that the average nitrogen content of proteins in food material is around 16% (http://www.fao.org/docrep/006/y5022e/y5022e03.htm). Glucose oxidase peroxidase kit Sigma-Aldrich was routinely used for determination of glucose after hydrolysis of various polysaccharide substrates (mannan, chitin, laminarin and pustulan) by chemical or enzymatic treatment. Periodate oxidation of carbohydrate was performed according to Hong et al. (1994).

**Results**

**Screening of enzymes that harbour hydrolytic activities specific on chitin, β-(1,3) and β-(1,6)-glucan**

As our goal was to find enzymes able to specifically hydrolyse β-(1,3)-glucan, β-(1,6)-glucan and chitin, we screened a large set of commercially available enzymatic preparations harbouring hydrolytic activities on laminarin, a linear β-1,3-glucan; pustulan, a β-1,6-glucan; chitin and mannans (Table 1). As the exact purity of these commercial polysaccharide products was either not available or questionable, these products were subjected to
Acid hydrolysis using the sulphuric acid hydrolysis method described earlier (Dallies et al., 1998), and the released glucose from β-glucan and mannose from mannans were quantified by HPAEC. With the exception of chitin whose hydrolytic yield with this method has been estimated to 45–55% (Aguilar-Uscanga & Francois, 2003), and assuming complete hydrolysis of the other polysaccharides and minor degradation of the released monomers in this highly acidic condition, the purity of laminarin, pustulan and mannans was estimated in the range of 65−73% of dry powder (Table 1). Also, it was necessary to establish assay conditions that would be common for all the enzymes tested. After preliminary assays following the recommendation of the manufacturers, we finally decided to use 50 mM potassium acetate buffer, at pH 5.0 and to run the reaction at 37 °C for 16 h, since in all cases, the hydrolysis reaction carried out with 1−5 U of enzyme in the presence of 1−10 mg mL−1 of substrate reached a plateau after 8−12 h (data not shown). Accordingly, we found that Glucanex®, a glucanase preparation obtained from Novozymes, hydrolysed all the different polysaccharides with yield very close to that obtained by chemical treatment, indicating that contrary to their commercial description, this enzyme preparation may also contain chitinase and manannases. Another enzymatic cocktail termed Glucazyme® from Megazyme was effective on laminarin and pustulan but almost inactive on chitin and mannans, indicating that they contain endo-β-(1,3)/β-(1,6)- and exo-β-glucanases in accordance with its commercial prescription, whereas the chitinase, that is, explicitly mentioned in this cocktail had, in our hand, no activity on chitin. This result confirmed that this enzymatic preparation could be used to hydrolyse β-glucans from yeast cell wall as proposed by Megazyme. The last enzymatic cocktail tested was Zymolysis 20T, commonly used to prepare yeast spheroplasts (Ovalle et al., 1998). This cocktail was found to only partially hydrolyse laminarin and had also a weak action on pustulan and chitin, showing that this enzyme preparation was not appropriate for our purpose.

We then assayed four enzymes with reported endo-β-(1,3) glucanase activity. Whereas purified laminarinase from Trichoderma sp. exhibited efficient hydrolytic action on both β-(1,3) and β-(1,6) glucan substrates, the purified enzyme from Hordeum was highly specific for β-(1,3) linkages and was unable to fully hydrolyse laminarin, according to its pure endo-β-(1,3)-glucanosidase activity. On the other hand, we found an exo-β-(1,3)-glucanase from Trichoderma that was highly efficient on laminarin with almost no action on pustulan. An endo-β-(1,3)-glucanase purified from this fungus was also tested and showed hydrolytic action on laminarin albeit with less glucose released than with the exo-β-(1,3)-glucanase. Interestingly, the combination of the endo and exo-β-(1,3)-glucanases led to a release of glucose units from laminarin that was about 20% higher than after acid treatment, which indicated higher polysaccharides recovered upon enzymatic hydrolysis than after acid hydrolysis (Table 2). Chromatography analysis showed mainly glucose after digestion of laminarin with both exo and

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**Table 2. Combination of selected enzymes to hydrolyse β-1,3 (laminarin), β-1,6-glucan (pustulan) and alkali-soluble yeast β-glucan**

<table>
<thead>
<tr>
<th>Type of hydrolysis action</th>
<th>Laminarin (Laminaria digitata) (μg glucose mg⁻¹ powder)</th>
<th>Pustulan (Umbilicaria pustulan) (μg glucose mg⁻¹ powder)</th>
<th>Yeast β-glucan* (S. cerevisiae) (μg glucose mg⁻¹ powder)</th>
<th>Chitin (Shrimp shells) (μg GINAc mg⁻¹ powder)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exo + endo-β-(1,3) glucanases + chitinase</td>
<td>898.1 ± 56.1 (1.24)</td>
<td>58.0 ± 12.1 (0.09)</td>
<td>474 ± 21.7 (0.76)</td>
<td>980.2 ± 20.0 (2.0)</td>
</tr>
<tr>
<td>Exo + endo-β-(1,3) glucanases + β-glucosidase</td>
<td>895.1 ± 44.1 (1.24)</td>
<td>358.0 ± 52.1 (0.09)</td>
<td>nd</td>
<td>0</td>
</tr>
<tr>
<td>Endo-β-(1,6)-glucanase</td>
<td>0</td>
<td>560 ± 42.2 (0.87)</td>
<td>nd</td>
<td>0</td>
</tr>
<tr>
<td>Endo-β-(1,6) glucanase + β-glucosidase</td>
<td>620.1 ± 23.7 (0.86)</td>
<td>868.8 ± 44.9 (1.24)</td>
<td>nd</td>
<td>0</td>
</tr>
<tr>
<td>Exo + endo-β-(1,3)-glucanases + endo-β-(1,6) glucanase + β-glucosidase</td>
<td>nd</td>
<td>nd</td>
<td>589 ± 4.9 (0.96)</td>
<td>nd</td>
</tr>
<tr>
<td>Acid hydrolysis</td>
<td>720.9 ± 25.4</td>
<td>695.6 ± 13.3</td>
<td>614.6 ± 3.5</td>
<td>491.3 ± 25.0</td>
</tr>
</tbody>
</table>

For enzymatic assay, the polysaccharides were resuspended at 1 mg mL⁻¹ in 50 mM acetate buffer pH 5.0. The value indicated in parentheses corresponded to that of the ratio of polysaccharides after enzymatic digestion to that after acid hydrolysis. The results shown are the mean ± SD of at least three independent experiments, each made in triplicate.

*Yeast β-glucan is an alkali-soluble fraction of yeast cell wall purchased from Megazyme and which contained 620 mg glucose units mg⁻¹ powder according to this manufacturer.
endo-β-(1,3)-glucanase. A tiny peak that eluted just before glucose was also detected that likely could be a contaminant in the polysaccharide solution as it was also present in the control reaction without enzyme. In contrast, the digestion of laminarin with endo β-(1,3)-glucanase alone resulted in the presence of two main peaks, one as glucose and a second that likely corresponded to a short oligosaccharide (see Supporting Information, Fig. S1). As indicated in Table 2, further addition of the β-glucosidase did not improve degradation yield of laminarin but it partially hydrolysed pustulan, suggesting that this commercial preparation may contain some β-(1,6)-glucanase activity.

Due to the absence of a commercially available β-(1,6)-glucanase, we produced the recombinant endo-β-(1,6)-glucanase from Trichoderma harzianum in Pichia pastoris according to the protocol of Bom et al. (1998). It is shown in Table 1 that this recombinant protein was only active on pustulan, releasing about 80% glucose, when compared to the amount released by acid hydrolysis. The chromatography profile of pustulan digestion with this endo-β-(1,6)-glucanase showed that the major product detected was gentiobiose (G2), a β-(1,6) disaccharide which agreed with the endo-β-glucanase action of this enzyme (Lora et al., 1995) (Fig. S2). Interestingly, the addition of a commercial preparation of β-glucosidase from Aspergillus led to a complete disappearance of gentiobiose and reduction in the intensity of the other peaks, with a concomitant increase of the glucose peak (Fig. S2). Overall, this enzymatic digestion caused a 25% higher hydrolysis of pustulan than the acidic method (Table 2). This result indicated that the commercial preparation of β-glucosidase from A. niger may also contain some β-(1,6)-glucanase activity in addition to the reported β-(1,3) and β-(1,4) hydrolytic activities. This higher value shows that the enzymatic hydrolysis is more efficient than the acidic method to hydrolyse pustulan, because this chemical treatment could destroy glucose residues during its release from the polymer. Finally, two chitinases were commercially available but only the enzyme purified from Streptomyces griseus was able to hydrolyse chitin with a yield close to 98% of the amount of the powder present in the reaction (Table 1), suggesting that this polymer was completely hydrolysed into its monomer N-acetylglucosamine. This result was verified by chromatography on HPAEC that showed only one peak corresponding to N-acetylglucosamine (see Fig. S3). In addition, this result confirmed that the acid hydrolysis method using sulphuric acid underdetermined chitin levels by about 50%. The reason why the enzyme from Trichoderma viride was unable to fully hydrolyse chitin, whatsoever the time of incubation was not explored further. To complete this analysis, we incubated a suspension of commercial yeast β-glucan with the mixture of endo and exo-β-(1,3)-glucanases for 16 h, at pH 5.0 and found that the amount of glucose released was about 76% of the total β-glucan estimated by acid hydrolysis (Table 2). After further addition of β-glucosidase and endo-β-(1,6)-glucanase to this mixture, the total glucose released was very close to the amount obtained after acid hydrolysis which was also in accordance with manufacturer’s indication (i.e. 62% of the dry powder consisted in β-glucan). From this total hydrolysis, we could deduce that the amount of β-(1,6)-glucan in this alkali-soluble yeast β-glucan was around 23%.

### Setting up the enzymatic procedure on mixed polysaccharides and on purified yeast cell wall

As a first step to set up the enzymatic hydrolysis of yeast cell wall, we evaluated the hydrolytic efficiency of our selected enzymes on a mix of the four polysaccharides components at proportion that mimicked their content in the yeast cell wall. We applied two different modes of enzymatic digestion to this mixture (Fig. 2). In mode 1, the digestion was started by adding chitinase from Streptomyces griseus, followed after 6 h by addition of endo and exo β-(1,3)-glucanase and then terminated with endo β-(1,6)-glucanase and β-glucosidase. Before each enzyme addition, a sample was withdrawn and the amount of monosaccharides released was determined by HPAEC. As reported in Fig. 2, the recovery of β-(1,3) and β-(1,6)-glucan after enzymatic hydrolysis was very close to the initial amount of total β-glucan initially added (550 mg), whereas that of chitin content was slightly above than initially added in the mixture. However, it is important to notice that the amount of chitin determined after whole incubation procedure with both β-(1,3) and β-(1,6)-glucanases enzymes was the same as after incubation with chitinase alone (data not shown), indicating that the action of the latter enzyme was not impaired by the presence of the other polysaccharides.

We then set out the enzymatic procedure on purified cell wall of the wild-type BY4741 strain. The purity of our cell wall preparation was checked qualitatively by the nearly absence of lipids (see Fig. S4). The amount of the proteins present was measured by a micro-Kjeldahl method (Miller & Houghton, 1945) and found to be around 14% of the cell wall dry mass (see Table 3). As depicted in Fig. 2, we evaluated the specific enzymatic digestion of chitin, β-(1,3) and β-(1,6)-glucan on the purified yeast cell walls according to two different sequential modes of enzymes addition. According to mode 1, the content of chitin determined after incubation with chitinase was around fourfold lower than after treatment of the cell wall with both chitinase and endo/exo-β-(1,3) and β-(1,6)-glucanase as performed in mode 2 (P < 0.01). This result confirmed previous findings that...
complete chitin hydrolysis required auxiliary polysaccharide hydrolases in addition to chitinase (Cabib & Sburlati, 1988) and agreed with the existence of cross-linkages between chitin and β-(1,3)-glucan (Sietsma & Wessels, 1990; Kollar et al., 1995; Cabib et al., 2007; Cabib, 2009). Also, this enzymatic procedure showed that the content of chitin in wild-type yeast cultivated on glucose was in the range of 5% of cell wall dry mass, rather than 1–2% as commonly reported (Lesage & Bussey, 2006; Orlean, 2012). This difference could be explained by incomplete hydrolysis of chitin by sulphuric acid treatment (see Table 1). In addition, the sequential addition of hydrolytic enzymes in mode 1 led to an apparent underestimation of β-(1,3)-glucan and an overestimation of β-(1,6)-glucan, in comparison to mode 2.

From these results, it can be estimated that the proportion of β-(1,6)-glucan in the total cell wall β-glucan was around 20–22% of the total β-glucan in yeast wall. This value is about 15–20% higher than the one reported in previous works that employed cell wall fractionation followed by chemical or enzymatic hydrolysis (Fleet & Manners, 1976; Brown et al., 1993; Hong et al., 1994; Magnelli et al., 2002; Aguilar-Uscanga & Francois, 2003). This difference could be due to the fact that the enzymatic mixture of endo-β-(1,6)-glucanase and β-glucosidase was able to hydrolyse not solely the linear β-(1,6)-glucose linked units but also glucose residues, that is, triply linked at C1, C3 and C6 on the β-(1,6)-glucan backbone, as initially described by Manners et al. (1973a, b) and confirmed by Aimananda et al. (2009). To verify this hypothesis, we subjected our purified cell wall to a chemical fractionation with sodium hydroxide, which yielded an alkali-insoluble fraction according to Hong et al. (1994). This fraction that contained the bulk of β-(1,6)-glucan was then treated with endo/exo β-(1,3)-glucanase, and the digested fraction was filtered through Microcon-10 membrane to separate a low-molecular-mass fraction (glucose units from β-(1,3) glucan hydrolysis) from the high-molecular-mass fraction retained on the membrane and which mainly consisted of β-(1,6)-glucan chains. Part of this latter fraction was treated with endo-β-(1,6)-glucanase and β-glucosidase, while the other part was subjected to periodate oxidation. As each glucose residue in the linear β-(1,6)-glucan has three hydroxyl groups on adjacent carbon atoms (C2, C3 and C4), periodate can oxidize and cleaves C-C bonds between C2 and C3 as well as C3 and C4, resulting in the liberation of the C3 atom in the form of formic acid that can be quantified by HPLC. Results of this experiment presented in Fig. S5 showed that the levels of β-(1,6)-glucan determined by the enzymatic hydrolysis were around 25% higher than those estimated after periodate oxidation. These results
Enzymatic method for determination of yeast cell wall polysaccharides

Table 3. Determination of polysaccharides and protein content in some selected yeast mutants affected in cell wall formation

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genetic defect and references</th>
<th>Chemical treatment</th>
<th>β(1,3)-glucan</th>
<th>β(1,6)-glucan</th>
<th>β(1,3)+β(1,6) glucan</th>
<th>Chitin</th>
<th>Protein (%)</th>
<th>Mannans</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4741</td>
<td>Wild type</td>
<td></td>
<td>30 ± 3</td>
<td>17 ± 3</td>
<td>80 ± 2</td>
<td>4.5</td>
<td>12.1 ± 0.9</td>
<td>2.0</td>
</tr>
<tr>
<td>BYgas1</td>
<td>1-(1,3)-glucan synthase isoform 1</td>
<td></td>
<td>2.6 ± 1.2</td>
<td>1.1 ± 0.1</td>
<td>3.7 ± 0.4</td>
<td>1.1</td>
<td>3.6 ± 0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>BYmnn9</td>
<td>Subunit of Golgi mannosyltransferase complex; (Jungmann &amp; Munro, 1998)</td>
<td></td>
<td>3.2 ± 1.4</td>
<td>1.1 ± 0.1</td>
<td>4.3 ± 0.5</td>
<td>1.1</td>
<td>4.2 ± 0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>BYChs3</td>
<td>Chitin synthase 3; required for synthesis of the majority of cell wall chitin</td>
<td></td>
<td>3.2 ± 1.4</td>
<td>1.1 ± 0.1</td>
<td>4.3 ± 0.5</td>
<td>1.1</td>
<td>4.2 ± 0.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Yeast strains were cultivated in YPD at 30°C and harvested at OD 600 nm of 1.0. The results shown are the mean ± SD from at least three experiments. Protein content was determined by micro-Kjeldahl method and calculated as N content 6.25 and expressed in % of cell wall dry mass. Statistical analysis was carried out using the Student’s t-test and one-way ANOVA, followed by Tukey’s test. *P < 0.05, **P < 0.01; ***P < 0.001; ****P < 0.0001. Non-parametric analysis and Mann-Whitney test were applied when appropriate. All results are statistically significant at the 1% level.

Yeast strains were used in the experiment, and the results showed that the enzymatic hydrolysis allowed the measurement of the so-called highly branched β-(1,6)-glucan (Manners et al., 1973b) from which about 75–80% corresponded to linear β-(1,6)-glucose linked units.

Validation of the combined chemical and enzymatic method on cell wall defective mutants

Based on the enzymatic protocol described above (Fig. 2, mode 2), and our previous procedure to purify cell wall (Dallies et al., 1998) from yeast culture, we set up an updated protocol (Fig. 1) that combines enzymatic and chemical hydrolysis to quantify mannans, chitin, β-(1,3) and β-(1,6)-glucan in the wall of wild-type and mutant strains harbouring defects in the synthesis of specific cell wall polymers. These results are summarized in Table 3. As a general rule, the hydrolysis of cell wall β-glucan by the enzymatic method yielded roughly 10–25% higher recoveries than acid hydrolysis (P < 0.01). Also, levels of chitin were underestimated with the acid method as already noticed in an earlier report (Aguilar-Uscanga & Francois, 2003). On the other hand, the enzymatic digestion with chitinase clearly reinforced previous data that these mutations, except chs3Δ, led to a 3- to 5-fold increase in chitin content (Popolo et al., 1997; Dallies et al., 1998; Klis et al., 2006), whereas the chs3Δ mutant defective in chitin synthase III contained < 10% of the wild-type levels of chitin. In addition, it is relevant to notice a good correlation between the sensitivity of these cell wall mutants to CFW and CR, two colloidal dyes that are known to bind preferentially to chitin microfibrils (Roncero et al., 1988; Kopecka & Gabriel, 1992; Hoch et al., 2005; Costa-de-Oliveira et al., 2013), and chitin content, as higher this content, greater is the sensitivity to these drugs (Fig. 3). Table 3 also shows that the content of β-(1,3)-glucan decreased by about 45% in a fks1Δ mutant that is defective in the major β-(1,3) glucan synthase (Eng et al., 1994). This reduction was to the same extent as that previously reported using chemical methods (Dallies et al., 1998) or fractionation followed by chemical method (Magnelli et al., 2002). The remaining production of β(1,3)-glucan is likely due to the activity of the minor β(1,3)-glucan synthase encoded by FKS2 that is activated in a fks1Δ null mutant (Mazur et al., 1995). An even higher reduction of β(1,3)-glucan (55%) was recorded in a gas1Δ mutant, which is defective in β-3,1-beta-glucanosyltransferase implicated in the elongation of β(1,3)-glucan chains (Mouyna et al., 2000), whereas β(1,6)-glucan content was barely modified in both fks1Δ and gas1Δ mutants. On the contrary, β(1,6)-glucan was decreased by about 4-times (P < 0.0001) in a kre6Δ mutant that is defective in β(1,6)-glucan biosyn-
be threefold higher than in wild type (Table 3).

decrease of mannans, and in a noticeable 10% in the Golgi (Orlean, 2012), resulted in a twofold decrease of mannans, which a reduction of \( \beta \)-(1,6)-glucan was accompanied by a 20% rise in \( \beta \)-(1,6)-glucan (\( P < 0.01 \)) and by a twofold increase of chitin (\( P < 0.001 \)). In contrast, exposure of a yeast culture to 9% ethanol did not cause any significant change of its cell wall composition, even though the nanomechanical properties as determined by AFM were dramatically altered (C. Eltsztein, C. Formosa, M. Schiavone, H. Martin-Yken, M. Morais, E. Dague & J. M. François, manuscript in preparation). Finally, we assessed the effects of caspofungin on cell wall composition in both S. cerevisiae and in the pathogenic yeast Candida albicans (Fig. 4), as we reported that the nanomechanical properties of both types of yeast determined using AFM technology were strongly altered after exposure to this drug (Formosa et al., 2013). While we already reported a rise of chitin that was roughly proportional to the dose of caspofungin added to the cells, here, we showed that this treatment also strongly impacted the \( \beta \)-glucan layers with a 50% decrease of \( \beta \)-(1,3)-glucan content that was compensated in part by a 20% increase of \( \beta \)-(1,6)-glucan at drug concentration corresponding to a MIC/2. One can notice that effects of caspofungin on the \( \beta \)-glucan content were more pronounced than in a \( fks1 \Delta \) mutant (Roemer & Bussey, 1991). Therefore, these results can be taken as direct means to validate our enzymatic method of \( \beta \)-(1,3) and \( \beta \)-(1,6)-glucan quantification. The cell wall composition of the \( mnn9 \Delta \) mutant which is defective in the elaboration step of protein mannosylation in the Golgi (Orlean, 2012), resulted in a twofold decrease of mannans, and in a noticeable 10–15% decrease of \( \beta \)-(1,3) and \( \beta \)-(1,6)-glucan as compared to wild-type cells. This result was apparently at variance to previous reports indicating a compensatory change in cell wall composition, as a reduction in mannans could be partially compensated by an increase of \( \beta \)-glucan (Dallies et al., 1998; Popolo et al., 2001; Klis et al., 2006). Although this statement was not totally incorrect, as seen for instance with \( fks1 \Delta \), \( gas1 \Delta \) and \( kre6 \Delta \) mutants for which a reduction of \( \beta \)-glucan was accompanied by an increase of mannans, we actually found that the protein content in the cell wall of a \( mnn9 \Delta \) mutant was found to be threefold higher than in wild type (Table 3).

**Effect of various stresses on levels of chitin, \( \beta \)-(1,3) and \( \beta \)-(1,6)-glucan**

To enlarge our study, we conducted a biochemical analysis of cell wall composition in response to various environmental stresses as well as to the treatment with caspofungin, an antifungal drug acting specifically on \( \beta \)-(1,3)-glucan synthase (Deresinski & Stevens, 2003). When yeast cells were transferred from 30 to 42 °C, we recently showed using atomic force microscopy (AFM) technology, the formation of a circular structure at the cell surface that takes its origins at a single punctuated source and propagates into concentric manner to reach a diameter of 2–3 \( \mu m \) (Pillet et al., 2014). In addition, as reported in Table 4, the heat shock at 42 °C caused a 45% reduction of the \( \beta \)-(1,3)-glucan that was accompanied by 20% rise in \( \beta \)-(1,6)-glucan (\( P < 0.01 \)) and by a twofold increase of chitin (\( P < 0.001 \)). Sensitivity of wild-type and cell wall defective mutants to ethanol was monitored in a series of experiments, with each sample analysis technically performed three times. Significance of the difference was statistically calculated by the t-test student to give \( P \) values which were represented by *\( P < 0.01 \) and **\( P < 0.001 \).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mannans</th>
<th>( \beta )-(1,3)-glucan</th>
<th>( \beta )-(1,6)-glucan</th>
<th>Chitin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>34.3 ± 2.9</td>
<td>38.7 ± 4.6</td>
<td>22 ± 9</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>Ethanol 9%</td>
<td>34.9 ± 1.4</td>
<td>38.0 ± 2.1</td>
<td>22 ± 2.0</td>
<td>4.9 ± 1.1</td>
</tr>
<tr>
<td>Heat shock</td>
<td>44.6 ± 2.6**</td>
<td>20.3 ± 3.5**</td>
<td>25 ± 3.2*</td>
<td>10.1 ± 0.5**</td>
</tr>
</tbody>
</table>

At the exponential phase of growth on YPD (OD\(_{600 \, nm}\) 1.5 unit), the BY4741 cells were exposed to 9% ethanol for 5 h exposure or subjected to a heat shock at 42 °C for 1 h or to 9. The cells were then collected to measure chitin, \( \beta \)-(1,3)- and \( \beta \)-(1,6)-glucan by the enzymatic method, and mannans by acid method as described in ‘Material and methods’. Results shown are the mean ± SD from three independent experiments, with each sample analysis technically performed three times. Significance of the difference was statistically calculated by the t-test student to give \( P \) values which were represented by *\( P < 0.01 \) and **\( P < 0.001 \).
mutant lacking the major β-(1,3)-glucan synthase. This can be explained in part by the action of the antifungal drug to also inhibit the activity of the minor β-glucan synthase encoded by FKS2. This significant modification of cell wall composition induced by caspofungin can be related with the increased mechanical resistance of the cells after treatment with this drug (Formosa et al., 2013).

Discussion

The yeast cell wall is a complex structure made of 4 types of polysaccharides: mannans, β-(1,3)-glucan, β-(1,6)-glucan and chitin whose proportions of each of them, and linkages between them can dramatically change according to growth conditions, stresses, etc. (Klis et al., 2006; Orlean, 2012). In addition, as several cell wall components may have biotechnological values, there is a crucial need to precisely quantify the content of each of these polymers. The method presented in this study was developed to respond to this question by combining specific enzymatic hydrolysis and chemical hydrolysis. While a similar approach was previously attempted by others to characterize cell wall structure and/or quantify cell wall polysaccharides composition (Fleet & Manners, 1977; Hong et al., 1994; Magnelli et al., 2002), we here provided additional inputs that allow conducting a simple protocol to achieve this goal, without requiring any fractionation, dialysis procedure or labelling of the yeast cells to trace the fate of the polysaccharides during their separation. The main inputs were (i) to identify and select a very active chitinase to fully hydrolyse chitin into its monomer N-acetylglucosamine, which can be thereafter determined by a simple colorimetric method. There were some previous attempts to employ chitinase from Serratia marcescens to hydrolyse chitin from the cell wall (Molano et al., 1977; Magnelli et al., 2002). While this enzyme is no longer commercially available, two other chitinases are available in the Sigma catalogue. We found that only the chitinase preparation from Streptomyces griseus was highly active on pure chitin and did not exhibit any side activity on β-glucan and mannans. In addition, we showed that maximal hydrolysis of chitin in the yeast cell wall required the incubation of this bacterial chitinase with endo/exo-β-glucanases, likely because the latter enzymes allowed better access of chitinase to chitin microfibrils, as proposed earlier (Cabib & Sburlati, 1988). Accordingly, we found that the chitin content in exponentially growing yeast cells on glucose was in the range of 5% of the cell wall dry mass, which is 2–3 times higher than commonly considered (Lesage & Bussey, 2006; Orlean, 2012). This data may suggest that previous methods based on acid hydrolysis were likely not effective enough to release all chitin that is bound to β-(1,3) and β-(1,6)-glucan, and which gives rise to a tightly knit mesh (Klis et al., 2006; Cabib & Arroyo, 2013). This new evaluation of the chitin content in the cell wall may better fit with the crucial role of this polymer in yeast morphogenesis (Blanco et al., 2012; Cabib & Arroyo, 2013). It remains unknown whether this enzymatic mixture comprising chitinase and β-glucanases can release the last N-acetylglucosamine resi-
dues that are linked at the nonreducing end of β-(1,3)-linked glucan through a β-(1,4) linkage (Kollar et al., 1995).

The second important accomplishment was to use a combination of ‘relatively pure’ endo and exo-β-(1,3)-glucanases from Trichoderma reesei that exhibited high specificity and efficient cleavage of the β-(1,3)-glycosidic bonds of the β-(1,3)-glucan polymers. This mix of enzymes was shown to completely hydrolyse laminarin, a linear β-(1,3)-glucosyl polymer and to release 80% of β-glucan present in the alkali-soluble fraction of purified yeast cell walls, in accordance with the level of β-(1,3)-glucan determined by previous chemical analysis of this fraction (Fleet & Manners, 1977). This combination of enzymes was more useful than the single recombinant endo-β-(1,3)-glucanase, which only releases laminaribiose and laminaritriose from linear β-(1,3)-glucan (Magnelli et al., 2002; Aimanianda et al., 2009). The last input of the method was to find an enzymatic treatment to quantify β-(1,6)-glucan as glucose units. To this end, the recombinant endo-β-(1,6)-glucanase from Trichoderma harzianum was combined with a β-glucosidase to split the disaccharide gentiobiose (G2) that is the major product of the endolytic action of β-(1,6)-glucanase on commercial β-(1,6)-glucan (pustulan). However, it turned out that the proportion of β-(1,6)-glucan in total β-glucan determined in purified yeast cell wall with our enzymatic method were around 22–27% (see Fig. 1 and Table 1). This value is about 15–20% higher than previously reported either by chemical analysis (Fleet, 1991) or by combination of enzymatic and acid hydrolysis of 13C-labelled yeast cells (Hong et al., 1994; Magnelli et al., 2002). This overestimation was confirmed by periodate oxidation of β-(1,6)-glucan fraction obtained after cell wall digestion with endo/ exo-β-(1,3)-glucanases. This chemical reagent is specific to attack vicinal hydroxyl groups on the glucose residues. Thus, in the case of linear β-(1,6)-linked glucose units, it specifically oxidizes and cleave C-C bonds between C2, C3 and C4, releasing a stoichiometric amount of formic acid. A simple explanation for this overestimation can be found in previous works of Manners et al. (1973b) and Aimanianda et al. (2009), showing that β-(1,6)-glucan is a highly branched polymer composed of 65–70% of glucose residues linked by C1 and C-6, 15% of nonreducing terminal glucopyranose residues, 5% of glucose linked by C-1 and C-3 and 10–15% of residues that are triply linked at C-1, C-3 and C-6. By subtracting the 15–20% glucose excess from this β-(1,3,6)-linked glucose units that is apparently cleaved by the β-glucosidase, the linear β-(1,6)-glucan content dropped to a proportion of 18–20% of total cell wall β-glucan, in agreement with data reported by Magnelli et al. (2002). As a conclusion, we could consider that our enzymatic method provides an overall quantification of the highly branched β-(1,6)-glucan that include β-(1,6)-linked glucose units and other glucose units linked at C1, C3 and C6 on the β-glucan backbone.

Using this novel method, we confirmed the huge rise of chitin levels in yeast mutants defective in the synthesis of β-(1,3)-glucan (fks1Δ), β-(1,6)-glucan (kre6Δ) and mannans (mnn9Δ) (Dallies et al., 1998), as well as in gas1Δ which lacks a major glucanosyltransferase that plays an important role in cell wall remodelling (Popolo & Vai, 1999; Carotti et al., 2004; Plotnikova et al., 2006). More interestingly, this method has highlighted significant changes in the relative proportion of β-(1,3) and β-(1,6)-glucan in these different mutants as well as in response to stresses. As expected, a mutant deleted for KRE6 encoding a protein needed in β-(1,6)-glucan synthesis (Roemer & Bussey, 1991; Kurita et al., 2011) has a low level of β-(1,6)-glucan, which supports the validation of our enzymatic method. However, in response to other mutations, as well as to heat shock and treatment with caspofungin, we noticed a significant reduction in the β-(1,3)-glucan content, whereas levels of the ‘highly branched’ β-(1,6)-glucan increased, with a concurrent increase of chitin. Taking into account that expression of genes encoding cell wall remodelling enzymes such as Crh1, Crh2, Cwp1, Gas1 and Bgl2 are strongly upregulated under these conditions (Lagorce et al., 2003; Reynolds-Martin et al., 2003; Rodriguez-Pena et al., 2010), this increased level of β-(1,6)-glucan is in accordance with an amplified cross-linkages between this polymer and chitin and between β-(1,6)-glucan and β-(1,3)-glucan (Kollar et al., 1995, 1997), in response to the cell wall compensatory mechanism that is triggered under these conditions (Klis et al., 2006; Lesage & Bussey, 2006; Orlean, 2012).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Chromatography profiles resulting from enzymatic hydrolysis of ß-(1,3)-glucan.

Fig. S2. Chromatography profiles resulting from enzymatic hydrolysis of ß-1,6-glucan.

Fig. S3. Chromatography profiles resulting from enzymatic hydrolysis of chitin.

Fig. S4. Evaluation of lipids contamination in purified yeast cell wall (Folch et al., 1957).

Fig. S5. Schematic diagram of yeast cell wall fractionation and analysis of the alkali-insoluble acid-soluble fraction for the content of ß-(1,6)-glucan content by periodate oxidation (fraction C) and by enzymatic hydrolysis with endo ß-(1,6)-glucanase and ß-glucosidase (fraction A).