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A new non-degrading isolation process for 1,3-β-D-glucan of high purity from baker's yeast *Saccharomyces cerevisiae*

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Abstract

1,3-β-D-glucan is a widespread polysaccharide in microorganisms, mushrooms and plants. Numerous benefits for the health of humans and animals have been described for more than 50 years. One readily available source for 1,3-β-D-glucan is the cell wall of the common baker's or brewer's yeast *Saccharomyces cerevisiae*. Conventional isolation processes involving treatments with hot alkali and acids, however, cause a certain degradation of the polymeric chains resulting not only in reduced yields but also a possible weakening of the beneficial effects. We have developed a new non-degrading process for the isolation of glucan from yeast cell walls comprising gentle extraction steps and enzymatic treatments without any extreme pH values. Depending on the quality of the utilized yeast cell walls, 1,3-β-D-glucan is obtained in a purity of up to 92% and a yield of 87%. During this process, the valuable byproduct mannoprotein—also beneficial for health—is easily obtained.

Keywords: 1,3-β-D-Glucan; Mannoprotein; Yeast cell walls; *Saccharomyces cerevisiae*

1. Introduction

Polysaccharides are a very important class of biopolymers, which consist of long chains of repeating sugar units. They are structurally characterized mainly by the type of monomer unit, the chain length, the kind of the glycosidic linkage and the degree of branching. Nature continuously synthesizes huge amounts of polysaccharides, which serve particularly as structural scaffolds like cellulose in plants and chitin in animals or as storage carbohydrates like starch and glycogen. A special group of β-1,3-linked polyglucoses-usually named 'glucan' or 'β-glucan' – is widespread in many bacteria, fungi, mushrooms, algae and higher plants and has attracted attention because of bioactive and medicinal properties, such as immune-stimulating, anti-inflammatory, antimicrobial, anti-infective, antiviral, antitumoral, cholesterol-lowering, radioprotective and wound-healing (Kogan, 2000; Stone & Clarke, 1992). The mechanism of the effect of 1,3-β-D-glucans is not yet fully understood and probably depends on the specific molecular structure, which is influenced by the molecular weight, branching the presence of charged residues and conformational features like the formation of helices. Different opinions concerning the structural requirements for a physiological effect have been discussed (Bohn & BeMiller, 1995; Kulicke, Lettau, & Thielking, 1997). Nevertheless, the 1,3-β-D-glucans lentinan (Maeda & Chihara, 1999; Wasser & Weis, 1999) and schizophyllan (Rau, 2002) are used clinically as adjuvants in cancer therapy, and the bacteria derived curdlan has found application as a thickener in the food industry (Lee, 2002). Other 1,3-β-D-glucans like scleroglucan (Giavasis, Harvey, & McNeil, 2002), pleuran, laminaran, pachyman or grifolan have a more or less great potential for a variety of applications (Jong, 2002).

One important source of 1,3-β-D-glucan is the cell wall of yeasts, particularly of the baker's and brewer's yeast *Saccharomyces cerevisiae* (Dijkgraaf, Li, & Bussey, 2002). Yeasts are unicellular fungi and are used for baking and ethanol production for thousands of years. Nowadays, the worldwide production exceeds 2.5 millions tons. Some yeast factories grow not only yeast for bakeries, but

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also produce so-called yeast extracts, which are obtained after mechanically or enzymatically supported autolysis. The inner parts of the cells are isolated and subsequently used as food supplements and flavour enhancers due to their high amounts of proteins and nucleotides. The outer parts of the yeast cells, the cell walls, remain as waste for which so far no commercial use has been established except as a supplement for animal feed. The cell walls are therefore an ideal raw material for the manufacture of 1,3-β-D-glucan. They are cheap and show a sufficiently high content of glucan, which accounts for 30–60% of the dry weight (Duffus, Levi, & Manners, 1982; Lipke & Ovalle, 1998; Magnelli, Cipollo, & Abeijon, 2002; Nguyen, Fleet, & Rogers, 1998; Northcote & Horne, 1952). First preparations from baker's yeast yielded a glucan-enriched product called zymosan (Hassid, Joslyn, & McCready, 1941; Pillemer, Blum, Lepow, Wurz, & Todd, 1956; Pillemer & Ecker, 1941), which showed health promoting properties and which was analysed to contain approximately 55% of β-D-glucan, 19% mannan, 15% protein and 7% lipid (DiCarlo & Fiore, 1958). In 1973, Manners et al. found the presence of two different glucans in the cell wall of yeast: 1,3-β-D-glucan containing 3% of β-1,6-glucosidic linkages (Fig. 1) as the major component (85%) and 1,6-β-D-glucan trivially named pustulan with β-1,3-glucosidic branches in minor amounts (15%).

Since the finding that glucan from yeast has beneficial properties for humans (Misaki & Kakuta, 1997; Wasser & Weis, 1999; Williams, Mueller, & Browder, 1996) and animals (Dalmo, 2000; Sakai, 1999; Sohn, Kim, Kim, & Han, 2000), many processes for the isolation and purification of the polysaccharide have been developed. Most of them use hot alkali, acids or a combination of both, which solubilize proteins and other polysaccharides. The remaining insoluble residue is designated as 'yeast glucan', but from carbohydrate chemistry it is well known that acidic as well as alkaline conditions lead to a more or less strong degradation of the glucose chains, particularly if oxygen is involved (Aspinall, Krishnamurthy, Furda, & Khan, 1975; Meller, 1960; Whistler & BeMiller, 1958; Young & Liss, 1978; Young, Sarkanen, Johnson, & Allan, 1972). Thus, the yields are often low resulting in high prices, or the purity of glucan is limited. In order to keep the native structure of the glucan ensuring a minimum of chain degradation, there has been a need for a mild manufacturing process, in which drastic, glucan-destroying conditions are avoided, but which are efficient in terms of the yield of glucan and the removal of undesirable components like proteins, lipids and other polysaccharides.

We here present a new process for the isolation of 1,3-β-D-glucan from baker's yeast (Sauter et al., 2002) combining gentle extractions by hot water and organic solvents with enzymatic treatments (Fig. 2). The process is simple, fast and easily to transfer to a large scale. Its efficiency is demonstrated with two kinds of industrially provided yeast cell walls. The resulting glucan is obtained in high yields and with high purity, and mannoprotein, also a biologically active compound of great interest, is obtained as a byproduct.

2. Experimental

2.1. Materials

Yeast cell walls YCW-1 and YCW-2 were provided by yeast factories from Germany and Switzerland, respectively. Both materials were supplied as spray-dried powders. Solvents used for analytical methods were of the purest grade, those used for processing of YCW of technical grade. All reagents were purchased from Aldrich and Fluka except for curdlan and glucose (HK) assay reagent (Sigma). The protease Savinase 16.0 L type EX from Novo-Nordisk (Denmark) was used.

2.2. Analytical methods

Determination of polysaccharides. (a) Acid hydrolysis: a dried sample (40 mg) was suspended in aqueous trifluoroacetic acid (75%, 6 ml) and stirred at 90 °C for 2.5 h. The mixture was evaporated and co-evaporated twice with H_2O. The residue was treated with H_2O (5–10 ml) and centrifuged. After separation of the supernatant, the sediment was suspended in H_2O (5–10 ml) and centrifuged. The supernatants were combined and evaporated yielding a mixture of monosaccharides. For the determination of

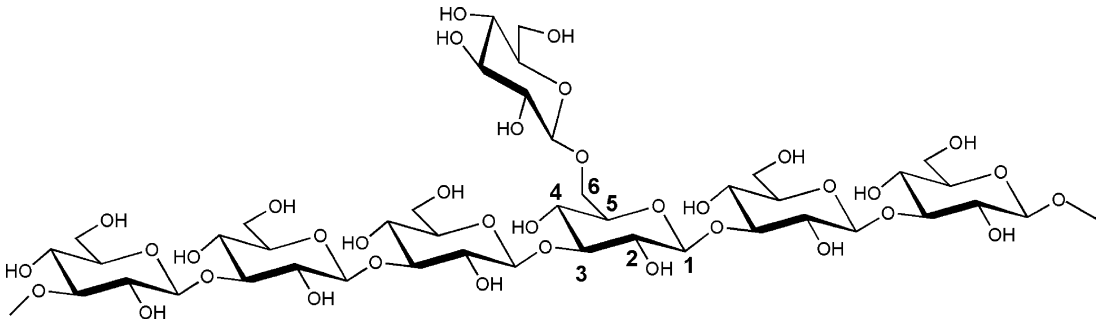

Fig. 1. Chemical structure of 1,3-β-D-glucan with 1,6-linked branches of glucopyranosyl units.

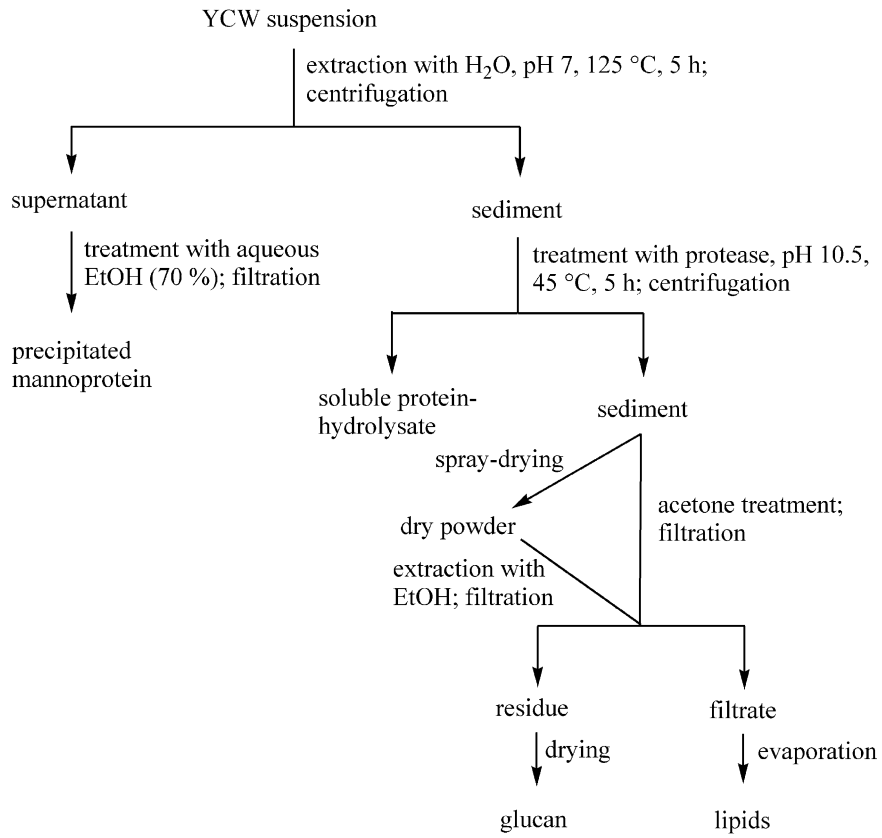

Fig. 2. Schematic process for the fractionation of yeast cell walls (YCW).

the correction factor, curdlan was used. (b) Enzymatic glucose determination: the residue was dissolved in H_2O (10 ml). An aliquot of the solution was diluted 1/40. 100 μl of this diluted solution was mixed with 1 ml of glucose (HK) assay solution. Glucose contents were determined spectrophotometrically according to the instructions of Sigma. (c) Ratios of glucose, mannose and chitin: the residue after acid hydrolysis was dissolved in D_2O, and the solution was measured by ^1H NMR. The ratios of glucose and mannose were determined by taking the sum of the integrated signals of the α- and β-anomers of both sugars. Traces of chitin were detected via the H-2 signal at 2.81 ppm. (d) Trimethylsilyl ethers: the residue after acid hydrolysis was dissolved in pyridine (20 ml). An aliquot (4 ml) was mixed with trimethylchlorosilane (600 μl) and hexamethyldisilazane (600 μl). After storing at 70 °C for 1 h, the mixture was evaporated. The residue was suspended in n-hexane and filtered over cotton wool. After evaporation of the filtrate, the residue was dissolved in 2-propanol, and a defined amount of a standard (3-nitrotoluol) was added. Gas chromatography yielded the amounts and the ratios of glucose, mannose and chitin as well resolved pairs of anomers.

Lipid content: dried material (10 g) was suspended in a mixture of n-hexane (160 ml) and MeOH (40 ml). The suspension was heated under reflux for 2 h. After cooling down to 40 °C, the mixture was filtered. The residue was washed with n-hexane, MeOH, acetone and ether. The combined filtrates were evaporated yielding a coloured oil, which was weighed.

Determination of protein content: protein contents were determined according to the procedure of Lowry as has been described by Peterson (1977).

NMR spectroscopy: NMR spectra were recorded on a Bruker AVANCE 500 spectrometer at 500.14 MHz for ^1H and 125.76 MHz for ^{13}C at 393 K for glucan and 300 K for mannoprotein. Chemical shifts δ are given in ppm and are referenced to internal acetone ($\delta = 2.030$ and 30.50 ppm) for D_2O and to the solvent peak ($\delta = 2.490$ and 39.50 ppm) for d_6-DMSO. Coupling constants J are given in Hz; 's' means singlet, 'd' doublet and 'AB' a AB-system with non-assignable coupling constants.

Fat analysis: samples were mixed with an internal standard (triundecanoin) and subsequently saponified with methanolic NaOH. After esterification in MeOH, the fatty acid methyl esters were quantitatively determined by gas chromatography using a Supelcowax-10 column and a FID.

Lipid extraction of spray-dried material derived from YCW-1: spray-dried powder (10 g) was suspended in an organic solvent or a solvent mixture (200 ml). The suspension was heated at reflux for 2 h. After cooling to 40 °C, the mixture was filtered. The residue was washed twice with the solvent (each 30 ml). The combined filtrates

were evaporated yielding a dark brownish oil, which was weighed. The results are shown in Table 2.

Electron microscopy: electron micrographs were recorded according to a procedure described by Walther and Müller (1997).

Elemental analyses were performed by the Microanalytical Laboratory of the Laboratory for Organic Chemistry (ETH Zürich).

Gas chromatography: trimethylsilylated carbohydrates were analysed on a HP 6890 GC system equipped with a HP-5 column ($30 \times 0.32 \times 0.25$ μm). A FID detector heated at 300 °C and a constant flow (1 ml/min) of helium as the carrier gas were used. After injection, the temperature started at 60 °C for 2 min and was subsequently increased to 290 °C with a rate of 20 °C/min. The retention times (min) for pentakis-*O*-trimethylsilyl-glucose were 10.85 (α) and 11.25 (β), for pentakis-*O*-trimethylsilyl-mannose 10.39 (α) and 10.89 (β), and for tetrakis-*O*-trimethylsilyl-glucosamin 10.75 (β) and 10.99 (α).

2.3. Procedures

2.3.1. Processing of YCW-1

For analytical purposes, YCW-1 was delipidized with *n*-hexane/MeOH. The evaporated extract was treated with *n*-hexane and filtered. The filtrate was evaporated and subjected to fat analysis. The results are shown in Table 3.

Hot water extraction: a suspension of YCW-1 (1.3 kg, washed free of water-soluble components) in H_2O (8.8 l) was adjusted to pH 7 with NaOH. The stirred suspension was heated to 125 °C in an appropriate vessel. After 5 h, the suspension was cooled to 45 °C and diluted with water (7.3 l). The insoluble residue was separated with a Sorvall RC 3B Plus centrifuge and washed twice with water. For analytical purposes, an aliquot of the sediment was lyophilized. Yield and composition are shown in Table 1.

Mannoprotein-1: an aliquot of the supernatant after the first centrifugation was added to EtOH under stirring until the water content reached 30%. The mixture was stored overnight at 5 °C giving to a precipitate, which was filtered, washed with EtOH/H_2O (70% w/w) and dried at 70 °C under vacuum yielding a colourless, amorphous powder. Yield and composition are shown in Table 1.

Protease treatment: the washed sediment after hot water extraction was diluted with H_2O yielding a total volume of 10 l. After heating to 45 °C and adjustment of the pH to 10.5 with NaOH, at $t = 0$, 1.5 and 3 h Savinase (each 7.5 ml) was added under stirring. After an overall duration of 5 h, the suspension was neutralised with conc. acetic acid. The insoluble residue was separated by centrifugation and washed twice with water. For analytical purposes, an aliquot of the sediment was lyophilized. Yield and composition are shown in Table 1.

Glucan-1a: the washed sediment after protease treatment was treated with an excess of acetone and filtered. The residue was washed three times with acetone and dried at 70 °C under vacuum yielding glucan-1a as a pale yellow powder. Yield and composition are shown in Table 1. $(C_6H_{10}O_5)_n$ (162.1): calcd C 44.45, H 6.22; found C 45.19, H 6.69, N 1.39.

Glucan-1b: the washed sediment after protease treatment was diluted with H_2O yielding a dry weight of 5%. The suspension was spray-dried yielding a fine powder (450 g). The powder was suspended in EtOH (4.5 l) and stirred for 2 h under reflux. After filtration, washing of the residue thrice with EtOH (each 0.7 l) and drying at 70 °C under vacuum, glucan-1b as a pale yellow powder was obtained. Yield and composition are shown in Table 1. The combined filtrates were evaporated yielding

Table 1
Yields and compositions [%] of fractions of the glucan isolation process

Fraction	Yield[a]	Glucose[b]	Mannose[b]	Lipids[c]	Proteins[d]
YCW-1		30 (100)	26 (100)	13 (100)	22 (100)
YCW-1 after hot water treatment	64	46 (98)	17 (40)	16 (74)	7 (20)
YCW-1 after hot water and protease treatment	35	64 (75)	5 (6)	28 (66)	3 (5)
Glucan-1a	26	85 (74)	6 (6)	tr	5 (6)
Glucan-1b	25	83 (69)	6 (6)	tr	5 (6)
Mannoprotein-1	14	5 (2)	78 (42)	tr	15 (9)
YCW-2		23	16	11	29
YCW-2 after hot water and protease treatment		60	<1	34	3
Glucan-2		92	1	tr	4
Mannoprotein-2		5	76	tr	14

All data represent weight ratios. Data in brackets refers to the contents [%] in the starting material (= 100%). 'tr' means traces. Chitin was detected as glucosamine only in traces.

[a] Referring to the weight of the starting material.

[b] Determination after acid hydrolysis of 1,3-, 1,6-β-D-glucan, glycogen (glucose) and mannan (mannose).

[c] Determination by extraction with *n*-hexane/MeOH.

[d] Determination according to Lowry.

a brownish oil. The oil was treated with *n*-hexane and filtered. The filtrate was evaporated and subjected to fat analysis. The results are shown in Table 3. $(C_6H_{10}O_5)_n$ (162.1): calcd C 44.45, H 6.22; found C 45.48, H 6.38, N 1.33.

2.3.2. Large scale processing of YCW-2

Hot water extraction: a suspension of YCW-2 (150 kg, washed free of water-soluble components) in tap H_2O (850 l) was adjusted to pH 7 with NaOH. The stirred suspension was heated to 125 °C in an appropriate vessel. After 5 h, the suspension was cooled to 45 °C. The insoluble residue was separated with a Westfalia SB 07 centrifuge and washed twice with water. A small amount of the sediment was taken for the recording of electron micrographs (Fig. 3).

Mannoprotein-2: an aliquot of the supernatant after the first centrifugation was added to EtOH under stirring until the water content reached 30%. The precipitate was filtered, washed with EtOH/H_2O (70% w/w) and dried at 70 °C under vacuum yielding a colourless, amorphous powder. The composition is shown in Table 1 and NMR spectra are shown in Figs. 6 and 7.

Protease treatment: the washed sediment after the hot water extraction was diluted with H_2O yielding a total volume of 470 l. After heating to 45 °C and adjusting the pH to 10.5 with NaOH, Savinase (3.5 l) containing Savinase adapted detergent solution (0.4 l) was added under stirring. After 3 h, pH had dropped to 9.5 and was readjusted to 10.5. Incubation was continued until pH remained constant (~2 h). After neutralization with conc. acetic acid, the insoluble residue was separated by centrifugation and washed twice with water. For analytical purposes, an aliquot of the sediment was lyophilized. The composition is shown in Table 1.

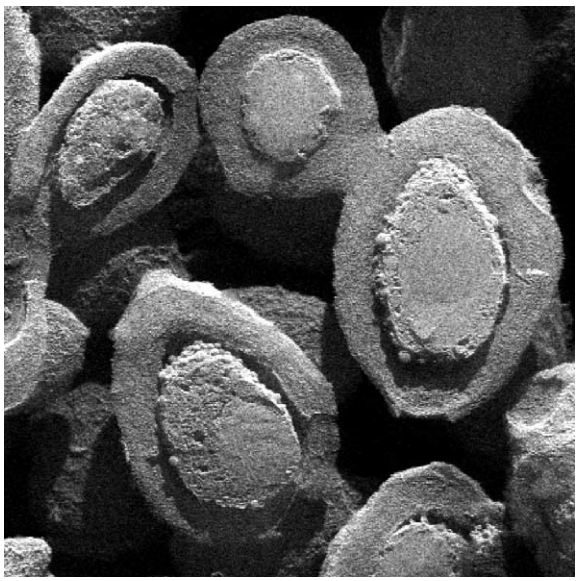

Fig. 3. Electron micrograph of YCW-2 after hot water treatment. Image size 8.1 × 8.1 Å.

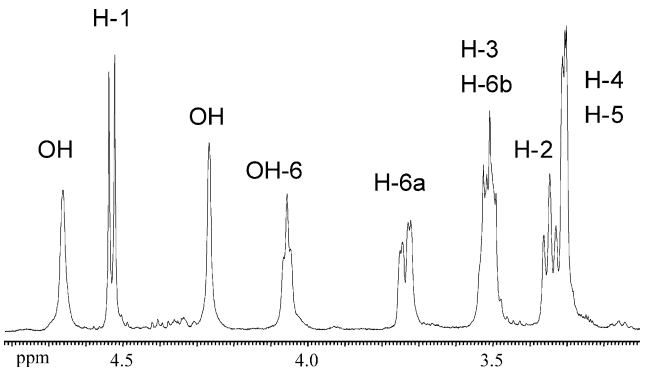

Fig. 4. ^{1}H NMR spectrum of 1,3-β-D-glucan derived from YCW-2 (500 MHz, d_6-DMSO, 393 K).

Glucan-2: an aliquot of the washed sediment after protease treatment was treated with an excess of acetone and filtered. The residue was washed three times with acetone and dried at 70 °C under vacuum yielding glucan-2 as a pale yellow powder. The composition is shown in Table 1. The combined filtrates were evaporated and subjected to fat analysis. The results are shown in Table 3.

^{1}H NMR (500 MHz, d_6-DMSO, 393 K, spectrum shown in Fig. 4): δ 4.553 (H-1, d, $J_{1,2} = 7.8$) 3.349 (H-2, dd, $J_{2,3} = 9.0$), 3.514 (H-3, dd, $J_{3,4} = 8.7$), ~3.31 (H-4, AB), ~3.31 (H-5, AB), 3.746 (H-6a, ddd, $J_{5,6a} = 1.4$, $J_{6a,6b} = 11.1$, $J_{6a,\ OH\text{-}6} = 3.8$), 3.518 (H-6b, dd, $J_{6b,OH\text{-}6} = 5.9$), 4.687, 4.285 (OH-2, s, OH-4, s), 4.071 (OH-6, dd). ^{13}C NMR (125.7 MHz, d_6-DMSO, 393 K, spectrum shown in Fig. 5): δ 102.49 (C-1), 72.46 (C-2), 85.76 (C-3), 68.18 (C-4), 76.02 (C-5), 60.67 (C-6). $(C_6H_{10}O_5)_n$ (162.1): calcd C 44.45, H 6.22; found C 45.08, H 6.25, N 1.18.

2.3.3. Spray-drying

Spray-drying of YCW-1 derived material was performed on a AT-4 Spray Drier (Bowen Engineering, USA) equipped with a CD-63 Atomizer (APV Anhydro A/S, Denmark). Aqueous suspensions (5% of dry weight) were applied with a rate of 2 l/h at an inlet temperature of 150 °C.

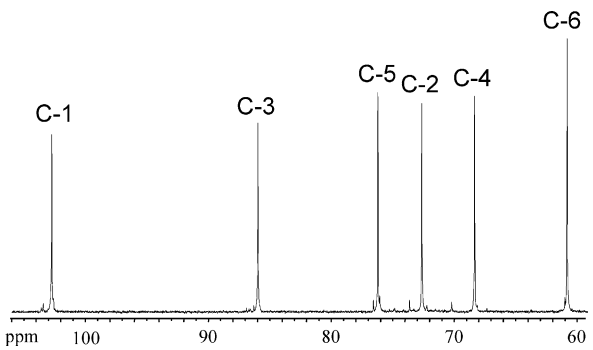

Fig. 5. ^{13}C NMR spectrum of 1,3-β-D-glucan derived from YCW-2 (125 MHz, d_6-DMSO, 393 K).

3. Results and discussion

3.1. Yeast cell walls

Although the yeast *S. cerevisiae* is an object of intensive research for many decades, only in the last years the structure of its cell wall has been elucidated in detail. Early work mainly developed methods for the quantitative determination of cell wall components and their chemical structure (Duffus et al., 1982; Northcote & Horne, 1952; Peat, Whelan, & Edwards, 1958), whereas in more recent papers, the fine structure including possible interconnections is described (Arvindekar & Patil, 2002; Kapteyn, Van Den Ende, & Klis, 1999; Kollár et al., 1997; Kollár, Petráková, Ashwell, Robbins, & Cabib, 1995; Lipke & Ovalle, 1998; Magnelli et al., 2002; Mŕsa, Ecker, Cappellaro, Teparic, & Tanner, 1999). Accordingly, 1,3- and 1,6-β-D-glucan, chitin, glycogen, lipids, mannoprotein–a protein–bound polymannose-and other proteins are the main components and are covalently linked to a great extent. The ratios of these compounds are strongly dependent on the conditions during growth and processing of the yeast. In order to obtain a pure 1,3-β-D-glucan in high yields and with high purity, an inexpensive and easily to perform removal of undesired components of the cell walls is a great challenge.

As a starting material, we used two kinds of industrially provided yeast cell walls termed YCW-1 (from Germany) and YCW-2 (from Switzerland). Although both materials are derived from baker's yeast, they show clear differences in the ratios of glucan, mannoprotein, proteins and lipids (Table 1). Whereas YCW-1 as well as YCW-2 has a little higher content of glucan than of mannan, YCW-1 consists of a significantly higher amount of these polysaccharides (56%) than YCW-2 (39%). The contents of lipids (11 and 13%) and proteins (22 and 29%) are in closer agreement. The reasons for the different compositions are most likely to be found in the growth and the conditions of autolysis of the yeasts. Our process was accomplished with YCW-1 on a laboratory scale, whereas with YCW-2, we wanted to demonstrate the feasibility on a pilot scale for the first two steps.

3.2. Glucan isolation process

Hot sodium or potassium hydroxide has been proven to be very efficient to remove proteins and alkali-soluble polysaccharides from yeast or yeast cell walls. Additionally, lipids are hydrolysed under basic conditions into glycerine and fatty acids. Thus, from the first reported glucan isolation methods (Hassid et al., 1941; Manners, Masson, & Patterson, 1973a; Misaki, Johnson, Kirkwood, Scaletti, & Smith, 1968; Northcote & Horne, 1952; Peat et al., 1958) until the recent years (Jamas, Rha, & Sinskey, 1989; Kelly, 1996; Rorstad, Robertson, & Raa, 1992; Williams et al., 1991; Zülli & Suter, 1998), numerous papers and patents are based on the treatment of yeast or yeast cell walls with hot NaOH or KOH, preferred at concentrations of 0.75–1.0 M and temperatures of 60–100 °C. The insoluble residue afterwards is usually washed with water or with acids serving for cleavage of the linkages between glucan, proteins and polysaccharides as well as for neutralization of the mixture. Subsequently, a lipid removal by the use of organic solvents is performed. Both hot alkali and acids have, however, the same effect on the glucan chains: they are degraded. As a result, the yields obtained represent a more or less distinctive loss of glucan compared to the original content in the cell walls (for example Kollár, Sturdik, & Sajbidor, 1992; Peat et al., 1958; Williams et al., 1991).

An alternative to the drastic conditions mentioned is the treatment with enzymes. Glucanases, chitinases and proteases have been used to solubilize the undesired components of yeast cell walls but predominantly for analytical and structural examinations and not for manufacture of glucan (Eddy, 1958; Fleet, 1994). Enzymatic treatments have the advantage of possibly preserving the native structure and of avoiding chain degradation, but on the other hand, they may not act quantitatively (Kath & Kulicke, 1999a; Wheatcroft, Langeris, Kulandai, Gilbert, Sime, & Smith, 1997).

Our process is characterized by steps comprising relatively mild conditions. Fig. 2 shows a summary of the whole procedure, which is described in detail in the following sections. The process starts with a hot water extraction in order to remove mannoprotein, pustulan and a part of the water-soluble proteins. After centrifugation, mannoprotein is obtained from the separated supernatant by precipitation in aqueous ethanol. The insoluble residue of the centrifugation is treated with Savinase, an enzyme used for the removal of proteins. Subsequently, after centrifugation and sufficient washing of the insoluble residue, there are two possibilities for removal of the lipids and obtaining the desired glucan. On a laboratory scale, the sediment can be washed with an excess of acetone, filtered and dried. For a pilot or production plant, spray-drying of the sediment and subsequent extraction with ethanol is more advantageous due to a much lower consumption of solvent.

3.3. Analytical methods

In order to determine the content of polysaccharides, i.e. particularly to clarify the important question of the glucan purity, our analyses started with an acid hydrolysis. Hot hydrochloric, sulfuric and trifluoroacetic acid are the most used reagents for the cleavage of polysaccharides leading to the monosaccharides, which build up the polymer chains and which are actually determined analytically. In each case, the chosen conditions are a compromise between the complete hydrolysis of the glycan chains and a preferably small rate of decomposition of the monosaccharides due to the influence of the acid. After finding the optimum

conditions for the polysaccharide hydrolysis, special correction factors are therefore usually necessary for each sugar depending on the particular stability under acidic conditions.

Dallies, Francois, and Paquet (1998) have recently reported a detailed study on the influence of different acids on the efficiency of hydrolysing yeast cell walls and the recovery of the monosaccharides released. Based on these results, we decided to use trifluoroacetic acid (TFA) for the determination of carbohydrates because of the simple work-up after hydrolysis. In contrast to Dallies et al., we had to use a much higher concentration of TFA, since from the starting yeast cell walls to the glucans obtained at the end, all samples were not hydrolysed completely with 2 N TFA but with 9 N TFA. Consequently, the loss of monosaccharides (~20% for glucose, ~50% for mannose) was significantly higher than reported by Dallies et al. taking 2 N TFA, although our conditions (90 °C, 2.5 h) are much less drastic.

The determination of glucose from glucans, mannose from mannoprotein and glucosamin from chitin was performed by three methods. Glucosamin was monitored in the NMR spectra of the hydrolysates, but was present in all samples examined only in traces. On the other hand, the hydrolyses in TFA yielded a small amount of a water-insoluble residue accounting for up to 3% of sample weight, which probably consisted of condensation products of carbohydrates and proteins, but which may also contain non-hydrolysed chitin. ^{1}H NMR spectra of the TFA hydrolysate additionally yielded the glucose/mannose ratio by means of the peak areas of the well-resolved signals of the anomeric protons. Glucose was easily quantified by the enzymatic conversion with glucose hexokinase, and the results were confirmed by gas chromatography after derivatization as trimethylsilyl ethers. The reproducibility of the three methods was quite satisfactory.

The protein content was determined according to the procedure of Lowry. This method is easily to perform and fast allowing the analysis of many samples in a short time. It is superior to a nitrogen method since a considerable part of the nitrogen does not originate from proteins but rather from chitin, nucleotidic residues and ammonium salts.

Content of lipids have been determined by an extraction with a hot mixture of *n*-hexane and methanol (4:1 v/v). Taking these two solvents of strongly different polarity allows the extraction of non-polar parts as well as more polar components of the lipids.

3.4. Hot water treatment

The extraction of water-soluble compounds from biological materials with hot water is an approved procedure for the isolation of valuable natural products. We used this step to remove water-soluble components of the yeast cell walls, i.e. particularly mannoprotein, other proteins and pustulan. We found a temperature of 125 °C and a stirring time of five hours to be the optimum conditions with respect to the separation of these compounds.

As shown in Table 1, the hot water extraction of YCW-1 resulted in a loss of weight of about one third which can be attributed mainly to mannoprotein and other proteins proven by the lowered protein and mannose content afterwards. About 80% of total proteins and 60% of mannoprotein have been removed from the starting material, whereas nearly all glucan (98%) was still present. Surprisingly, even a considerable part (26%) of the lipids have been depleted, what is probably due to water-soluble components in the lipid fraction like phospholipids or to hydrolysed glycerides. YCW-2 was hot water treated in a 1000 l scale causing certain differences to the small scale concerning stirring, duration of heating and cooling down and centrifugation. The hot water treatment seemed to work, however, with a comparable efficiency.

The chosen conditions of the hot water treatment lead to a significant swelling of the cell wall demonstrated by electron micrographs of the resulting washed and lyophilised material (Fig. 3). Whereas yeast cell walls show a thickness of 0.1–0.4 μm (Charpentier, Nguyen Van Long, Bonaly, & Feuillat, 1986; Northcote, 1963), we measured the average thickness of hot water treated YCW-2 by means of electron micrographs to be 0.5–0.7 μm.

3.5. Mannoprotein precipitation

The supernatant obtained by centrifugation of the hot water treated sample was supposed to consist predominantly of mannoprotein. Similar to 1,3-β-D-glucan, this compound has also been described as health-promoting, immune-stimulating and antitumoral (Matsumoto, Takanohashi, Okubo, Suzuki, & Suzuki, 1980; Mikami et al., 1982). Additionally, mannoprotein from yeast was reported to be an effective bio-emulsifier (Cameron, Cooper, & Neufeld, 1988; Kunst, van Schie, Schmedding, & Veenema, 1997). Therefore, an isolation step for a purified mannoprotein was of great interest for us. Precipitation in aqueous ethanol (Northcote & Horne, 1952; Peat, Whelan, & Edwards, 1961) was successful and gave colourless and amorphous products (mannoprotein-1 and mannoprotein-2, Table 1). Referring to the dry weight of YCW-1, mannoprotein-1 was obtained in a yield of 14%. Thus, 70% of the mannoprotein in the supernatant has been precipitated. This amount can probably be increased by fitting the conditions for the precipitation. For the characterization of mannoprotein-1 see Section 3.8.2.

3.6. Protease treatment

In order to remove the remaining proteins, which have not been extracted by hot water, we used an enzymatic treatment of the sediment obtained after centrifugation. Thus, the chain degradation of β-glucan due to the alkali-treatment obtained in most of the conventional procedures

can probably be avoided. We have studied several commercial proteases and found Savinase to be the most efficient. This enzyme has it highest activity at a pH of about 10 and a temperature around 50 °C. We treated the insoluble residues from the hot water extractions of YCW-1 and YCW-2 with Savinase at 45 °C at pH 10.5 for 5 h and subsequently centrifuged the mixture. Analysis of the sediment derived from YCW-1 (Table 1) revealed a loss of 25% of glucan that is predominantly due to a release of pustulan. Additionally, 34% of the original mannan has been transferred in the liquid phase and could possibly be obtained by another precipitation. Considering the proteins, the effect of the protease becomes clear: only 5% of the original protein content of YCW-1 is now left which means that Savinase has hydrolysed approx. 75% of the residual proteins after the hot water extraction. It is reasonable to assume that the enzyme has a hampered access to the remaining proteins, which may be shielded by glucan or the lipids. Again, also a part of the lipids (8%) was removed during the protease treatment probably due to saponification under the slightly alkaline conditions. Despite this partial depletion of lipids, their content in the insoluble residue has increased compared to the initial yeast cell walls: to 28% for the YCW-1 derived material and to 34% for the residue derived from YCW-2.

3.7. Removal of lipids and drying

The insoluble residue after hot water extraction and protease treatment contained in principle only glucan and lipids. Removal of the lipids can be performed in two ways. For small amounts on a laboratory scale, stirring the residue with a large excess of acetone and subsequent filtration transfers all lipidic compounds into the filtrate indicated by a dark-yellow to brown colour. An excess of acetone is necessary for two reasons: first for dissolving completely the lipids and secondly for displacing the water contained in the glucan particles and being responsible for the swelling. After exchange of the water by acetone, the residue after filtration can easily be dried even at higher temperatures yielding a fine homogenous powder. Agglutination, which usually happens when water-swollen glucan is dried at higher temperatures is thus prevented. After acetone treatment, we obtained the desired products glucan-1a and glucan-2, the former one in a yield of 26% referred to the starting material YCW-1.

On an industrial scale, the use of acetone in such amounts is not feasible, therefore, spray-drying prior to a treatment with an organic solvent is the method of choice. YCW-1 derived material was spray dried at an inlet temperature of 150 °C. Structural changes possibly shown by a brownish colour have not been observed under these conditions.

Due to the fine consistency of the obtained spray-dried powder, organic solvents have ready access to the lipidic components resulting in a reasonable amount required for the extraction and the washing. In order to keep the costs low, we examined some common cheap solvents. As shown in Table 2, methanol and ethanol of technical grade are well suited for the extraction of the lipids, which is in agreement with the early works for the delipidization of zymosan and glucan (Hassid et al., 1941; Manners, Masson, & Patterson, 1973b; Misaki et al., 1968; Northcote & Horne, 1952; Peat et al., 1958). Surprisingly, hexane, acetone and isopropanol, which are known as efficient lipid-dissolving solvents can only partly remove the lipids. This might be the reason why Jamas, Easson and Ostroff (1996) need six treatments with acetone and isopropanol and Zülli and Suter (1998) four treatments with isopropanol for complete delipidization.

Table 2
Extraction efficiency of various organic solvents and solvent mixtures for spray-dried material derived from YCW-1. The absolute lipid content was 28%

Solvent	Extracted lipids (%)
n-Hexane/methanol 4:1 (v/v)	100
Methanol (abs.)	100
Ethanol (tech.)	98
Ethanol (abs.)	96
Acetone/H_2O 4:1 (v/v)	96
2-Propanol	43
Acetone (tech.)	37
n-Hexane/2-propanol 4:1 (v/v)	17
n-Hexane	7

We accomplished the extraction of the spray-dried powder derived from YCW-1 containing 28% of lipids with ethanol under reflux. After this treatment, we filtered the suspension and washed the residue with further ethanol. It became obvious that hot solvents are necessary, since after cooling down, waxy-like precipitates appeared in the filtrate. The filtration residue was dried in an oven under vacuum yielding glucan-1b as a fine and nearly colourless powder in an over-all yield of 25%.

Recently, the advantages of spray-drying of glucan with respect to its immunomodulatory properties have been described (Hromádková, Ebringerová, SasinKová, Šandulá, Hříbaloví, & Omelkoví, 2003). Accordingly, spray-dried glucan showed a twofold higher immune stimulating activity compared to lyophilization or drying after solvent exchange. Besides the advantage of saving organic solvents, this is another reason to use spray-drying rather than acetone treatment for an industrial production of yeast glucan.

3.8. Characterization of products

3.8.1. 1,3-β-D-Glucan

Several features, which characterize glucan obtained by our process are important in order to evaluate and compare its quality. Both sources YCW-1 and YCW-2 yielded glucans with a similar appearance, i.e. fine powders with a pale yellow colour showing a tendency to form dust independent whether acetone or spray-drying/ethanol have been used. Suspensions in water have a pH of 7–8 but

exhibit a significant difference when heated to more than 80 °C. Whereas glucan-2 forms an stable gel, an usual behaviour for yeast glucans (Kogan, Alföldi, & Master, 1988), glucan-1a and glucan-1b swell markedly but do not gelatinise. A reason for the lack of gel formation may be found in the few remaining interconnections between glucan, mannan and proteins, which are not broken during the process and which are dependent on the specific yeast cell walls. Glucan derived from yeast has been described to be soluble solely in dimethyl sulfoxide (DMSO), but not in water or other organic solvents (Kogan et al., 1988). In fact, our glucans show a limited solubility in DMSO. With the aid of heating and vigorously stirring, up to 5 g/l can be dissolved. DMSO is an common solvent for NMR spectroscopy, structural examinations in solution are therefore possible.

The NMR spectra of our glucans have the highest resolution not at room temperature but at 120 °C. The spectra of glucan-2 (Figs. 4 and 5) prove the structure and relative purity indicated by distinctive and well resolved signals for the protons and carbon atoms of the glucose unit of the main chain. The assignment of all signals is in accordance with published spectra (Ensley et al., 1994; Kim et al., 2000). Additionally, signals with small intensities are visible which might partly be due to slight impurities, but most probably originate from the glucopyranosyl moiety linked in position 6 (Fig. 1). The NMR spectra of glucan-1a and glucan-1b are quite similar to those of glucan-2 (data not shown), although some slight differences occur for example in the resolution of the signals and the chemical shifts of the hydroxyl protons.

The elemental analyses of glucan-1a, glucan-1b and glucan-2 show a satisfactory agreement with the calculated values for carbon and hydrogen, but additionally have nitrogen contents of 1–2%. Determination of protein contents resulted in 4–5%, which is less than calculated, if all nitrogen came from the proteins. Possibly, inorganic salts like ammonium or residual traces of nucleotides are responsible for the nitrogen not originating from proteins.

After acid hydrolysis, the amount of released glucose represents the purity of glucan. As shown in Table 1, glucan-1a and glucan-1b yielded glucan contents of 85 and 83% and a mannan content of 6% meaning that 94% of the original mannan has been removed. The NMR spectra do not show any signals for the 1,6-linked pustulan; thus, from YCW-1, 1,3-β-D-glucan with a purity of up to 85% was obtained in a yield of 26% of dry weight. If the total glucan content of the yeast cell walls is assumed to be composed of 85% 1,3- and 15% of 1,6-linked β-glucan (Manners et al., 1973a), we thus have isolated 87% of the whole 1,3-β-D-glucan of YCW-1.

Compared to glucan-1a and glucan-1b, glucan-2 shows a significantly higher purity with a content of 92% of glucan and 1% of mannan. Obviously, our process worked with a better efficiency for YCW-2 than for YCW-1, what might probably be due to the different growth and work-up of the specific yeasts. The lack of gelatinisation and the lower resolution of the NMR spectra confirm the result that YCW-1 is a less appropriate source for the manufacturing of 1,3-β-D-glucan of high purity than YCW-2, although the quality of glucan-1a and glucan-1b are satisfactorily high for most applications.

3.8.2. Mannoprotein

Mannoprotein from yeast is a proteoglycan and accordingly composed of a minor part of proteins, which is covalently linked to the major part of a mannan polymer. The mannan part consists of 1,2-, 1,3-, and 1,6-linked α-D-mannopyranosyl units bearing additionally some phosphate groups. The whole structure has recently been elucidated (Vinogradov, Petersen, & Bock, 1998).

Table 1 shows the composition of both mannoproteins derived from YCW-1 and YCW-2. Thus, the precipitated compounds consist of about 15 and 14% of protein, respectively, and 83 and 81% of carbohydrates, respectively, mainly composed of mannose (94%) besides a small amount of glucose (6%). The ^{1}H and ^{13}C NMR spectra of mannoprotein-2 show signals originating from the protein part and typical sugar signals (Figs. 6 and 7) which have a great similarity to recently published NMR spectra (Gonçalves, Heyraud, De Pinho, & Rinaudo, 2002; Kath & Kulicke, 1999b; Vinogradov et al., 1998). One conspicuous signal with a coupling constant of 7.9 Hz is present at $\delta = 4.36$ ppm. By comparison with published data (Kawagishi et al., 1989; Monteiro et al., 2000; Santos, Marquina, Barroso, & Peinado, 2002), the doublet can be assigned to the H-1 signal of pustulan. Obviously, a small amount of this 1,6-linked β-glucan has also been precipitated and is responsible for the glucose content of 5% noted in Table 1. The NMR spectra of mannoprotein-1 are quite similar to those of mannoprotein-2, therefore they are not shown here.

3.8.3. Lipids

After solvent extraction of hot water and protease treated yeast cell walls, we evaporated the filtrates and obtained orange to brown oily residues with a more or less distinctive odour of yeast. The composition of the lipids including possible changes during our process were of interest for us. Therefore, we carried out a fat analysis of a solvent extract of YCW-1 and the extracts at the end of the process obtained by an treatment of the spray-dried powder with *n*-hexane/methanol as well as treatment of the moist YCW-2 derived sediment with acetone. Table 3 summarizes the results which are obtained by a usual method of fat analysis comprising the cleavage of all fatty acid esters with alkaline methanol followed by gas chromatographic determination of fatty acids as methyl esters.

One surprising fact is the relatively high content of non-cleaved compounds. Already the extract of the starting material YCW-1 contained after saponification only 78% of fatty acids. The extracts obtained at the end

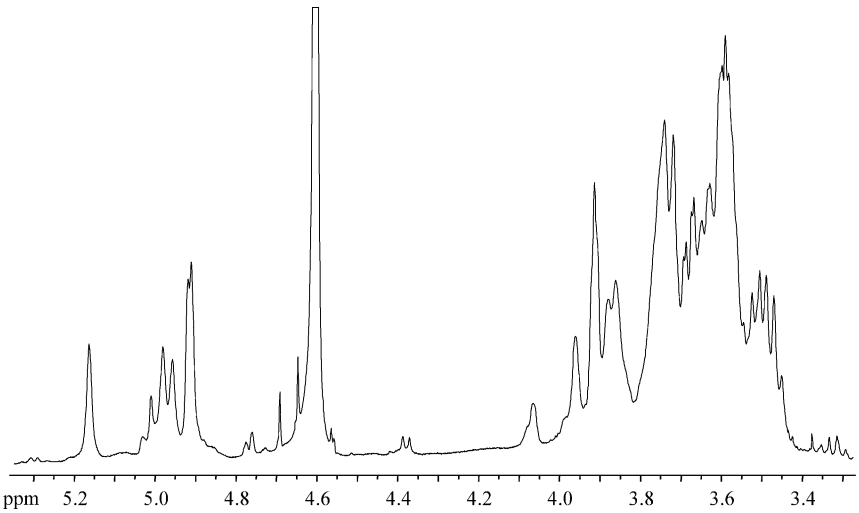

Fig. 6. ^{1}H NMR spectrum of mannoprotein derived from YCW-2 (500 MHz, D_2O, 300 K). The carbohydrate part is shown.

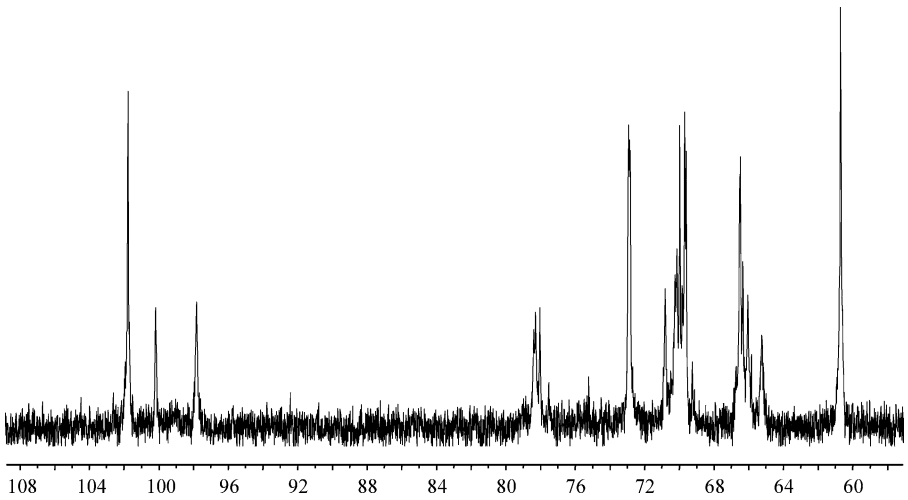

Fig. 7. ^{13}C NMR spectrum of mannoprotein derived from YCW-2 (125 MHz, D_2O, 300 K). The carbohydrate part is shown.

of the process consisted of less than 70% of glycerides. This means that considerable parts of the lipids were not typical fats. In fact, it is known that besides glycerides, several sterols and squalene are minor components of the lipid fraction in yeasts (Peña & Sandra, 1995). This might explain the portion of non-saponifiable compounds in our extracts, although the amounts would be unexpectedly high.

Only a few papers deal with the composition of lipids of yeast cell walls (Müller et al., 1994; Peña & Sandra, 1995; Šajbidor, Kopecká, Šandula, & Kačuráková, 1991). They are, however, consistent with our data shown in Table 3 in that only four fatty acids account for more than 90% of all fatty acids detected. Of these, palmitoleic (C16-1) and oleic acid (C18-1) are prevailing besides smaller amounts of palmitic (C16-0) and stearic acid (C18-0). Other fatty acids are present only in traces. Differences exist in the ratios of these four fatty acids reported in the cited papers as well as in our analyses

Table 3
Fatty acid content and composition (%) of solvent extracts after saponification and determination as methyl esters

	YCW-1	YCW-1 after hot water and protease treatment and spray-drying	YCW-2 after hot water, protease and acetone treatment
Fatty acids[a]	78.3	69.5	66.5
Saturated	19.5	19.1	13.8
Monounsaturated	79.7	79.9	84.5
Polyunsaturated	0.8	1.0	1.7
C16-0	12.1	11.9	7.1
C16-1	46.0	43.1	33.2
C18-0	6.1	5.4	6.2
C18-1	32.7	35.4	49.5

All data represent weight ratios. Other fatty acids found (C10-0-C24-0, C14-1-C22-1) account for $<0.6\%$ each.

[a] Referring to total sample weight.

depending on the starting material and the specific conditions of the respective glucan isolation process. Not only growth and processing of yeast and yeast cell walls but moreover pH, time, temperature and reagents influence to a great extent the composition of the fat and thus the ratios of the fatty acids.

4. Conclusions

In this paper, we have described a new process for the isolation of valuable 1,3-β-D-glucan starting from industrially manufactured cell walls of *S. cerevisiae*. In contrast to previous published or patented processes, which predominantly use drastic conditions for the removal of the undesired contaminants, our process steps comprise harmless treatments with water, enzymes and organic solvents avoiding chain degradation and resulting in remaining of the native glucan structure. Furthermore, the whole process seems to be industrially accomplishable with relatively low costs allowing possibly a decrease of the current high prices for yeast glucan of high purity.

We carried out the process on a kilogram scale and examined in detail with respect to yields and compositions the proceeding of the glucan purification by profound analytical methods. Based on these results, we accomplished the first two process steps starting from 150 kg of yeast cell walls thus demonstrating the feasibility on a large scale. 1,3-β-D-Glucan was obtained in a yield of 87% of the original ratio in the yeast cell walls and a purity of up to 92% besides small amounts of mannan and proteins. The lipids have been removed completely and the proteins and mannan to an extent of 94% referred to the starting yeast cell walls. As a second product, mannoprotein with a mannose content close to 80% was obtained.

We are now in the process of proving that glucan isolated by our process is in fact non-degraded. Particularly the determination of molecular weight in comparison to glucans being manufactured by conventional processes should substantiate the effectiveness of our treatments with respect to preserving the original glucan structure of the cell wall. The insolubility in nearly all common solvents, however, make the application of usual methods for the determination of molecular weight difficult. Solublization by non-degradative chemical derivatization is a possible way and is currently examined.

Further work will concern the physiological effects of glucan obtained by our process in comparison to the more or less degraded glucans derived from alkali and acid treatments. The structural requirements for example for an immunostimulation in humans or animals are still under discussion, but we think that a non-degraded, preferably native yeast glucan is very likely to be the most effective. Animal trials and studies with humans are necessary to clarify this important question.

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