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Optimized methodology for extraction of (1 → 3)(1 → 6)-β-D-glucan from *Saccharomyces cerevisiae* and *in vitro* evaluation of the cytotoxicity and genotoxicity of the corresponding carboxymethyl derivative

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ABSTRACT

The cell wall of *Saccharomyces cerevisiae* is an important source of β-D-glucan, a glucose homopolymer with immunostimulant properties. The standard methodologies described for its extraction involve acid and alkaline washings, which degrade part of its glucose chains and reduce the final yield. In the present study, an optimized methodology for extraction of β-D-glucan from *S. cerevisiae* cells, involving sonication and enzyme treatment, with a yield of 11.08 ± 0.19%, was developed. The high-purity (1 → 3)(1 → 6)-β-D-glucan was derivatized to carboxymethyl-glucan (CM-G). *In vitro* tests with CM-G in Chinese hamster epithelial cells (CHO-k1) did not reveal any cytotoxic or genotoxic effects or influences of this molecule on cell viability. The method described here is a convenient alternative for the extraction of (1 → 3)(1 → 6)-β-D-glucan under mild conditions without the generation of wastes that could be potentially harmful to the environment.

1. Introduction

β-D-Glucans are glucose homopolymer constituents of the cell wall of cereals, fungi and yeasts, which differ by the type of glycosidic linkage between glycopyranose residues. An important source of this polysaccharide is the cell wall of *Saccharomyces cerevisiae*, which represents up to 20% of the cell dry weight. Around 60% of this total corresponds to the β-glucans, which occur as long chains with about 1500 residues of β(1 → 3) glucose (Klis, Mol, Hellingwerf, & Brul, 2002) and short chains with approximately 190 β(1 → 6) glycosidic units (Aimanianda et al., 2009). β-D-Glucan from *S. cerevisiae* has been studied extensively, especially for its immunostimulatory potential. *In vitro* and *in vivo* studies have revealed that the immunomodulatory properties of β-D-glucan are related to the structure of the polymer, its molecular weight and side chains (Bohn & BeMiller, 1985). The methods usually employed to extract β-D-glucans from the cell wall of *S. cerevisiae* involve acid and alkaline washings, which lead to degradation of glucose chains (Liu, Wang, Cui, & Liu, 2008). Its exposure to these conditions, even for short periods of time, results in unsatisfactory yields and limited purity, and generates waste potentially harmful to the environment (Freimund, Sauter, Kappeli, & Dutler, 2003). Thus, less aggressive methods have been described in an attempt to replace the harsh conditions of alkaline extraction.

Freimund et al. (2003) reported extraction with hot water and enzyme treatment as an alternative method for obtaining β-D-glucan from *S. cerevisiae*. Later, Liu et al. (2008) proposed an additional high-pressure homogenization step to aid in disrupting the yeast cell wall, and observed not only satisfactory yields, but also preservation of the glucose chains of the polymer. A limiting and possibly the most important factor in the use of β-D-glucan extracted from *S. cerevisiae* as an immunostimulating agent is its insolubility in water. According to Mantovani et al. (2008), this characteristic limits β-glucan application and the extrapolation of experimental *in vitro* data. A technique frequently used to increase its solubility is the derivatization of the glucan molecule (Slamenová et al., 2003) to carboxymethyl-glucan (CM-G), one of its most studied derivatives. The aim of this study was to develop a method to extract (1 → 3)(1 → 6)-β-D-glucan from the cell wall of *S. cerevisiae* via optimized steps involving sonication, removal of lipids and enzyme treatment. To assess the potential for the use of the immunostimulatory potential of β-D-glucan, CM-G was obtained and evaluated *in vitro* for its cytotoxicity, genotoxicity and cell viability effects in Chinese hamster ovary epithelial cells (CHO-k1).

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2. Experimental

2.1. Materials

Saccharomyces cerevisiae cells were obtained from pressed mass of yeast, 28% on dry basis (ITAIQUARA®). The protamex enzyme was provided by Novozymes® Latin America Ltda. All reagents used were of analytical grade.

2.2. Analytical methods

2.2.1. Carbohydrate determination

Total carbohydrate content was determined via the phenol-sulfuric method according to Dubois, Gilles, Hamilton, Rebers, and Smith (1956), using glucose for the standard curve. The qualitative analysis of carbohydrates in the proteolysis optimization was achieved by thin layer chromatography (TLC), according to the methods of Moreira, Souza, and Vendruscolo (1998), in reference to glucose and mannose standards from Sigma®.

2.2.2. Protein determination

Total soluble protein content was determined via the method described by Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin (Sigma®) for the standard curve. Total nitrogen (N) was measured via the micro-Kjeldahl method (Tecnal® (TE-036/1)), using 6.25 as conversion factor.

2.2.3. Fat determination

Total fat content was determined according to the AOAC method (1990) (Soxlet Tecnal® (TE-188)), with petroleum ether as the organic solvent.

2.2.4. Analysis of macro- and micro-elements

Analyses were conducted by plasma atomic emission spectrometry (ICP-ICAP 61E, Thermo JARREL Ash Corporation), or with a Flame Photometer (Micronal®).

2.2.5. Infrared spectroscopy

The infrared spectra of β-D-glucan and CM-G were obtained in an FT-IR spectrophotometer Shimadzu model 3300. KBr pellets were used for the preparation of samples, and the readings were considered to have a deviation of ±2 cm^{-1}.

2.2.6. Nuclear magnetic resonance spectroscopy (NMR)

The ^{13}C and ^{1}H NMR spectra were obtained from 50 mg of (1 → 3)(1 → 6)-β-D-glucan dissolved in d_6-DMSO or 50 mg of CM-G in D_2O by a Gemini–Varian Spectrometer equipped with a NMR300-OXFRORD magnet, operating at 75.449 MHz for ^{13}C and 300.059 MHz for ^{1}H. The chemical shifts were expressed in ppm in reference to the peaks of the solvents (δ_H 2:49 and δ_C 39.50 ppm for d_6-DMSO and δ_H 4.78 for D_2O).

2.2.7. Statistical analysis

The results were analyzed with Statistica 7.0, with $p \leqslant .05$ representing statistically significant data, and experimental validations performed in triplicate.

2.3. Procedures

2.3.1. Extraction of (1 → 3)(1 → 6)-β-D-glucan

2.3.1.1. Autolysis and hot water treatment. Autolysis and hot water treatment were performed according to the methods of Liu et al. (2008) with modifications. A 30% (w/w) cell suspension, pH 5.0, was added to 3% NaCl (w/v) and incubated for 24 h at 55 °C in a water bath with mild agitation. Then, the material was heated to 85 °C, maintained at that temperature for 15 min, and cooled down to 25 °C; the mixture was then centrifuged at 4500*g* for 10 min. The autolysis rate was defined as the total loss of biomass during the process.

The material resulting from the autolysis was adjusted to a 30% dilution in sodium phosphate buffer, 0.02 M, pH 7.5, to which glass spheres (0.4 mm) were then added. The suspension was heated to 121 °C in an autoclave and maintained at that temperature for 4 h. The insoluble residue was separated and washed three times, with centrifugation at 4500*g* for 7 min at room temperature after each wash, and was subsequently maintained at 4 °C.

2.3.1.2. Sonication. To optimize the sonication time and concentration of the cell suspension, tests were conducted at 20 kHz and 150 W in an ice bath via a 3^2 full factorial design, with three replicates of the central point. The times studied, in minutes, were 2, 4 and 6 for cell dilutions of 10%, 15% and 20% in distilled water, respectively. After centrifugation at 4500*g* for 15 min at 10 °C, the slides were prepared and submitted to Gram-staining to differentiate intact cells (violet) from those with ruptured walls (pink) (Liu et al., 2008). The material was observed under 100× magnification using a Photonic microscope coupled to a video camera and displayed using the Motic Images Plus 2.0 program. The centesimal count of treatments was performed in three fields in triplicate to obtain the mean. Control counts were performed before sonication at each cell concentration tested. The results were submitted to ANOVA and Tukey tests, and the best conditions were experimentally validated.

2.3.1.3. Lipid extraction. After sonication, the lipids were extracted with isopropanol and petroleum ether. For extraction with isopropanol, the procedure was conducted according to methods of Liu et al. (2008). For extraction with petroleum ether, the sample was wrapped with filter paper and positioned so that the petroleum ether passed throughout the sample for 2 h under reflux. The recovered residue was washed two times with acetone 1:1 (w/v), centrifuged at 4500*g* for 5 min, and then stored at 4 °C. The tests were performed in triplicate and were analyzed using the ANOVA and Tukey tests.

2.3.1.4. Proteolysis of the cell wall. The protamex enzyme has been reported as effective for the removal of remaining proteins from the hot water extraction (Liu et al., 2008), with optimum conditions of 5 h at 55 °C and pH 7.5. However, the amount of protease that allows for the total removal of proteins during extraction of β-D-glucan was not reported. Moreover, considering the protein nature of protamex, the necessary number of washes to ensure its total removal after hydrolysis was determined.

To determine the optimum amount of enzyme necessary, duplicate tests with 0.05 U, 0.1 U, 0.2 U, 0.3 U, 0.4 U and 0.5 U of enzyme per gram of cell wall in a 20% aqueous suspension were randomly conducted. At the end of the reaction time, the enzyme was inactivated at 85 °C for 20 min. The material was cooled to 25 °C and washed with centrifugation at 4500*g* for 5 min. The supernatants were collected for detection of soluble proteins, and the precipitate of each treatment was used for total nitrogen determination. The results were analyzed via ANOVA and Tukey tests.

2.3.2. Dialysis and lyophilization

Dialysis was performed for 48 h against distilled water, under mild agitation, with frequent water replacements. After dialysis, the material was frozen at −20 °C and dried under vacuum at −80 °C. The residual moisture after lyophilization was determined by drying at 102 °C until a constant weight was achieved.

2.3.3. Carboxymethylation of β-D-glucan

The derivatization of non-dialyzed water-insoluble (1 → 3)(1 → 6)-β-D-glucan was performed as described by Sandula, Kogan, Kacuraková, and Machová (1999), using monochloro acetic acid. The substitution degree (DS) of the carboxymethylated product was determined by potentiometric titration with potassium hydroxide solution, according to the methods described by Rinaudo and Hudry-Clergeon (1967).

2.3.4. In vitro tests with (1 → 3)(1 → 6)-β-D-glucan and CM-G

The *in vitro* assays to evaluate the cytotoxicity, genotoxicity and cell viability modulation of (1 → 3)(1 → 6)-β-D-glucan and CM-G were performed with CHO-k1 cells cultivated at 37 °C in a BOD incubator in HAM-F10 and Dulbecco modified Eagle (1:1) culture media (Gibco) supplemented with 15% fetal bovine serum and antibiotics (0.01 mg/ml streptomycin and 0.005 mg/ml penicillin) (Gibco). As agent for inducing damage to DNA and negative controls, doxorubicin (DXR – 1 μg/ml) and culture medium (10 μl/ml) were used, respectively. For each test, three independent experiments were performed.

2.3.4.1. Cytotoxicity. The cytotoxicity of water-insoluble (1 → 3)(1 → 6)-β-D-glucan and CM-G was evaluated using the MTT test (Thiazolyl Blue Tetrazolium Bromide), conducted according to methods described by Mosmann (1983). CHO-k1 cells were treated with (1 → 3)(1 → 6)-β-D-glucan or CM-G at the following concentrations (in μg/ml): 6.25, 12.5, 25, 50, 75, 100, 125, 150, 175 and 200, with eight replicates per treatment. Exposure times of 3 h and 24 h were studied. The results of treatment were analyzed by ANOVA and compared to the control with the Dunnet test. Based on the results, the CM-G concentrations were defined for the genotoxicity test.

2.3.4.2. Genotoxicity. The genotoxic effects of CM-G were evaluated with the comet assay (Electrophoresis test in single-cell gel), according to the methods of Speit and Hartmann (1999) after treatment with 12.5 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml and 200 μg/ml CM-G for 24 h in medium free of fetal bovine serum. Slides of each replicate were coded, and 100 cells from each treatment were visually analyzed (Kobayashi, Sugiyama, Morikawa, Hayashi, & Sofundi, 1995) and classified according the following criteria: Class 0: no tail; Class 1: tail up to the diameter of the comet head, Class 2: intermediate-sized tail, with two times the diameter of the comet head; Class 3: long tail, longer than two times the diameter of the comet head. Cells with fully fragmented nuclei were not accounted. The scores were obtained according to Manoharan and Banerjee (1985) with modifications, as shown by Eq. (1). The results were analyzed via the ANOVA.

$$\text{Score} = (1 \times n_1) + (2 \times n_2) + (3 \times n_3) \quad (1)$$

where n = number of cells tested in each class.

2.3.4.3. Cell viability tests. The cell viability, apoptosis and necrosis indexes, after treatment with CM-G concentrations of the comet assay, were determined by differential staining with acridine orange (AO) and ethidium bromide, as described by McGahon et al. (1995). A total of 200 cells were visually examined and classified as: (I) live, with a functional membrane and uniform green color in the nucleus; (II) in an early apoptosis stage with a functional membrane, but fragmented DNA, showing a green color in the nucleus and cytoplasm with an apparent marginalization of its nuclear content; (III) in the final apoptotic stage, showing orange-stained areas in the cytoplasm and in regions where the chromatin was condensed in the nucleus, but distinct from necrotic cells; (IV) necrotic and showing a uniform orange color in the nucleus. Each examination was performed in triplicate. The means of the indexes of each treatment were analyzed and compared to controls via ANOVA and the Dunnet test.

3. Results and discussion

3.1. Extraction of water-insoluble (1 → 3)(1 → 6)-β-D-glucan

3.1.1. Autolysis and hot water treatment

The autolysis rate in this study was 53 ± 3%, higher than that reported by Liu et al. (2008) (48%), under the same conditions. However, those researchers worked with a brewer's *S. cerevisiae* slurry, which may explain this difference, since the yeast cell changes with the environment in which it is found (Kapteyn, Van Oen Enoe, & Klis, 1999).

Generally, obtaining *S. cerevisiae* β-D-glucan includes alkaline treatments for the removal of proteins that are covalently linked to mannans in the cell wall. Although alkali treatment efficiently removes the protein, it also results in loss of carbohydrates (Thanardkit, Khunrae, Suphantharika, & Verduyn, 2002). In this study, the proteins, especially the mannoproteins, were removed in the extraction with hot water, and the glass spheres helped to rupture the cells during the high-pressure agitation.

The total crude protein determined before treatment with hot water was 18.02 ± 1.28%, and after treatment, the percentage decreased to 3.25 ± 0.96%, with approximately 82% of the total protein being extracted, similar to that reported by Freimund et al. (2003).

3.1.2. Breaking of the cell wall through sonication after treatment with hot water

Saccharomyces cerevisiae cells are very resistant, and due to the thickness and rigidity of the cell wall, sonication is not enough to rupture them (Mendes-Costa & Moraes, 1999). However, the structure of the cell wall changes after the removal of mannoproteins, which contribute to its mechanical strength and confer resistance to external pressure. Additionally, after the removal of mannoproteins, the β-D-glucan swells, altering its compactness, which also leads to a decrease in the mechanical strength of the cell wall. Thus, at the end of the treatment with hot water, the cell wall is more susceptible to breakage (Liu et al., 2008). In addition to being fast and inexpensive, sonication also removes the need for chemicals potentially harmful to the environment. According to Sandula et al. (1999), sonication helps in the removal of impurities, such as the amorphous portions of (1 → 6)-β-D-glucan, which are trapped between the fibrils of the glucan in the cell wall. Analyses of the effects of cell concentration and sonication time show that both affect the disruption of the cell wall. The adjusted determination coefficient (R^2) of the model was 0.90, and the lack of fit was not significant (0.19). The coefficients derived for the cell wall breaking rate enabled the design of Eq. (2), which is adjusted for the experimental data. The best results were observed after 6 min at 10% and 15% (Table 1).

$$y = 86.57 - 4.10x_1 - 1.36x_1^2 + 5.05x_2 + 2.37x_2^2 - 1.86x_1 \cdot x_2 \quad (2)$$

The analysis of the surface response curves revealed that the increase in cellular concentration negatively affected the breakage of the cell wall through sonication of *S. cerevisiae* cells. The results suggest an increase in the rupture of the cell wall after over 6 min of sonication at a 10% concentration. However, this fact should be carefully examined, since longer sonication times have been used in the depolymerization of glucan and its derivatives (Chorvatovicová, Machová, & Sandula, 1996). In the present study, sonication is proposed to rupture the cell wall of intact cells remaining in the extraction with hot water. In the experimental validation of the 10% and 6 min conditions, values of 97 ± 0.18%

Table 1
Full factorial design (3^2) with absolute and codified values of sonication time and cell concentration, and results predicted and observed for the breaking rate of the *S. cerevisiae* cell wall.

Test	Concentration (%)	x_1	Time (min)	x_2	% Breaking rate	
					Observed[a]	Predict[b]
1	10	−1	2	−1	81	83.08
2	10	−1	4	0	90	87.97
3	10	−1	6	+1	97	96.96
4	15	0	2	−1	81	80.31
5	15	0	4	0	85	85.20
6	15	0	6	+1	94	94.08
7	20	+1	2	−1	80	78.59
8	20	+1	4	0	82	83.41
9	20	+1	6	+1	85	84.98
10	15	0	4	0	86	85.20
11	15	0	4	0	84	85.20

[a] Results observed for the experimental data.
[b] Results predicted by the model based on the equation $y = 86.57 - 4.10x_1 - 1.36x_1^2 + 5.05x_2 + 2.37x_2^2 - 1.86x_1 \cdot x_2$.

Table 2
Results observed in the cell wall proteolysis step for each test with the protamex enzyme.

Test	Enzyme units[a] (U)	x_1	% Total N[b]	Proteins[c] (μg)
1	0.05	A	4.61	318.18
2	0.05	A	4.59	316.12
3	0.1	B	4.50	361.87
4	0.1	B	4.48	364.50
5	0.2	C	4.43	380.06
6	0.2	C	4.44	375.56
7	0.3	D	3.91	511.87
8	0.3	D	4.43	506.43
9	0.4	E	0.02	536.87
10	0.4	E	0.02	542.62
11	0.5	F	0.01	542.37
12	0.5	F	0.02	537.06

[a] Units of protamex, Novozymes®.
[b] Determined by the micro-Kjeldahl method using a 6.25 conversion factor.
[c] Detected by the method of Lowry in the supernatant of the first wash after proteolysis.

were observed. However, the small difference between the 10% and 15% breaking rates suggests that higher concentrations may be used to speed up the process.

3.1.3. Extraction of lipids from the cell wall after sonication

When alkaline treatment is used, lipids are hydrolyzed into glycerol and fatty acids (Freimund et al., 2003). According to Jamas, Easson, and Ostroff (1997), polar lipids are solubilized and discarded in the supernatant after treatment with alkali; therefore, most of the β-D-glucan extraction processes do not include lipid extraction. In this study, after sonication, the precipitate contained 1.93 ± 0.61% lipids. Before the enzyme treatment, lipids should be extracted because they may interfere in the protease action (Liu et al., 2008). Several organic solvents have been proposed for extraction of lipids during the removal of the β-D-glucan from *S. cerevisiae*. Liu et al. (2008) reported extraction under reflux with isopropanol; however, Freimund et al. (2003) extracted only part of the lipids with the same solvent. Previous studies have reported that many treatments with isopropanol were required for complete delipidization (Jamas, Easson, & Ostroff, 1996). In this study, the percentage of lipids after extraction with isopropanol and petroleum ether was 0.1 ± 0.01% and 0.09 ± 0.01%, respectively, with $p \geqslant .05$, as determined by the Tukey test. Having the option to use either solvent is very interesting because it allows for the selection of the most appropriate solvent for the process flow diagram.

3.1.4. Proteolysis of the cell wall

The protamex enzyme has been widely used in the food industry and has proven to be safe (Shen, Wang, Wang, Wu, & Chen, 2008). In addition, it is inexpensive, easy to handle and easy to store, and does not invalidate the large-scale β-D-glucan extraction process.

After five washings, it was not possible to detect proteins in the supernatant of the treatments. Table 2 describes the total soluble proteins present in the supernatant of the first washing of each treatment. As expected, the largest quantities of soluble proteins were detected in the supernatant of treatments with 0.4 U and 0.5 U. The total N mean of treatments revealed differences by the Tukey test between 0.4 U of enzyme and treatments with lower amounts of the protease; however, no difference was observed between 0.4 U and 0.5 U. Such enzymatic treatments have been used in the extraction of β-D-glucan (Freimund et al., 2003; Kath & Kulicke, 1999; Liu et al., 2008), but there are no previous studies focusing on optimizing the amount of enzyme used and the washings required for its total removal.

The TLC analysis of carbohydrates from the insoluble portion of each treatment revealed a retention factor (Rf) of 0.43, corresponding to glucose, and an absence of an Rf value corresponding to mannose (Moreira et al., 1998). In the experimental validation of proteolysis with 0.4 U of enzyme 0.02 ± 0.01% total N was observed.

3.2. Experimental validation of the process, lyophilization and residual moisture

The (1 → 3)(1 → 6)-β-D-glucan obtained in optimized conditions contained 96.82 ± 0.81% total carbohydrates, higher than the 92% observed by Xiaozhong, Jie, Baogui, and Wangxiang (2000) for β-D-glucan obtained via alkaline and acid extraction.

After lyophilization the residual moisture was 4.8 ± 0.9%, which is important for avoiding the compaction of the (1 → 3)(1 → 6)-β-D-glucan during storage. These results differ from those of Hromádková et al. (2003), who reported a moisture content of 12.7% for *S. cerevisiae* β-D-glucan that was lyophilized and extracted through alkaline treatment. Considering that the structure and organization of glucan particles, which is directly related to water holding capacity, changes with the extraction process, our results showed that the methodology proposed here allows for obtaining of (1 → 3)(1 → 6)-β-D-glucan for long-term use.

3.3. Extraction yield

The extraction yield of (1 → 3)(1 → 6)-β-D-glucan was 11.08 ± 0.19% from *S. cerevisiae* cells, similar to the 11% observed by Liu et al. (2008) with similar methods, but higher than the yield related to alkaline extractions.

Considering the yield obtained in relation to the amount of total β-glucan in *S. cerevisiae*, around 94% of β-glucan was recovered. The polymer obtained was totally insoluble in water and was an ivory color. According to Mantovani et al. (2008), the solubility of β-glucan is associated with the degree of polymerization (DP), which is higher than 100 for those completely insoluble in water.

The solubility of β-glucans also depends on the degree of branching (DB), with more branched glucan being more soluble. In their study, Kogan, Alföldi, and Masler (1988) reported low degrees of branching for water-insoluble β-D-glucan from *S. cerevisiae*, which had long side chains. According to Chorvatovicová et al. (1996), modestly branched glucan has DB < 0.25 and Chen and Seviour (2007) related that DBs up to 0.33 are optimal for biological activity. However, these data are not entirely clear because several factors are involved in the immunomodulatory activity of glucan,

and considerable variations may occur due to the side chains. Moreover, in recent study, Aimanianda et al. (2009) reported that the *S. cerevisiae* (1 → 6)-β-glucan have on average branching at every fifth residue with one or two β-(1 → 3)-linked glucose units in the side chain and this can have a great influence on recognition of β-D-glucan by specific cellular receptors.

3.4. Carboxymethylation of (1 → 3)(1 → 6)-β-D-glucan

The derivatization of water-insoluble β-D-glucan facilitates and allows its use in medicine, since adverse effects, such as formation of granuloma, hypersensitivity and pain, are associated with parental use of insoluble β-D-glucan (Maeda, Watanabe, Chihara, & Rokutanda 1988). Moreover, the derivatization may increase its biological activity, and the derivatives are more efficient than β-D-glucan itself (Chen & Seviour, 2007). CM-G is one of the most studied soluble derivatives; it is commercially available in several countries and has many proven beneficial effects (Miadoková et al., 2005).

The solubility of β-D-glucan derivatives depends on and is directly proportional to the degree of substitution (DS). Water-soluble CM-G with a DS between 0.4 and 1.15 has been reported. The CM-G obtained here had a DS of 0.8, displayed a whitish color and formed an opalescent solution at a concentration of 2 mg/ml. These same characteristics were observed by Sandula, Machová, and Hrıbalová (1995) for CM-Gs with a DS between 0.56 and 0.89, which is considered an optimum DS for CM-G. The DS reached in the derivatization of this study is identical to that obtained by Miadoková et al. (2005) for CM-G with antimutagenic, anticlastogenic and antigenotoxic effects. The derivatization yield from water-insoluble β-D-glucan was 94 ± 2%, similar to the 90–95% reported by Sandula et al. (1999).

3.5. Characterization of (1 → 3)(1 → 6)-β-D-glucan and CM-G

3.5.1. Analysis of macro- and micro-elements

The values of macro- and micro-elements detected in water-insoluble (1 → 3)(1 → 6)-β-D-glucan and CM-G before and after dialysis are shown in Table 3. The (1 → 3)(1 → 6)-β-D-glucan contained total macro- and micro-nutrient concentrations of 8.82 ± 0.2 mg/g before and 1.21 ± 0.1 mg/g after dialysis (Table 3). These values are considerably lower than the 38.70 mg/g, reported by Liu et al. (2008) for non-dialyzed β-D-glucan extracted from *S. cerevisiae* spent from a brewery. Yamada, Alvim, Santucci, and Sgarbieri (2003) described which brewery by-products are rich in macro- and micro-nutrients, so that the differences in the amount of minerals may be associated with the yeast itself. Another factor that may have influenced the results is the increase in the solubility of minerals due to the number of washings proposed here. Supposedly, the low amount of minerals even before the dialysis step suggests that for a large-scale extraction, or for any application of β-D-glucan, this step can be eliminated. However, it is an important step in studies involving the biological activity of water-insoluble β-glucan, due to the interference of salts in vital processes.

In CM-G, the amount of macro- and micro-elements detected was 9.02 ± 0.11 mg/g before and 1.20 ± 0.05 mg/g after dialysis. However, the dialysis of CM-G is necessary for the removal of residual salts from the derivatization process, which can interfere with clinical performance of CM-G. However, this is the first report of analysis of macro- and micro-elements in CM-G before and after dialysis.

3.5.2. Structural characterization

Infrared spectroscopy (IR) is often applied for the structural characterization of β-glucans, and according to Sandula et al. (1999), it can provide rapid and reliable information on the quality of glucan preparations. In this study, the spectrum obtained for (1 → 3)(1 → 6)-β-D-glucan showed typical patterns, with absorption at 891 cm^{-1}, which is characteristic of β-glycosidic linkages (Hromádková et al., 2003; Schmid et al., 2001). In contrast, the absence of absorption at 841 cm^{-1} provided evidence of the absence of α linkage in the polysaccharide.

The bands at 1040 cm^{-1} and 1080 cm^{-1}, relating to the stretching of CO and CC, are described for (1 → 3)-β-D-glucan and (1 → 6)-β-D-glucan, respectively. The intense band due to the axial deformation of OH at the region from 3100 to 3600 cm^{-1}, centered at 3415 cm^{-1}, has also been reported for β-D-glucans in addition to the vibrations of the CH linkage with absorption at the region of 2925 cm^{-1}, indicating the frequencies of the functional groups of these polysaccharides (Sandula et al., 1999). There was no formation of bands in the region from 1650 to 1550 cm^{-1}, corresponding to amide CO stretching.

In the IR spectrum of the CM-G, bands at 1595 and 1421 cm^{-1} were observed, referring to the COO– stretching due to the introduction of carboxymethyl anions in the molecule during carboxymethylation. These data are in line with those of Sandula et al. (1999), who reported vibration at the region near 1600 and 1421 cm^{-1} for the IR spectrum of the CM-G from *S. cerevisiae*. Compared to the spectrum of the water-insoluble (1 → 3)(1 → 6)-β-D-glucan, a considerable decrease of the band at the region from 3100 to 3600 cm^{-1} was observed, corresponding to OH stretching, due to its link with carboxymethyl anions, forming $–OCH_2COOH$ and $–OCH_2CH_2COOH$.

The NMR spectrum revealed no evidence of the α configuration of the anomeric carbons (δ 100.0), showing only the presence of anomeric carbons in the β configuration, with signals close to 103 ppm (Schmid et al., 2001).

^{13}C NMR spectra of (1 → 3)(1 → 6)-β-D-glucan in this study (Fig. 1) are identical to those published by Kogan et al. (1988), corresponding to a (1 → 3)(1 → 6)-β-D-glucan composed of a main chain of glycosidic units linked in β(1 → 3), with branching points at position 6 and side chains with at least four glucose units binding at β(1 → 6). The observed signals corresponding to side-chain carbons, such as δ 75.51, referring to the C5 of side glycosidic units

Table 3
Values of the macro- and micro-elements detected in the (1 → 3)(1 → 6)-β-D-glucan and carboxymethyl-glucan (CM-G) before and after dialysis, in milligrams (mg) per gram (g).

Polymer		Macro- and micro-elements (mg/g)									Total
		Ca	K	P	Mg	Cu	Zn	Fe	Al	Na	
β-Glucan	BD[a]	0.91	0.15	2.94	0.10	0.05	1.51	0.25	0.91	2.00	8.82
	AD[b]	0.14	0.05	0.43	0.02	nd[c]	0.13	0.02	0.02	0.40	1.21
CM-G*	BD	0.95	0.14	2.95	0.09	0.05	1.50	0.26	nd	3.08	9.02
	AD	0.08	0.02	0.05	0.02	0.11	0.74	0.03	nd	0.15	1.20

[a] Before dialysis.
[b] After dialysis.
[c] Non-detected.
* Obtained from (1 → 3)(1 → 6)-β-D-glucan non-dialyzed.

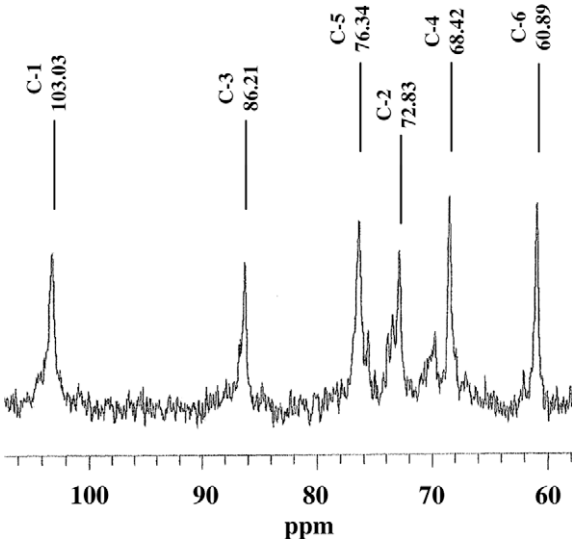

Fig. 1. ^{13}C RMN spectrum of water-insoluble (1 → 3)(1 → 6)-β-D-glucan from *S. cerevisiae* (d_6-DMSO 75.449 MHz) with indicated main chain carbons.

in a β(1 → 6) linkage, have also been previously reported (Kogan et al., 1988). Other signals assigned to the O^- substituted glucose unit that are related to the presence of side chains identified in this study are in agreement with previous reports (Barbosa, Steluti, Dekker, Cardoso, & da Silva, 2003; Schmid et al., 2001).

The assignment of signals obtained from the ^{1}H NMR spectrum agreed with the reports of Freimund et al. (2003) for β-glucan extracted from *S. cerevisiae*, under similar conditions. There was no δ_H 4:36 signal corresponding to pustulan, or any δ signal corresponding to mannoprotein carbohydrates.

Signals of the ^{13}C and ^{1}H NMR spectra of CM-G agree with those published by Machová, Kogan, Alfoldi, Soltes, and Sandula (1995), which characterized glucans carboxymethylated with different DS, even 0.8.

3.6. In vitro tests with CM-G

In vitro tests do not reflect the *in vivo* behavior, but provide valuable information on compounds with potential for use in humans (Takahashi, 2003, chap. 6). Yeast β-D-glucan is assigned as safe by the Food and Drug Administration (FDA); however, preclinical assays are important because a new methodology is proposed for its extraction here.

3.6.1. Cytotoxicity

The colorimetric MTT test is based on the reduction of yellow tetrazolium salts by mitochondrial reductases in the metabolically active cells. Intracellularly, blue crystals are formed, which are solubilized and then analyzed through UV visible spectrophotometry. Thus, the lower the MTT reduction is, the lower the spectrophotometric signal, and hence, the lower the mitochondrial activity will be (Mosmann, 1983). Fig. 2 shows the results of the MTT tests for (1 → 3)(1 → 6)-β-D-glucan and CM-G in CHO-k cells, after exposure times of 24 h.

Only 200 μg/ml (1 → 3)(1 → 6)-β-D-glucan after an exposure time of 24 h had cytotoxic effects (Fig. 2), decreasing mitochondrial activity by 36.8%. In a study with β-glucan from barley, Angeli, Ribeiro, Angeli, and Mantovani (2009) also reported cytotoxic effects for the same concentration. It is important to emphasize that after an exposure of 3 h, which is generally the time estimated in MTT assays, no cytotoxic effects were observed for all the concentrations of (1 → 3)(1 → 6)-β-D-glucan tested in this study.

In the CHO-k1 assays with CM-G, no cytotoxic effects were observed in both exposure times of 3 h and 24 h for the concentrations evaluated (Fig. 2).

3.6.2. Comet assay

The comet assay is widely used for evaluation of genotoxic effects, and its advantages include sensitivity in detecting low levels of DNA damage, collection of data at the level of individual cells, use of a small number of cells for analysis and the possibility of application in any population of isolated eukaryotic cells (Tice

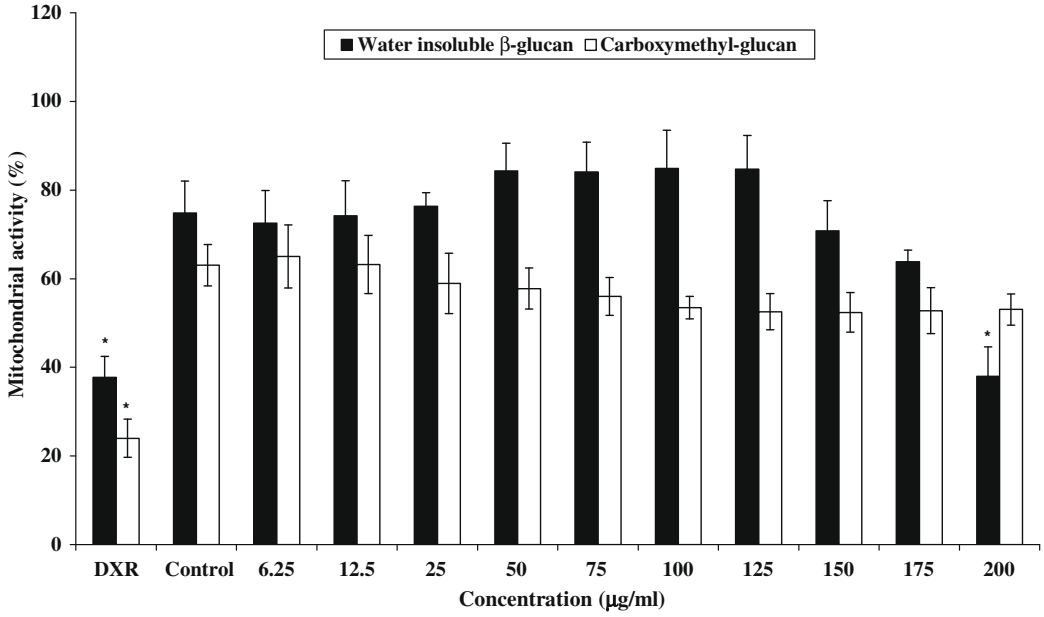

Fig. 2. MTT test in CHO-k1 cells treated with (1 → 3)(1 → 6)-β-D-glucan and CM-G for 24 h. The data are expressed as means of mitochondrial activity (%) and bars denote standard deviation corresponding to three individual experiments; DXR: doxorubicin; * denotes $p \leqslant .05$, a statistically significant difference from the control, as determined by the Dunnet test.

Table 4
Distribution of different classes of damage, scored in reference to the genotoxicity and frequency of lesioned cells observed in the comet assay, after CHO-k1 cell exposure to CM-G for 24 h.

Treatment	Class of damage ($X \pm SD$)				Score	Frequence of DC
	0	1	2	3		
Control	91.67 ± 3.21[a]	7.67 ± 2.08[a]	0.67 ± 1.15[a]	0.00[a]	9.00 ± 4.36[a]	0.08 ± 0.03[a]
DXR (1 μg/ml)	4.67 ± 2.08[b]	66.00 ± 13.89[b]	20.33 ± 10.12[b]	9.00 ± 1.73[b]	133.67 ± 11.55[b]	0.95 ± 0.02[b]
CM-G (μg/ml)						
12.5	98.33 ± 0.58[c]	1.67 ± 0.58[a]	0.00[a]	0.00[a]	1.67 ± 0.58[a]	0.02 ± 0.01[c]
25	97.67 ± 1.15[c]	2.33 ± 1.15[a]	0.00[a]	0.00[a]	2.33 ± 1.15[a]	0.02 ± 0.01[c]
50	95.67 ± 1.15[a,c]	4.33 ± 1.15[a]	0.00[a]	0.00[a]	4.33 ± 1.15[a]	0.04 ± 0.01[a,c]
100	94.67 ± 1.53[a,c]	5.33 ± 1.53[a]	0.00[a]	0.00[a]	5.33 ± 1.53[a]	0.05 ± 0.02[a,c]

DXR = doxorubicin; $X \pm SD$ = (mean ± standard deviation); DC = damage cells.
[a,b,c] Values of the same column with different cases differ statistically by Dunnet test.

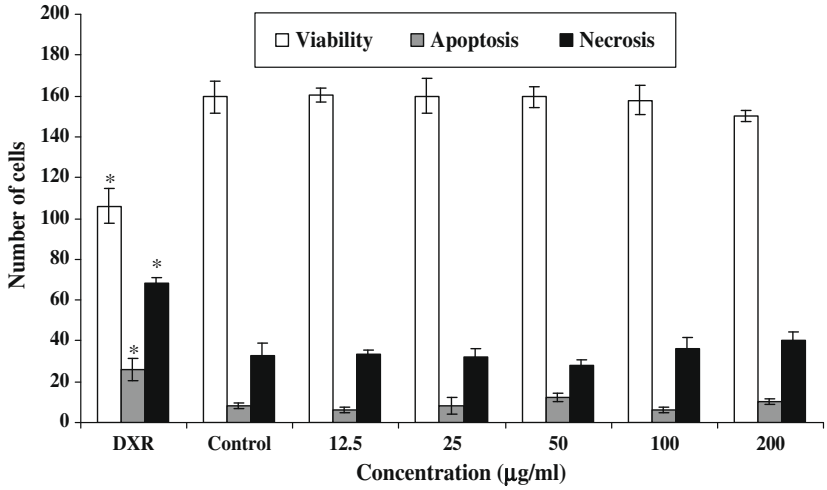

Fig. 3. Mean indexes of viability, apoptosis and necrosis of CHO-k1 cells after a 24-h exposure to CM-G; bars denote standard deviation of three individual experiments; * denotes $p \leqslant .05$, a statistically significant difference from the control, as determined by the Dunnet test.

et al., 2000). Table 4 shows the results of the comet assay for the different CM-G concentrations studied.

Statistical analyses have shown that none of the concentrations had genotoxic effects because there was no increase in the number of cells with DNA damage in relation to the control. Similar data were observed by Oliveira et al. (2007) in their genotoxicity evaluation of the *S. cerevisiae* β-D-glucan.

The values of scores and cells with damage observed after CM-G treatments were lower than those obtained in negative control groups, and denote that the administration of CM-G, a potential adjuvant in therapy of various diseases, including cancer, will not damage the DNA of patients.

3.6.3. Cell viability

Fig. 3 shows the data on cell viability, apoptosis and necrosis indexes for CHO-k1 cells treated with CM-G.

The CM-G concentrations evaluated did not interfere with the viability, apoptosis or necrosis indexes compared with the control. These results corroborate those obtained by Oliveira et al. (2007), who observed that β-D-glucan extracted from *S. cerevisiae* does not decrease cell viability or increase apoptosis or necrosis.

4. Conclusion

This study describes a high-performance optimized process that enables the extraction of 94% of β-glucans from the cell wall of *S. cerevisiae*. In several steps, it is possible to obtain the polymer with a high degree of purity without the use of drastic conditions or generation of waste potentially harmful to the environment. The carboxymethyl derivative obtained from water-insoluble (1 → 3) (1 → 6)-β-D-glucan had a DS of 0.8 and lacked any cytotoxic or genotoxic effects and did not interfere with cell viability. Due to the potential use of CM-G in therapy for various diseases, additional *in vivo* studies involving the administration of CM-G as an adjuvant in prostate cancer therapy are in progress.

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