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In Vitro Potential Antioxidant Activity of (1→3),(1→6)-β-D-Glucan and Protein Fractions from *Saccharomyces cerevisiae* Cell Walls

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(1→3),(1→6)-β-D-Glucan, a cell wall polysaccharide in many microorganisms, fungi and algae, is a well-known biological response modifier. Recently, it was found that (1→3)-β-D-glucan from *Saccharomyces cerevisiae* also exhibits antioxidative capabilities. In this study the antioxidative activity of the cell wall fractions of brewer's yeast was investigated. Particular emphasis was put on the question to which extent glucan is responsible for the antioxidative activity of the cell walls and how the other cell wall components might contribute. For the experiments yeast cell walls from brewery fermentations were used. Glucan was isolated by a three-step extraction procedure including a combination of hot water and enzymatic treatment. The level of (1→3),(1→6)-β-D-glucan in the cell walls was analyzed enzymatically. The antioxidant activity was determined by electron paramagnetic resonance spectrometry and Trolox equivalent antioxidant capacity assay. The results show that the antioxidative activity of yeast cell wall proteins exceeds that of β-glucan greatly. Especially aromatic side chains and free thiols from denatured proteins seem to work as antioxidants.

KEYWORDS: Yeast glucan; cell wall proteins; antioxidative activity; electron paramagnetic resonance spectrometry; Trolox equivalent antioxidant capacity

INTRODUCTION

Plenty of spent brewer's yeast (*Saccharomyces cerevisiae*) is produced as a byproduct of the brewing process every year. The yield comprises about 15 L from leftovers and 3−4 L from deposits per cubic meter of beer, or roughly 2−3% of total beer production (*1*, *2*).

The cell wall of *Saccharomyces cerevisiae* consists of approximately 29−64% β-glucans, 31% mannans, 13% proteins, 9% lipids, and 1−2% chitin (*3*−*5*). However, the exact structure and composition of the yeast cell wall depends strongly on the cultivation conditions. The cell wall consists of two layers: on the outside there is a mannoprotein complex; on the inside is (1→3)-β-D-glucan as a structural polymer. The structure of the glucan is composed of 1,3-glycosidic basic chains which are linked via 1,6-glycosidic intermediate chains to form a three-dimensional network. Glucan can be subdivided into two components: one that is insoluble in alkali and accounts for ca. 80−85% of the entire content and one that is alkali-soluble (*3*).

Most of the spent yeast is sold as low-price animal feed or has to be disposed with costs. However, yeast β-glucan shows a certain bioactivity and thus a potential physiological value. Various publications describe the strong positive influence of these glucans on the immune system, resulting in antibacterial, wound-healing, and antitumor activities (*6*−*10*). Hence, producing a high-value-added product, e.g., functional food from yeast glucan, could benefit breweries and the yeast industry by receiving an additional source of income and eliminating the costs of waste disposal.

In recent years evidence suggested that biopolymers (like β-glucan) also exhibit antioxidative activity (*11*). The exploration of antioxidants is of great interest because they are able to protect living organisms from the attack of reactive oxygen species (ROS), and in this way to decrease the risk of several degenerative diseases (*12*, *13*). *Saccharomyces cerevisiae* contains several endogenous substances which act as antioxidants (*14*, *15*). There are enzymatic components such as superoxide dismutase, catalase, or glutathione (GSH) reductase as well as nonenzymatic compounds like GSH, ubiquinone, sulfhydryl amino acid, or mineral ions (*16*). In addition to antioxidants within the cell, yeasts also contain potential antioxidants in their cell walls. The antioxidative capability of yeast (1→3)-β-D-glucan in terms of free radical scavenging was

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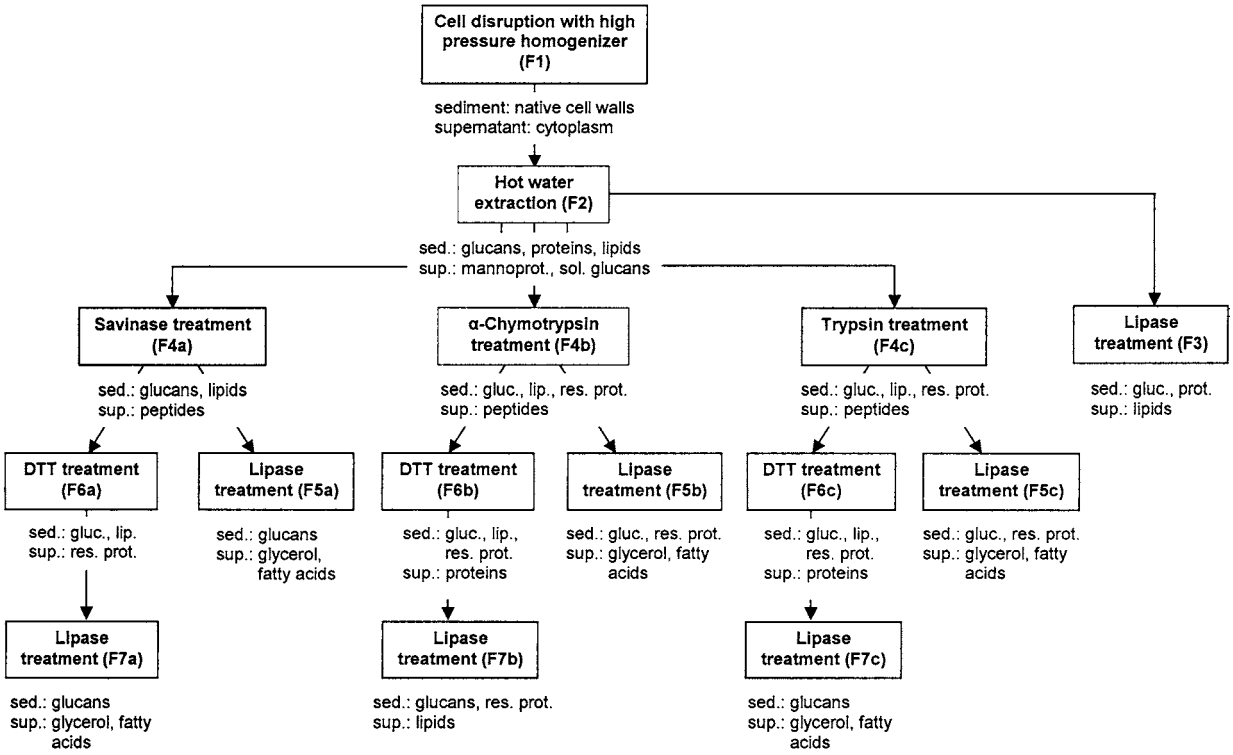

Figure 1. Schematic process for the fractionation of yeast cell walls; sed. = sediment; sup. = supernatant; sol. = soluble; gluc. = glucans; lip. = lipids; prot. = proteins; res. prot. = residual proteins.

investigated by Kogan et al. (*17*). The EPR technique was applied to assay the antioxidative activity of a derivative of alkali-insoluble (1→3)-*β*-D-glucan rendered water-soluble by carboxymethylation. Its radical scavenging activity was compared to that of water-soluble D-mannitol, frequently considered as a reference for carbohydrate-type antioxidants (*11*, *18*). For the (1→3)-*β*-D-glucan derivative the scavenging activity was much stronger than that for D-mannitol. These findings correlate with those of Tsiapali et al. (*11*) who suggest that increased antioxidant activity of polysaccharides in comparison with monosaccharides is due to the fact that polysaccharides contain multiple anomeric hydrogen atoms which are primarily abstracted by the active free radicals, while monosaccharides possess only one such anomeric hydrogen.

The aim of the present work was to evaluate the antioxidative activity of (1→3),(1→6)-*β*-D-glucan and protein fractions of *Saccharomyces cerevisiae* cell walls. In contrast to previous works (*17*, *19*) underivatized glucan was investigated since unmodified (1→3),(1→6)-*β*-D-glucan from the yeast cell wall of baker's yeast is classified as "Generally Recognized as Safe" or GRAS by the FDA (FDA Specifications 184.1983). For the prospective application as a food additive or dietary supplement it is useful to leave the *β*-glucan chemically unmodified to maintain its classification as GRAS. Furthermore, employing drastic conditions in the chemical extraction and the derivatization can degrade the glucan severely (*20*, *21*). However, as the chain length seems to have a significant effect on the antioxidative activity (*11*), this study uses a mild enzymatic method for isolating the glucans (*22*).

In addition, the yeast cell wall contains more potentially interesting constituents than just glucan (e.g., mannans (*23*) and proteins). However, there has been no methodical evaluation of the antioxidative capabilities of the yeast cell wall components individually (apart from glucan) or in combination with each other so far.

For this reason a fractionation of the cell wall was conducted yielding fractions differently enriched with glucan and containing varying amounts of the other cell wall components. EPR measurements and TEAC assay of the different fractions were performed to elucidate a potential contribution of the other cell wall components to the antioxidative activity of yeast cell wall glucan.

MATERIALS AND METHODS

Yeast. The yeast used in this study was a *Saccharomyces cerevisiae*, strain H31, provided by the Berliner Pilsner brewery, Berlin, Germany. Since the yeast was obtained from brewery fermentation, it was washed three times to remove beer residues. Therefore, 3 kg of yeast with a dry substance of 21% was washed with 18 L of water.

Yeast Cell Disruption. The separation of the cell walls was carried out by mechanical disruption in a high-pressure homogenizer (type Panda, 1000 bar, Niro Soavi, Parma, Italy). Therefore, the dry mass of the yeast suspension was adjusted to 7.5% (w/w) with water. Three homogenization cycles at 700 bar were needed. The yeast cell walls were separated in a Beckman J2-HS lab centrifuge yielding a sediment consisting of disrupted cell walls (fraction F1) and a supernatant containing the cell content. Subsequently, the sediment was washed three times with water. For analytical purposes, an aliquot of the sediment was lyophilized in an Alpha 2-4 freeze dryer (Christ GmbH, Osterode, Germany). The supernatant was stored at −20 °C.

Processing of the Yeast Cell Walls. Glucan was enriched in the yeast cell walls by a modified procedure of Freimund et al. (*22*). The schematic process for the fractionation is shown in **Figure 1**. After cell disruption in a high-pressure homogenizer (fraction F1), mannoproteins and water-soluble glucans were extracted from the cell walls by a hot water treatment (F2). Afterward, the remaining cell wall proteins were removed by either the commercially available protease Savinase (F4a) or by more selective proteases as α-chymotrypsin (F4b) and trypsin (F4c), respectively. After protease treatment each sample was split into two halves. While one-half was treated with a commercially available lipase to remove the cell wall lipids (F5a, F5b, and F5c), the other half was treated with dithiothreitol (DTT) to improve

Table 1. Percentage of Initial Cell Wall Material of All Sediments with Respect to Cell Dry Mass before Cell Disruption and Protein Content of the Sediments

fractions	percentage of initial cell wall material [% dry weight]	protein content [% dry weight]
F1	20	22.23
F2	9.7	18.17
F3	7.6	16.93
F4a	7.6	2.50
F5a	6.3	2.45
F6a	6.4	1.75
F7a	5	1.80
F4b	9.7	17.16
F5b	8.9	16.78
F6b	8.3	17.22
F7b	7	15.22
F4c	9.4	18.59
F5c	9.4	15.84
F6c	9.3	17.17
F7c	7.9	16.86

protein extraction (F6a, F6b, and F6c). Cell wall lipids were also extracted by a commercial lipase (F7a, F7b, and F7c).

For the purpose of analysis, a sample was taken after every step of the extraction procedure. Glucan-rich sediment was separated by centrifugation from a supernatant which contained the cell components extracted in the previous step. Thus, different fractions of glucan-enriched yeast cell walls (sediments) and supernatants containing cell wall components were gained. The sediments were lyophilized and ground to powder; the supernatants were filtered through a membrane filter with 0.2 μm pore width to remove solids. The recovery of the different fractions obtained during the fractionation process is given in **Table 1**.

Hot Water Extraction. After cell disruption the washed sediment (fraction F1) was diluted with water to 13% dry mass (w/w) and adjusted to pH 7 with NaOH (30% w/w), according to Freimund et al. (*22*). The suspension was heated to 125 °C in a VARIOKLAV steam autoclave (H + P Labortechnik AG, Oberschleissheim, Germany) for 5 h. Afterward, the suspension was cooled down to room temperature and was diluted with 72.5 mL water per 100 g suspension. The sediment was separated by centrifugation and washed twice with water. An aliquot of the sediment (fraction F2) was lyophilized and the supernatant was stored at −20 °C.

Savinase Treatment. Following hot water extraction, the washed sediment (fraction F2) was diluted with water to 4% (w/w) dry mass. After the mixture was heated to 45 °C and the pH adjusted to 10.5 with NaOH (30% w/w), the protease Savinase 16.0 L type EX (Novozymes, Bagsvaerd, Denmark) was added under continuous stirring at three times (t = 0, 1.5, and 3 h); 0.075 mL of Savinase per 100 mL suspension was needed each time. After 5 h, the sediment was separated by centrifugation and washed twice with water. An aliquot of the sediment (fraction F4a) was lyophilized and the supernatant was stored at −20 °C.

α-Chymotrypsin Treatment. Subsequent to hot water extraction, the washed sediment (fraction F2) was diluted with water to 4% (w/w) dry mass. After the mixture was heated to 45 °C and the pH adjusted to 8 with NaOH (30% w/w), an α-chymotrypsin solution was added under continuous stirring at three times (t = 0, 1.5, and 3 h). The α-chymotrypsin (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) solution was prepared with water, yielding an activity of 5 U mL^{-1}; 1.15 U per 100 g cell wall suspension was needed each time. After 5 h the sediment was separated by centrifugation and washed twice with water. An aliquot of the sediment (fraction F4b) was lyophilized and the supernatant was stored at −20 °C.

Trypsin Treatment. Following hot water extraction, the washed sediment (fraction F2) was diluted with water to 4% (w/w) dry mass. After the mixture was heated to 45 °C and the pH adjusted to 8 with NaOH (30% w/w), a trypsin solution (1.56 U mL^{-1}) was added under continuous stirring at three times (t = 0, 1.5, and 3 h). The trypsin (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) solution was prepared with water, yielding an activity of 150 U mL^{-1}; 1.15 U per 100 g cell wall suspension was needed each time. After 5 h the sediment was separated by centrifugation and washed twice with water. An aliquot of the sediment (fraction F4c) was lyophilized and the supernatant was stored at −20 °C.

Dithiothreitol (DTT) Treatment. After protease treatment the washed sediment (fractions F4a, F4b, and F4c) was diluted with water to 4% (w/w) dry mass and further treated with dithiothreitol (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) (0.003 mol per L of suspension) at room temperature under stirring for 15 min. The sediment was separated by centrifugation and washed twice with water. An aliquot of the sediment (fractions F6a, F6b, and F6c) was lyophilized and the supernatant was stored at −20 °C.

Lipase Treatment. Subsequent to either hot water extraction (fraction F2), protease treatment (fractions F4a, F4b, and F4c), or DTT treatment (fractions F6a, F6b, and F6c), the washed sediment was diluted with water to 4% (w/w) dry mass and further treated with Lipolase 100 L EX (Novozymes, Bagsvaerd, Denmark) at 45 °C and pH 10.5 with stirring for 3 h (1 g of Lipolase per 100 mL suspension). The glucan particles were harvested by centrifugation, washed twice, and lyophilized. An aliquot of the sediment (fractions F3, F5a, F5b, F5c, F7a, F7b, and F7c) was lyophilized and the supernatant was stored at −20 °C.

Determination of (1→3),(1→6)-β-D-Glucan Content. The amount of (1→3),(1→6)-β-D-glucan in the cell wall fractions was determined enzymatically by the commercial assay "YEAST BETA GLUCAN ASSAY KIT" (Megazyme Int., Bray, Ireland). For the analysis the lyophilized, ground sediments of each fraction were used. The measurements were carried out with a Novaspec II spectrophotometer (Pharmacia LKB, Sweden). The absorption of all solutions was measured at 510 nm against a blank and the β-glucan content was calculated.

Determination of Mannoprotein Content. Determination of the mannoprotein content of the native cell walls was carried out by ethanol precipitation. Nine grams of the supernatant after hot water extraction (F2) were added to ethanol under stirring until the water content reached 30%. The mixture was stored overnight at 4 °C. The precipitate was centrifuged, washed with ethanol/H_2O (70% w/w), and dried at 105 °C in an incubator until no residual moisture was left. The dried precipitate was weighed and mannoprotein content was calculated with respect to cell wall dry mass (dry mass of F1).

Determination of Protein Content. Protein contents of the cell wall fractions were determined by a Kjeldahl method (Kjeldahtherm-Turbosog-Vapodest 33, C. Gerhardt GmbH & Co. KG, Bonn, Germany). The factor 6.25 was used to convert for protein concentration.

Determination of Antioxidative Activity. Two methods were applied to measure the antioxidative activity: electron paramagnetic resonance spectrometry (EPR) and Trolox equivalent antioxidant capacity assay (TEAC).

Electron Paramagnetic Resonance Spectrometry (EPR). The ability of the samples to donate a hydrogen atom or electron to a free radical was monitored by electron paramagnetic resonance spectrometry (EPR) (*24*) using a synthetic stabilized radical.

Both sediments and supernatants of the cell wall fractions were analyzed with a Miniscope MS 100 (Magnettech GmbH, Berlin, Germany). For the sediments the lipid soluble radical TEMPO (2,2,6,6-tetramethyl-piperidine-1-oxyl) was chosen since the sediments were dissolved in DMSO. For the aqueous supernatants the water-soluble Fremy's salt (potassium nitrosodisulfonate) was used as a radical, according to Roesch et al. (*25*).

A 10-fold dilution of the supernatants was added to an equal volume of Fremy's salt radical solution (1 mM in phosphate buffered saline, pH 7.4). The spectrum of the low-field resonance of Fremy's salt was recorded from 90 s after mixing the sample with the radical for a duration of 25 min by EPR. Signal intensity was obtained by double integration and the concentration calculated by comparison with a control reaction (phosphate buffer instead of supernatant). Spectra were obtained at 20 °C. The antioxidative activity (mM Fremy's salt per L of supernatant) was expressed as the percentage of Fremy's salt radicals reduced by the samples and was calculated by eq 1.

$$AA = \frac{\text{reduction of Fremy's salt [\%]} \times \text{initial amount of Fremy's salt [mM]}}{100 \times \text{initial amount of sample (undiluted) [L]}} \quad (1)$$

One hundred microliters of dissolved sediment (50 mg of lyophilized, ground sediment per 5 mL of dimethyl sulfoxide) was added to an equal volume of TEMPO radical solution (1 mM in DMSO). The spectrum of the low field resonance of TEMPO was recorded from 90 s after mixing of the sample with the radical for a duration of 25 min by EPR. Signal intensity was obtained by double integration and the concentration calculated by comparison with a control reaction with DMSO instead of the dissolved sediment. Spectra were obtained at 20 °C. The antioxidative activity (mM TEMPO per g of sediment) was expressed as the percentage of TEMPO radicals reduced by the samples and was calculated by eq 2.

$$AA = \frac{\text{reduction of TEMPO [\%]} \times \text{initial amount of TEMPO [mM]}}{100 \times \text{initial amount of sample [g]}} \quad (2)$$

Trolox Equivalent Antioxidant Capacity (TEAC). As a second method, a modified TEAC assay (*26*) was used to measure the antioxidant activity of the supernatants. Originally described by Miller et al. (*27*), the TEAC assay is based on scavenging of the long-living 2,2-azinobis-3-ethylbenzthiazolin-6-sulfonic acid (ABTS) radical anions. The radicals are generated by potassium persulfate and can easily be detected with a spectrophotometer at 734 nm (*28*). Present antioxidants reduce the absorption according to their concentration. As a reference substance, Trolox (a water-soluble analogue of vitamin E, Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) was used. Absorbance was measured exactly 6 min after the reaction was started by addition of persulfate to a mixture of ABTS and the supernatants (10-fold diluted with PBS buffer, pH 7.2−7.4). The modification used here was to measure absorbance after exactly 6 min. The exact time period between starting the reaction and measuring the absorbance is very important because of the stoichiometric reaction of ABTS and persulfate, which leads to an incomplete, time-dependent formation of the ABTS radical (*26*).

The antioxidative activity of the sample is quantified by comparison with Trolox (constructing a calibration curve) and is given in Trolox equivalents (mM Trolox per L).

Statistical Analysis. The fractionation of the yeast cell walls was done once. All analyses were carried out in duplicate and the standard deviation was calculated. The averaged values along with the standard deviations are documented in the respective figures.

RESULTS AND DISCUSSION

Content of (1→3),(1→6)-β-D-Glucan. The content of (1→3),-(1→6)-β-D-glucan in the different cell wall fractions is shown in **Figure 2**.

The β-glucan content of the native yeast cell wall (F1) amounts to 42.6% dw which correlates with the literature data (*3*). For the hot water extracted cell wall fraction (F2) the glucan content rises to 64.5% dw. This enormous increase (more than 20% dw) accounts for mannoproteins as confirmed by ethanol precipitation and is less than that quoted in the literature (*3*). Enzymatic lipid extraction, resulting in fraction F3, hardly altered the glucan content in comparison to fraction F2. The slight difference in glucan content (2.5% dw) corresponds with the lipid content of the native cell wall lying below the 9% dw quoted in the literature (*3*). However, the composition of the cell wall depends strongly on growth conditions of the yeast; thus, deviation from literature data is not unusual.

The treatment of fraction F2 with Savinase (major enzymatic activity is subtilisin which hydrolyzes protein with low specificity (*29*)) leads to a glucan content of 83% dw in fraction F4a. This is an 18% dw rise which is due to the protein content of the cell wall. However, the proteins of the mannoprotein complex also contribute to the cell wall proteins. Since mannoproteins, which make up 20% dw of the cell wall, have a protein content of 20% dw (Kjeldahl), their protein contribution to the cell wall amounts to 4% dw and their carbohydrate contribution (mannans) to 16% dw. Thus, protein makes up approximately 22% dw of the whole cell wall. This value corresponds very well with the results of protein determination according to Kjeldahl (**Table 1**). Therewith, protein content of the cell walls applied in this study is much higher than the 13% dw quoted in the literature (*3*). Further treatment of fraction F4a with a lipase and dithiothreitol (resulting in F5a, F6a, and F7a) did not offer results that could be evaluated since glucan contents of F5a and F7a are similar to those of F4a if the standard deviations are taken into account and glucan content of F6a is even lower.

Treatment of the hot water extracted fraction F2 with the highly specific proteases α-chymotrypsin and trypsin did not change the glucan content significantly (F4b and F4c). Obviously, both enzymes removed very few proteins from the cell wall as verified by additional Kjeldahl protein determination (**Table 1**). Protein content of the cell wall fractions treated with α-chymotrypsin and trypsin did not differ much from that of the hot water extracted fraction. An explanation might be found in the way the two proteases work. α-Chymotrypsin is known to have specificity for those peptide bonds which contain aromatic amino acid residues such as tryptophan, tyrosine, and phenylalanine. Trypsin preferentially splits those peptide linkages, which contain either lysine or arginine as protein side chains (*29*). Both enzymes are highly specific in contrast to Savinase and thus can only release a small number of peptides.

After α-chymotrypsin treatment the application of lipase and dithiothreitol treatments result in a small increase of the glucan content (F5b, F6b, and F7b). For the trypsin treated cell wall fractions glucan content rises only after lipase treatment (F5c and F7c). The increase of the glucan content after lipolytic treatment is probably due to a proteolytic coactivity of the Lipolase as demonstrated by a decrease in protein content. DTT reduces disulfide bonds in proteins resulting in two free thiol groups (*30*). However, protein determination shows no significant decrease in protein content of the DTT treated cell wall fractions, indicating that application of DTT did not improve protein release any further.

The protein contents of all cell wall fractions are listed in **Table 1**.

EPR Measurements. According to the methods of Roesch et al. (*25*), both, sediments and supernatants of the cell wall fractions were analyzed with EPR to determine the antioxidative activity.

Exemplarily, the degradation kinetics of TEMPO and Fremy's salt are shown in **Figure 3A** for the native yeast cell wall (sediment F1), for dissolved mannoproteins and glucans (supernatant F2), and for peptides in aqueous solution (supernatant F4a).

As illustrated by the degradation kinetics, the native yeast cell wall hardly reduces the radical concentration. In contrast, supernatant F2 (mannoproteins and soluble glucans) and F4a (peptides) show a much stronger radical degradation. This effect is caused by the protein content of the two supernatants. The side chains of the amino acids phenylalanine, tryptophan, and tyrosine may display antioxidative activity due to their aromatic character. The low antioxidative activity of the native cell wall can be explained by looking at its composition. The potentially antioxidative (1→3),(1→6)-β-D-glucan network is located on

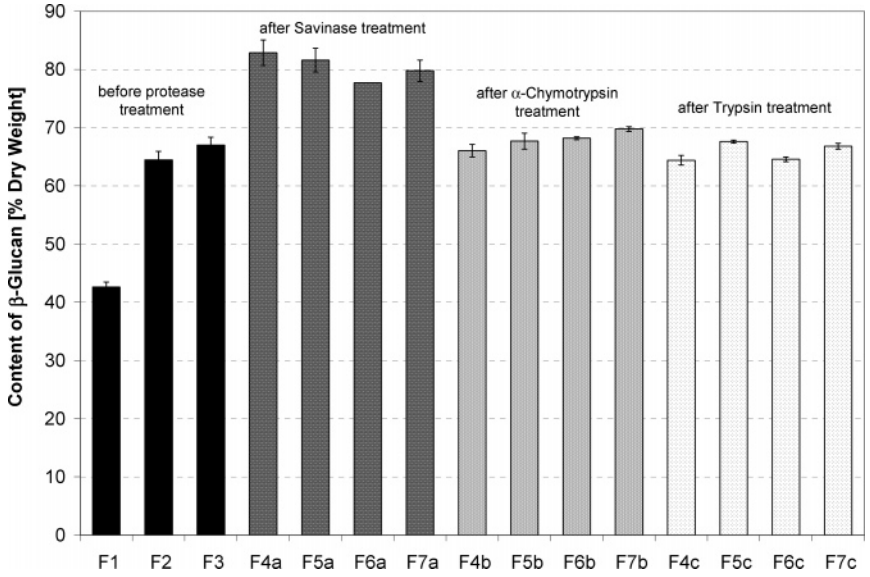

Figure 2. (1→3),(1→6)-β-D-Glucan content of the cell wall fractions (sediments). Fraction numbers refer to Figure 1.

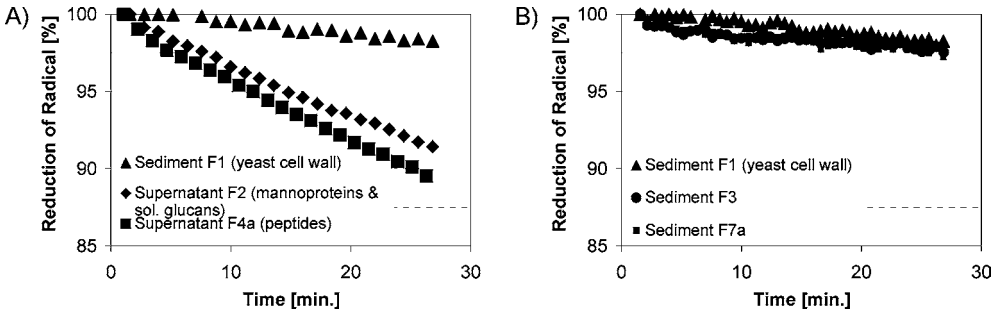

Figure 3. EPR assay. Radical degradation kinetics of selected fractions. Radicals used: TEMPO for sediments (F1, F3, and F7a); Fremy's salt for supernatants (F2 and F4a). Fraction numbers refer to Figure 1.

the inside and is covered by a mannoprotein complex (*3*). Furthermore, in its unprocessed form, β-glucan from baker's yeast is entangled with fat and protein (*31*). For these reasons the (1→3),(1→6)-β-D-glucan might just not be accessible. However, as it seems that the proteins have a significant impact on the antioxidative activity, denaturing the protein structure with proteases and DTT seems to be an important step to gain antioxidative activity of the β-glucan and protein fractions.

For the sediment fractions the scavenging of TEMPO was too low to be evaluated. The sediments represent the gradually purified residues of the cell wall. Exemplarily, the degradation kinetics of three selected sediment fractions (F1, F3, and F7a) are shown in **Figure 3B**. Even fractions after Savinase, lipase, and DTT treatment, which show the highest β-glucan contents and thus represent a relatively pure (1→3),(1→6)-β-D-glucan (devoid of larger amounts of fat or proteins), do not show a significant antioxidative activity (**Figure 3B**, F7a). There is only one explanation: (1→3),(1→6)-β-D-glucan is hardly responsible for the antioxidative activity of the yeast cell wall. However, for carboxymethylated yeast β-glucan antioxidative activity was detected (*17*).

The antioxidative activities of the supernatants could be easily determined and were compared with the results of the TEAC assay. Since the EPR values correlate very well with the data of the TEAC assay, they are not shown in an extra diagram.

TEAC Assay. The total antioxidative activity of the different supernatants is shown in **Figure 4**.

High antioxidative activities were observed for the fractions F1 and F2. Fraction F1 consists of the cytoplasm obtained after cell disruption and contains several endogenous antioxidative substances, e.g., superoxide dismutase and ubiquinone (*16*). Supernatant F2, obtained after hot water extraction, consists mainly of mannoproteins and glucans dissolved in water. This combination seems to be highly antioxidative whereas pure mannoproteins did not show high antioxidative activity (data not shown).

The fractions F4a, F4b, and F4c result from protein removal of sediment F2. This sediment was treated with three different proteases, which were chosen according to different specificities. The most unspecific is the commercial Savinase whereas the enzymes α-chymotrypsin and trypsin are highly specific. F4a is the supernatant after Savinase treatment and shows the highest antioxidative activity of all fractions. Savinase cleaves proteins unspecifically, thus releasing many peptides. These peptides contain amino acids whose aromatic side chains may act as antioxidants (see above). The low antioxidative activities of the supernatants after treatment with the enzymes α-chymotrypsin and trypsin can be explained with their specificity. Trypsin only cleaves at arginine and lysine residues, whereas chymotrypsin cleaves at tyrosine, phenylalanine, tryptophan, methionine, and leucine residues (*29*). This could explain that the lowest antioxidative activities of all protease treated fractions are observed for the supernatants after trypsin treatment (F4c). In contrast, the comparatively less specific α-chymotrypsin can release more peptides, resulting in higher antioxidative activity (F4b). A contribution of the proteases to the antioxidative activity of the supernatants is not expected since they underwent self-proteolysis during the incubation with the cell wall material.

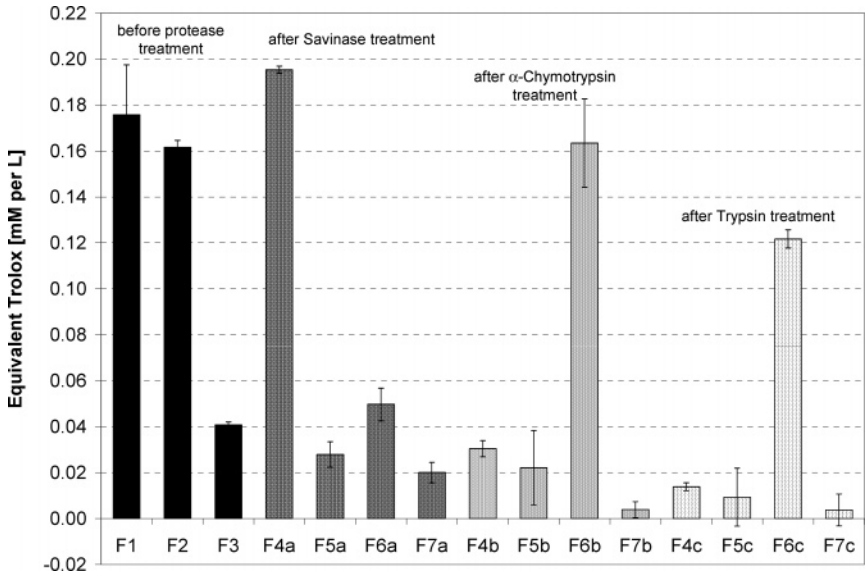

Figure 4. TEAC assay. Total antioxidative activity of the supernatants obtained after every extraction step. Fraction numbers refer to Figure 1.

Moreover, the proteases were applied in a very low concentration which revealed no significant antioxidative effect in control experiments.

After lipase treatment all supernatants (F3, F5a, F5b, F5c, F7a, F7b, and F7c) show low antioxidative activity. The commercially available lipase Lipolase hydrolyzes fat by cleaving the ester bonds in the 1 and 3 positions of triglyceride molecules. The resulting products are mono- and diglycerides, glycerol, and free fatty acids (*32*). All these do not have any groups which could act as antioxidants. The residual antioxidative activity probably accounts for a slight proteolytic coactivity of the Lipolase preparation, cleaving proteins and thus releasing peptides.

In contrast to the protein determination described above, the TEAC values illustrate that the application of DTT leads to an increase in the antioxidative activity. Especially for the specific proteolytic treatments (α-chymotrypsin and trypsin) an enormous increase was observed (F6b and F6c). Here it seems that DTT treatment improves the accessibility of the radicals by denaturing the proteins (splitting of disulfide bonds). Further, the resulting free thiol groups may contribute to the high antioxidative activity. When following the Savinase treatment, DTT does not give a remarkable impact (F6a) as, due to the unspecificity of Savinase, many peptides have already been released. The results from the EPR measurements correlate well with the TEAC assay.

To put these data in context, they are compared with the findings of Krizkova et al. (*19*) who investigated the antioxidative activities of water-soluble carboxymethylated glucans. They found that carboxymethylated glucan from *Saccharomyces cerevisiae* exhibits an almost 5 times lower antioxidative activity than trolox. In the present work the TEAC result of the most active supernatant fraction F4a (Figure 4) also shows activity that is 5 times lower than that of trolox. However, in this case the 10-fold dilution of the supernatants must be taken into account. Thus, even supernatant fractions with rather low TEAC values exhibit equal or even higher antioxidative activities (related to trolox) than the carboxymethylated glucan in the publication of Krizkova et al. (*19*).

All these observations lead to the conclusion that proteins play a very important role for the antioxidative activity of yeast cell walls, maybe even more than (1→3),(1→6)-β-D-glucans. After processing, the supernatants partly show very strong antioxidative activity, especially the supernatants after protein extraction. It can be assumed that the cell wall proteins act as antioxidants. More precisely, aromatic side chains and maybe thiol groups of the present proteins seem to be antioxidative whereas the intensity apparently depends on the accessibility of these groups.

For the brewing industry spent yeast is still a waste product, although the yeast cells are extremely valuable due to their high bioactivity. In the food industry yeast cell walls accumulate as byproducts during the production of yeast extract. However, the cell walls are of great interest, too, since they exhibit immunomodulating capabilities (*6*, *7*) and a high antioxidative activity after a few inexpensive and easy extraction steps (disruption, hot water extraction, and proteolytic treatment). Thus, the industrial waste products yeast and yeast cell walls could become high-value-added products, giving the yeast processing industries an extra source of income and decreasing their problems with waste disposal.

LITERATURE CITED

(1) Bock, M.; Oechsle, D. Beer recovery from spent yeast with Keraflux-membranes. *Brewer* **1999**, July, 340−345.

(2) United Nations Industrial Development Organization (UNIDO). Sectorial Profile of Brewing Industry, 2000.

(3) Kath, F.; Kulicke, W. M. Mild enzymatic isolation of mannan and glucan from yeast *Saccharomyces cerevisiae*. *Angew. Makromol. Chem.* **1999**, *268*, 59−68.

(4) Bacon, J.; Farmer, V.; Jones, D.; Taylor, J. The glucan component of the cell wall of baker's yeast (*Saccharomyces cerevisiae*) considered in relation to its ultrastructure. *Biochem. J.* **1969**, *114*, 557−567.

(5) Fleet, G. H.; Manners, D. J. Isolation and composition of an alkali-soluble glucan from cell-walls of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **1976**, *94*, 180−192.

(6) Fitzpatrick, F. W.; Haynes, L. J.; Silver, N. J.; Dicarlo, F. J. Effect of glucan derivatives upon phagocytosis by mice. *J. Reticuloendoth. Soc.* **1964**, *15*, 423−428.

(7) Bohn, J. A.; BeMiller, J. N. (1→3)-β-D-Glucans as biological response modifiers: a review of structure-functional activity relationships. *Carbohydr. Polym.* **1995**, *28*, 3−14.

(8) Engstad, C.; Engstad, R.; Olsen, J.; Osterud, B. The effect of soluble β-1,3-glucan and lipopolysaccharide on cytokine production and coagulation activation in whole blood. *Int. Immunopharmacol.* **2002**, *2*, 1585−1597.

(9) Pelizon, A. C.; Kaneno, R.; Soares, A. M. V. C.; Meira, D. A.; Sartori, A. Immunomodulatory activities associated with beta-glucan derived from *Saccharomyces cerevisiae*. *Physiol. Res.* **2005**, *54*, 557−564.

(10) Li, J.; Li, D. F.; Xing, J. J.; Cheng, Z. B.; Lai, C. H. Effects of β-glucan extracted from *Saccharomyces cerevisiae* on growth performance, and immunological and somatotropic responses of pigs challenged with *Escherichia coli* lipopolysaccharide. *J. Anim. Sci.* **2006**, *84*, 2374−2381.

(11) Tsiapali, E.; Whaley, J.; Kalbfleisch, H.; Ensley, H.; Browder, W.; Williams, D. Glucans and related natural polymers exhibit weak solution free radical scavenging activity, but stimulate free radical activity in a murine macrophagen cell line. *Free Rad. Biol. Med.* **2001**, *30*, 393−402.

(12) Babincova, M.; Sourivong, P. Free radical scavenging activity of *Scoparia dulcis* extract. *J. Med. Food* **2001**, *4*, 179−181.

(13) Wang, P.; Kang, J.; Zheng, R.; Yang, Z.; Lu, J.; Gao, J.; Jia, Z. Scavenging effects of phenylpropanoid glycosides from *Pedicularis* on superoxide anion and hydroxyl radical by the spin trapping method. *Biochem. Pharmacol.* **1996**, *51*, 687−691.

(14) Demasi, A. P.; Pcrcira, G. A.; Netto, L. E. Cytosolic thinredoxin peroxidase I is essential for the antioxidant defence of yeast with dysfunctional mitochondria. *Fed. Eur. Biochem. Soc. Lett.* **2001**, *509*, 430−434.

(15) Pinheiro, R.; Belo, I.; Mota, M. Oxidative stress response of *Kluyveromyces marxianus* to hydrogen peroxide, paraquat and pressure. *Appl. Microbiol. Biotechnol.* **2002**, *58*, 842−847.

(16) Santiago, L. A.; Mori, A. Antioxidant defenses of baker's yeast against free radicals and lipid peroxides in rat brain. *Arch. Biochem. Biophys.* **1993**, *306*, 16−21.

(17) Kogan, G.; Stasko, A.; Bauerova, K.; Polovka, M.; Soltes, L.; Brezova, V.; Navarova, J.; Miihalova, D. Antioxidant properties of yeast (1→3)-β-D-glucan studied by electron paramagnetic resonance spectrometry and its activity in the adjuvant arthritis. *Carbohydr. Polym.* **2005**, *61*, 18−28.

(18) Tsou, T.-C.; Yang, J.-L. Formation of reactive oxygen species and DNA strand breakage during interaction of chromium(III) and hydrogen peroxide in vitro: Evidence for a chromium(III)-mediated Fenton-like reaction. *Chem. Biol. Interact.* **1996**, *102*, 133−153.

(19) Krizkova, L.; Durackova, Z.; Sandula, J.; Slamenova, D.; Sasinkova, V.; Sivonova, M.; Krajcovic, J. Fungal β-(1-3)-D-glucan derivatives exhibit high antioxidative and antimutagenic activity in vitro. *Anticancer Res.* **2003**, *23*, 2751−2756.

(20) Mueller, A. Experimentelle Studien zur Struktur und zu ausgewaehlten Eigenschaften von Polysaccharidfraktionen aus Hefezellwaenden. Ph.D. Thesis, Humboldt University, Berlin, Germany, 1993.

(21) Mueller, A.; Ensley, H.; Pretus, H.; McNamee, R.; Jones, E.; McLaughlin, E.; Chandley, W.; Browder, W.; Lowman, D.; Williams, D. The application of various protic acids in the extraction of (1→3)-β-D-glucan from *Saccharomyces cerevisiae*. *Carbohydr. Res.* **1997**, *299*, 203−208.

(22) Freimund, S.; Sauter, M.; Kaeppeli, O.; Dutler, H. A new non-degrading isolation process for 1,3-β-D-glucan of high purity from baker's yeast *Saccharomyces cerevisiae*. *Carbohydr. Polym.* **2003**, *54*, 159−171.

(23) Krizkova, L.; Durackova, Z.; Sandula, J.; Sasinkova, V.; Krajcovic, J. Antioxidative and antimutagenic activity of yeast cell wall mannans in vitro. *Mutat. Res.* **2001**, *497*, 213−222.

(24) Rohn, S.; Kroh, L. W. Electron spin resonance—A spectroscopic method for determining the antioxidative activity. *Mol. Nutr. Food Res.* **2005**, *49*, 898−907.

(25) Roesch, D.; Bergmann, M.; Knorr, D.; Kroh, L. W. Structure-antioxidant efficiency relationships of phenolic compounds and their contribution to the antioxidant activity of sea buckthorn juice. *J. Agric. Food Chem.* **2003**, *51*, 4233−4239.

(26) Rohn, S.; Rawel, H. M.; Kroll, J. Antioxidant activity of protein-bound quercetin. *J. Agric. Food Chem.* **2004**, *52*, 4725−4729.

(27) Miller, N. J.; Rice-Evans, C.; Davies, M. J.; Gopinathan, V.; Milner, A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci.* **1993**, *84*, 407−412.

(28) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. A. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231−1237.

(29) Bond, J. S. Commercially available proteases. In *Proteolytic Enzymes*; Beynon, R. J., Bond, J. S., Eds.; IRL Press: Oxford, U.K., 1989; pp 232−240.

(30) Ruegg, U. T.; Rudinger, J. Cleavage of disulfide bonds in proteins. *Methods Enzymol.* **1977**, *47*, 111−116.

(31) Ross, G. D.; Cain, J. A.; Myones, B. L.; Newman, S. L.; Lachmann, P. J. Specificity of membrane complement receptor type three (CR3) for beta-glucans. *Complement Inflammat.* **1987**, *4*, 61−74.

(32) Jensen, G.; Gormsen, E.; Jensen, B. H.; Christensen, T.; Boel, E.; Stentebjerg-Olesen, B. Lipolase A microbial lipase for detergents developed by application of r-DNA technique. *Comun. Jorn. Com. Esp. Deterg.* **1990**, *21*, 23−37.

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