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Thermal and surface behavior of yeast protein fractions from *Saccharomyces cerevisiae*

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ABSTRACT

An easy and inexpensive method of fractionation of a yeast homogenate was proposed and it is based on differential centrifugation steps of insoluble components and subsequent precipitations of soluble fractions. In this fractionation, the effect of addition of protease inhibitor was studied. The procedure, which was performed in mild conditions in order to minimize protein denaturation, produced four fractions that proceed from distinct parts of the yeast cell and with a different chemical composition: Fr I, Fr II, Fr III and Fr IV. Thermal and surface behavior of these samples was also analyzed. Fr I and Fr II, mainly composed by cell wall debris and membrane cell components, respectively, exhibited an adsorption rate ($\Delta\gamma/\Delta t^{1/2}$) ten-fold higher than Fr III and Fr IV, composed by nucleoproteins and cytoplasmic proteins. All fractions exhibited a unique DSC endotherm with different peak temperature (T_p) and enthalpy values (ΔH). Fr IV exhibited the highest T_p value (74 °C) and less affected by inhibitor absence. Fr I and Fr II showed the highest ΔH values (27–47 J/g protein) but they were markedly affected reducing their enthalpy values and increasing their surface properties in absence of protease inhibitor.

1. Introduction

Yeasts, specifically the *Saccharomyces* genus, have historically been recognized for their fermentative capabilities. Traditionally, these unicellular fungi have been used by the food industry principally for their production of ethanol and carbon dioxide which are important to the brewing, wine, distilling and baking industries. At present, yeasts are acquiring increasingly more attention for their other uses (Dzeziak, 1987a). Yeast biomass, as a byproduct of food industry, is a world-spread commodity and contains about half of its dry weight as proteins and other commercially important components, as polysaccharides that could be isolated for the upgrading of yeast production (Dzeziak, 1987b; Otero, Wagner, Vasallo, García, & Añón, 2000; Otero et al., 2002). The limiting factors in the utilization of yeast biomass are its high nucleic acid content, mainly ribonucleic acid (RNA) and its poor functionality (Otero et al., 2000). Therefore the isolation of yeast proteins is an attractive alternative for the utilization of yeast biomass through its use as emulsifying, gelling and foam stabilizing agents in food systems (Dzeziak, 1987b). An important number of works on yeast protein functionality (water holding capacity, viscosity, gelling and emulsifying properties) have been reported, mainly on *Saccharomyces cerevisiae* and other yeast, as *Kluyveromyces* (Barriga, Cooper, Idziak, & Cameron, 1999; Cameron, Cooper, & Nufeld, 1988; Pacheco & Sgarbieri, 1998; Torabizadeh, Shojaosadati, & Tehrani, 1996; Vasallo et al., 2006). The aim of the procedures of fractionation generally employed is the isolation of yeast protein fractions with a reduced content of nucleic acids and with a high yield and solubility. In order to achieve a high yield and facilitate the cell rupture, the homogenization processes are usually performed in alkaline medium. This treatment produces yeast samples with non-defined composition of protein species and with an advanced degree of denaturation. In addition, the most of the fractionation methods of yeast components have been accomplished, focusing on the isolation of bioactive molecules such as β-glucans and enzymes.

From a functional viewpoint, a considerable work has been carried out on yeast protein isolates and isolated cell wall proteins (Pacheco & Sgarbieri, 1998; Vasallo et al., 2006). Nevertheless, there is scarce information and more investigation is needed to assess the

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potentialities of yeast fractions as food ingredients (Otero, Vasallo, Verdecia, Fernández, & Betancourt, 1996).

The present article focuses on a new protein fractionation from baker's yeast biomass. We proposed an inexpensive method of fractionation to obtain samples containing the totality of proteins of *S. cerevisiae*. This procedure, based on differential centrifugation steps of insoluble components and subsequent precipitation of soluble fractions (in acidic medium and with acetone) was carried out in mild conditions in order to avoid protein denaturation. Moreover, the addition of a protease inhibitor before cell rupture was also evaluated. Chemical composition, thermal properties and surface behavior of obtained fractions were studied.

2. Materials and methods

2.1. Materials

Sample of *S. cerevisiae* pressed bakers' yeast (Calsa, Lanús, Argentina) was purchased in a local supermarket. Chemical reagents of analytical grade were obtained from Merck and Sigma Chemical Co (St. Louis, MO, USA).

2.2. Preparation of yeast protein samples

The schematic process for the isolation of yeast protein samples is shown in Fig. 1. Commercial baker yeast (125 g, 30 g/100 g dry matter) was washed with 375 mL of 50 mmol/L sodium phosphate buffer pH 7.5. Then, the pellet (23 g/100 g dry matter) was resuspended in 375 mL of 50 mmol/L sodium phosphate buffer pH 7.5, with and without protease inhibitor (1 mmol/L phenylmethylsulfonyl fluoride, PMSF). The cells were broken in a continuous glass bead grinder (Dyno-mill). Glass beads (diameter 0.5 mm) were previously washed with 1 mol/L hydrochloric acid and then with distilled water. Homogenization was made in the grinding chamber with 35 g of yeast suspension and 15 g of glass beads, five times for 1 min. During the process, temperature was set at values below 10 °C. The rupture of yeast cell was confirmed by optical microscopy. Glass-free yeast homogenate was centrifuged at 5000 rpm (3840*g*, Beckman Coulter Avanti J25 centrifuge, JA14 rotor, Beckman Coulter, Fullerton, USA) for 15 min and the pellet (pellet 1) was washed with distilled water and freeze-dried (Thermovac freeze-dryer, Thermovac Industries Corp., USA). This fraction was called Fraction I and it was obtained as a flake-like brownish sample (**Fr I**). The turbid supernatant 1 was further centrifuged at 12,000 rpm (22,100*g*, Beckman Coulter Avanti J25 centrifuge, JA14 rotor, Beckman Coulter, Fullerton, USA) for 20 min, yielding a yellow precipitate labelled as Fraction II (**Fr II**), which was resuspended in distilled water and lyophilized. The clean supernatant after the centrifugation (supernatant 2) was treated at 40 °C for 1 h under magnetic stirring and NaCl was added (3 g/100 mL). Then, this supernatant was adjusted to pH 4.5 with HCl and subsequently centrifuged at 22,100*g* for 20 min. The precipitate (pellet 3) was washed with water and freeze-dried (Fraction III, **Fr III**). The supernatant 3 was treated with cold acetone (−20° C) at 50 mL/100 mL. The precipitation was performed in a water-ice bath and the addition of acetone was carefully performed to avoid temperature increase. The precipitate (pellet 4) was washed and resolubilized in water, and freeze-dried. This fraction was named Fraction IV (**Fr IV**). Fr III and Fr IV were obtained as pale yellow powders.

2.3. Determination of RNA

Nucleic acid content was determined by hot perchloric acid extraction by following the experimental procedure of Rut (1973). Freeze-dried samples were treated with $HClO_4$ 0.5 mol/L for 30 min at 90 °C. Then, the samples were cooled in a water-ice bath and subsequently centrifuged at 22,100*g* (Beckman Coulter Avanti J25 centrifuge, JA14 rotor, Beckman Coulter, Fullerton, USA) for 15 min. Finally, the supernatants were adequately diluted with $HClO_4$ 0.5 mol/L and the absorbances at 270 and 290 nm were determined in a T60 UV–visible spectrophotometer (PG Instruments Limited, UK). The RNA content (g/100 g) was determined as:

$$\text{RNA}(\text{g}/100\ \text{g}) = [(A_{270} - A_{290}) \times F \times 100/(16.3 \times M)]$$

where A_{270} and A_{290} are the absorbances at 270 and 290 nm, respectively, F is the dilution factor and M is the mass of sample, expressed in mg. Assays were conducted at least in triplicate.

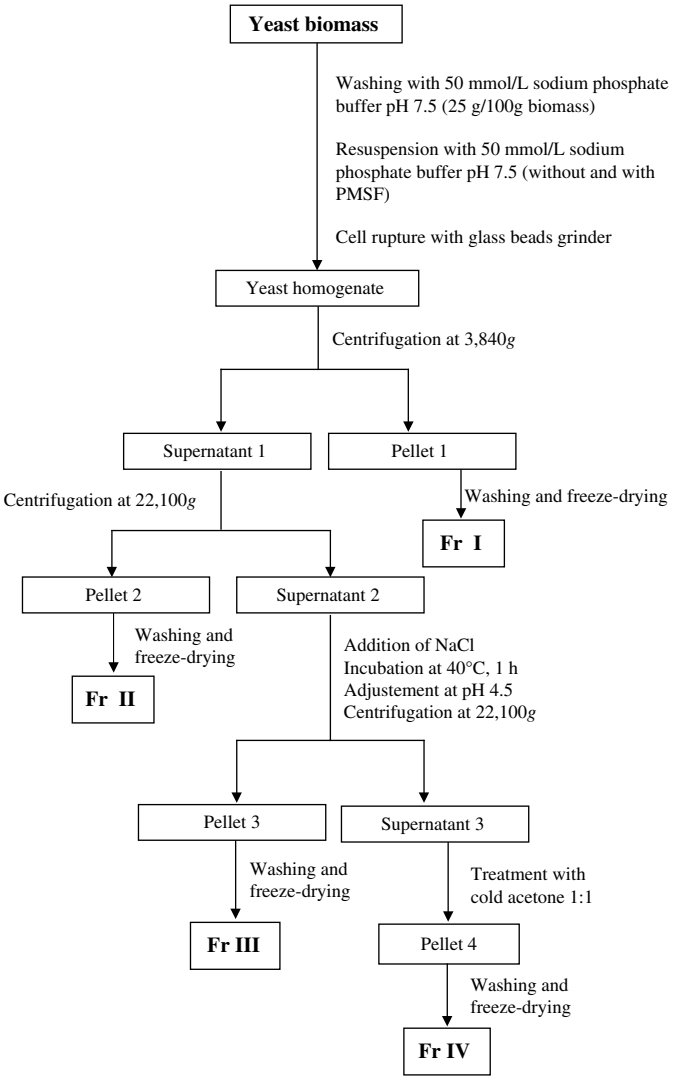

Fig. 1. Schematic process for the fractionation of yeast protein samples from *Saccharomyces cerevisiae*.

2.4. Determination of crude and corrected proteins

Protein content was determined by Kjeldahl method. Kjeldahl digestions were carried out in micro-Kjeldahl flasks with 50 mL calibration marks. Total nitrogen (TN), which includes nitrogen from protein, peptides, free amino acids and nucleic acids, was determined in the digested samples by the colorimetric method of Nkonge and Murray Ballance (1982). Standard nitrogen solutions were prepared by using oven-dried (3 h at 105 °C) ammonium

sulfate. Blank digest was prepared in an identical manner by using only catalyst and sulphuric acid. Nucleic acid nitrogen (NAN) was determined from nucleic acid content by using the factor 5.7, which takes into account the mean composition of purines and pyrimidines. Crude protein and corrected protein content values were determined as TN × 6.25 and (TN − NAN) × 6.25, respectively.

2.5. Determination of soluble protein

Aqueous dispersions were prepared by dissolving the lyophilized samples in 10 mmol/L sodium phosphate buffer, pH 7.0 (10 mg sample/mL) for at least 2 h under magnetic stirring to ensure the complete dispersion of the sample. A high speed blender (Ultraturrax T-25, SN-25-10G dispersing tool, IKA Labortechnik, GmbH & Co, Staufen, Germany) at 20,000 rpm for 30 s was further used to obtain a fine dispersion. Then, the samples were centrifuged at 10,000*g* for 15 min (IEC Centra MP4R centrifuge, International Equipment Company, USA). The protein concentration in the supernatants was determined by the Biuret method (Gornall, Bardawill, & David, 1949) using bovine serum albumin as standard protein. Solubility was expressed as grams of soluble protein/100 g dry sample.

2.6. Determination of carbohydrates

Total carbohydrates were measured by phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) with 5.0 g/100 mL phenol and assayed at 490 nm. Glucose was utilized as standard.

2.7. Determination of lipids

Total fat was determined by Soxhlet extraction using *n*-hexane and methanol as organic solvents. Taking these two solvents of strongly different polarity allows the extraction of non-polar parts as well as more polar components of the lipids. The mixture *n*-hexane–methanol assures total extraction of fat, as was reported by Freimund, Sauter, Käppeli, and Dutler (2003).

2.8. Determination of dry matter and moisture

Dry matter and moisture were determined by heating the samples for 3 h at 105 °C to constant weight.

2.9. Determination of adsorption behavior at air–water interface

To evaluate the surface properties of protein samples at the air/water interface, the supernatants obtained by centrifugation of fine aqueous dispersions were utilized. In all cases, the supernatants were diluted in order to obtain a soluble protein concentration of 0.05 g/100 mL. The surface tension (γ, in mN/m) was measured at 20 °C according to dynamic method using an automated drop volume tensiometer Lauda TVT 2 (Lauda-Königshofen, GmbH & Co., Germany). The principle of the drop volume technique is to measure the volume (or weight) of a drop detaching from a capillary with a circular cross-section. The dosing rate allows the control of the formation of drops in a drop time range of 0.3 s up to 30 min per drop. From the volume of the drop and the capillary diameter, γ can be calculated by using the Gauss–Laplace equation (Miller, Hoffmann, Hartmann, Schano, & Halbig, 1992). As a result of these experiments, γ values were recorded as a function of time square root ($t^{1/2}$, in $s^{1/2}$). The initial slope ($\Delta\gamma/\Delta t^{1/2}$) was calculated from the linear portion of the γ vs $t^{1/2}$ curve. These values are in agreement with the rate of absorption, especially in the first step of process. Assays were conducted in duplicate.

2.10. Electrophoresis

Samples were extracted during 2 h at 20 °C with a buffer containing 1.5 mol/L Trizma, 0.4 g/100 mL SDS, pH 8.0 in the presence of 5 g/100 mL of β-mercaptoethanol. Protein dispersions were diluted with an equal volume of a pH 6.8 buffer 0.125 mol/L Tris–HCl, 0.1 g/100 mL SDS, 40 mL/100 mL glycerol and 0.05 g/100 mL bromophenol blue. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) modified by Puppo, Lupano, and Añón (1995) using a continuous gel of 10 g/100 mL in polyacrylamide. A continuous dissociating buffer system was used, containing 0.375 mol/L Tris–HCl, pH 8.8, and 0.1 g/100 mL SDS for the separating gel and 0.025 mol/L Tris–HCl, 0.192 mol/L glycine, and 0.1 g/100 mL SDS, pH 8.3, for the running buffer. The gel slabs were fixed and stained overnight by addition of 0.1 g/100 mL Coomassie Blue R-250 solution (40 mL/100 mL methanol, 10 mL/100 mL acetic acid). Electrophoresis was carried out at constant voltage (200 V). Low MW markers (Pharmacia calibration kit, Pharmacia Biotech, Uppsala, Sweden) used included: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa); α-lactalbumin (14.4 kDa). Molecular weight of different polypeptides was achieved by analysis of bands using a Bio Rad Molecular Analyst Program (Bio Rad Laboratories Inc., USA).

2.11. Differential scanning calorimetry

Samples (10–15 mg) of 25 g/100 mL dispersions in water were hermetically sealed in aluminium pans and heated from 30 to 100 °C at heating rate of 10 °C/min (DSC Polymer Rheometric Scientific, Piscataway, NJ, USA). A double empty pan was used as reference. Denaturation enthalpies and peak temperatures (T_p) were drawn from the corresponding thermograms (Software Plus V5.4l, USA). Enthalpies values (ΔH) were expressed as J/g protein, taking into account the dry weight (determined by perforating the pans and heating overnight at 105 °C) (Wagner & Añón, 1990) and the protein content of sample. All assays were conducted in duplicate.

3. Results and discussion

According to procedure of yeast protein fractionation previously described in Fig. 1, four samples (Fr I, Fr II, Fr III and Fr IV) were obtained. The amount of each sample expressed as ratio (R) is shown in Table 1. Fr I was significantly more abundant than other fractions ($R = 0.4$–0.5). The comparison of R values, obtained from the assays performed without and with PMSF, showed that the

Table 1
Ratio (R) of yeast fractions obtained by cell rupture and fractionation in the absence (+) and presence (−) of protease inhibitor (phenylmethylsulfonyl fluoride, PMSF). The ratio for each fraction was determined as: R = weight of fraction (g)/weight of all fractions (g).

Sample	PMSF	R
Fr I	+	0.51 ± 0.01
Fr II	+	0.17 ± 0.01
Fr III	+	0.18 ± 0.01
Fr IV	+	0.14 ± 0.01
Fr I	−	0.43 ± 0.01
Fr II	−	0.21 ± 0.01
Fr III	−	0.19 ± 0.01
Fr IV	−	0.17 ± 0.01

All the data were expressed as the mean and S.D. of duplicate measurements.

presence of protease inhibitor increased the *R* value of Fr I, in detriment of the other fractions.

According to the fractionation procedure (Fig. 1), the sample Fr I, which is the fraction of highest density after cell rupture, is supposed to be mainly cell wall debris. On the other hand, Fr II, could correspond to insoluble cell and organelle membrane components which are responsible of turbitidy of supernatant at 3840*g* (supernatant 1) and therefore required a higher centrifugal force to sediment (22,100*g*). Samples Fr III and Fr IV, resultant from the two first step of centrifugation, are composed mainly by cytoplasm soluble proteins. When the supernatant 2 is adjusted at pH 4.5 by acid, Fr III is obtained, so it is composed by acid insoluble proteins. The proteins that remain soluble at pH 4.5, are further precipitated with acetone and constitute the fraction IV (Fr IV). To corroborate that the obtained samples are different and contain the above mentioned cell components, a composition study was carried out. Table 2 shows the content in protein, nucleic acids, carbohydrates and lipids in comparison with the whole cells. In the absence of PMSF, Fr I showed an remarkable high content of carbohydrates (>70 g/100 g), due to the presence of cell wall polysaccharides, mainly glucans and mannans (Kapteyn, Van den Ende, & Klis, 1999; Walker, 1998). The pronounced enrichment of polysaccharide content of Fr I respect to whole cells reveals that this fraction is mainly composed by cell wall debris. Freimund et al. (2003) studied the compositions of two yeast cell walls' (YCW) fractions from different yeast factories; their content of polysaccharides was very variable (39–56 g/100 g) whereas the lipid and protein contents were in close agreement (11–13 and 22–29 g/100 g, respectively). Fr I showed only a similar content of protein respect to YCW fractions, whereas the composition of carbohydrates and lipids exhibited clear differences (Table 2). The reasons for these results are likely due to growth and processing conditions. The molecular architecture of the cell wall is not constant. Cells could exhibit significant differences in cell wall composition and structure as a function of environmental conditions, such as temperature and pH. In the proposed fractionation procedure, a slightly alkaline medium (pH 7.5) and room temperature were used. Therefore, Fr I would contain the main components of the cell wall of *S. cerevisiae*: cell wall proteins (CWPs, phosphorylated mannoproteins), β-(1,6)-glucan and β-(1,3)-glucan. These components are all interconnected by covalent bonds (Kapteyn et al., 1999; Walker, 1998). However, some of these mannoproteins are solubilized during the preparation of aqueous dispersions (soluble protein content ≈ 8 g/100 g, Table 2). On the other hand, nucleic acid content of Fr I was significantly lower than the other fractions and whole yeast (2.32 g/100 g), which was within the range normally found in food products like organ meats, seafoods, lentils and beans (Pacheco & Sgarbieri, 1998). In this fraction, nucleic acids are a contamination. Fr II has similar crude and corrected protein contents than whole cells, while lipid content was the highest among all fractions. These protein would be present in both plasmatic and organelle membranes. In addition, the soluble protein content of Fr II, determined by Biuret method, was approximately four times higher than that corresponding to Fr I. The presence of lipid components, phospholipids and sterols, which are effectively extracted by a mixture of two organic solvent of different polarity (*n*-hexane–methanol) would explain the high fat content obtained in this fraction (≈30 g/100 g, Table 2). The experimental fractionation procedure (see Fig. 1) included two steps of centrifugation at different centrifugal force and hence, the separation is based on size and density. It is probable that smaller cell wall fragments are also present in Fr II. Carbohydrate and nucleic acid contents of Fr II were approximately 10 and 6 g/100 g, respectively (Table 2). Although it is possible that cell wall polysaccharides are present in this sample, the contribution of ribose residues of nucleic acid to total carbohydrate content must also be considered.

As was previously mentioned, a limiting factor of utilization of yeast biomass is its high nucleic acid content, basically ribonucleic acid. Some reagents and techniques used for the isolation of yeast protein fractions with low nucleic acid content may cause alteration in protein structure and have been scarcely used in treatment for a variety of reasons that range from the impracticability of the operation scale needed, to potential toxicity of the solvent used (Guzmán-Juárez, 1982, chap. 7; Pacheco & Sgarbieri, 1998). In the present work, the clean supernatant after the second step of centrifugation was treated in mild conditions, at 40 °C with the addition of NaCl for 1 h in order to activate the endogenous ribonuclease. Yeast RNases exhibit optimal temperatures very close to 50 °C. Otero et al. (2000) reported that in *S. cerevisiae* protein samples, thermal denaturation starts below 50 °C. Therefore, a lower incubation temperature (40 °C) was chosen to preserve native structure of proteins. Fr III, obtained by acid precipitation of this supernatant is supposed to be composed mainly by cytoplasm proteins, especially nucleoproteins, which are released to the bulk of suspension once the external structures are disrupted. According to Table 2, this fraction exhibited a relative protein enrichment respect to whole cells, similar to yeast protein concentrates. These concentrates are obtained by isoelectric precipitation of soluble fraction of yeast homogenate after a centrifugation step. Pacheco and Sgarbieri (1998) reported that these protein concentrates are composed mainly by cytoplasm enzymes and nucleoproteins and their RNA was extremely high (>15 g/100 g on dry basis). Fr III, however, exhibited nucleic acid content significantly lower (≈5 g/100 g), although it was only

Table 2
Chemical composition of whole yeast and its protein fractions. With exception of moisture, all values are expressed in dry basis.

Sample		Crude protein (g/100 g)	Corrected protein (g/100 g)	Soluble protein (g/100 g)	Nucleic acids (g/100 g)	Total carbohydrates (g/100 g)	Lipids[a] (g/100 g)	Moisture (g/100 g)
Whole yeast		48.1 ± 0.9	40.5 ± 0.9	n.d.	6.9 ± 0.1	40.2 ± 1.4	7.0 ± 1.2	69.5 ± 0.1
Fr I	With PMSF	22.6 ± 1.4	20.0 ± 1.4	7.7 ± 0.2	2.3 ± 0.1	72.2 ± 2.8	5.1 ± 0.6	9.1 ± 1.0
Fr II		48.8 ± 2.2	42.0 ± 2.1	27.4 ± 0.4	6.2 ± 0.1	9.7 ± 0.5	29.4 ± 1.7	13.1 ± 0.5
Fr III		64.3 ± 2.1	58.8 ± 2.0	14.5 ± 0.3	5.0 ± 0.1	7.5 ± 0.5	Negligible	6.5 ± 0.4
Fr IV		65.0 ± 2.2	54.1 ± 2.1	54.9 ± 0.3	10.0 ± 0.1	15.6 ± 3.0	Negligible	11.1 ± 0.1
Fr I	Without PMSF	19.7 ± 0.3	18.2 ± 0.3	10.0 ± 0.3	1.4 ± 0.0	77.4 ± 3.3	n.d.	9.2 ± 0.1
Fr II		45.1 ± 3.5	39.4 ± 3.4	37.2 ± 0.4	5.2 ± 0.1	11.8 ± 0.9	n.d.	12.2 ± 0.6
Fr III		68.3 ± 3.3	60.9 ± 3.3	14.9 ± 0.2	6.8 ± 0.1	4.4 ± 0.7	n.d.	6.7 ± 0.2
Fr IV		55.5 ± 1.1	45.8 ± 1.2	50.6 ± 0.6	8.8 ± 0.2	15.5 ± 1.8	n.d.	10.8 ± 0.9

All the data were expressed as the mean and S.D. of triplicate measurements.
[a] n.d. = Non determined.

slightly lower than RNA in intact cells. The soluble protein content of Fr III is close to 15 g/100 g (Table 2). A value slightly lower than what reported by Pacheco and Sgarbieri (1998) for yeast protein concentrates in a previous paper. Fr IV is a fraction composed by cytoplasm proteins soluble at acid pH (pH 4.5). This fraction was the most easily dispersible in the buffer during the preparation of aqueous dispersions; this fact became evident since a similar content of corrected and soluble protein was observed. In addition, this fraction exhibited the highest content of nucleic acid among all fractions. Then, it is highly probable that proteins were also bound to nucleic acid. According to previously mentioned results, a more drastic treatment to reduce nucleic acid content is required for Fr III and Fr IV. In both samples (Fr III and Fr IV) carbohydrate content was probably overestimated due to the presence of nucleic acids. Moreover, no fat was practically extracted when these samples were treated with a mixture of *n*-hexane–methanol (Table 2).

When cell rupture was performed in absence on PMSF, a slight decrease of crude and corrected proteins was observed in Fr I and Fr II, while soluble proteins, determined by Biuret method, and were higher in these fractions, mainly in Fr II. This fact could be attributed to the action of endogenous proteases. Moreover, both fractions showed a slight increase in the carbohydrate composition, whereas the nucleic acid contents exhibit a small reduction. Among the samples obtained by precipitation during the fractionation procedure, the absence of protease inhibitor affected the crude and corrected protein contents of Fr IV, showing in both cases a pronounced decrease. In contrast, the protein content of Fr III was not significantly changed with respect to the sample obtained by fractionation in the presence of PMSF. In this sample, a small increase of nucleic acid content was observed (Table 2).

SDS-PAGE patterns showed that all samples have a different polypeptide composition (Fig. 2). As was mentioned above, an exogenous serine protease inhibitor (PMSF) was added prior to cell rupture in order to study the effect of their addition on the characteristics of samples obtained. The patterns did not exhibit important differences among samples obtained in absence or presence of PMSF. It seems to be that in PMSF absence, some bands on SDS-PAGE patterns of Fr I and Fr II are weaker than those corresponding to the experiments with protease inhibitor. A wide variety of proteases is present in yeast cells. The major proteases are vacuolar proteases A, B and C, the latter is also named carboxypeptidase A (Jazwinski, 1990; Walker, 1998). Protease A is an acidic endoproteinase that is largely inactive below pH 6, and therefore it is inactivated during the acid precipitation (pH 4.5) in Fr III isolation. Although PMSF is quite effective with proteases B and C, fortunately these enzymes (and also protease A) are found as complexes with their natural inhibitors in yeast homogenates around neutral pH (Jazwinski, 1990); in this work, the cell rupture and centrifugation steps at pH 7.5 were carried out at pH 7.5. Therefore, it is highly probable that proteolysis during the fractionation procedure was not sufficiently extensive. Fr I showed a main band at 35 kDa, which is only slightly less intense for the sample obtained without PMSF (Fig. 2). The presence of high molecular species that could not penetrate the separating gel was also evident; it is possible that these proteins are linked with cell wall polysaccharides. Mrsa, Seidl, Gentzshm, and Tanner (1997) were able to identify approximately 20 CWPs. Nine of them were extractable with hot SDS-β-mercaptoethanol, where 11 of them remained as insoluble protein. Two main classes of CWPs (GPI-CWPs and Pir-CWPs) can be distinguished in detergent-soluble fraction, which are extractable with β-glucanase (GPI-CWPs) and by treatment in mild alkali condition or by β-glucanase digestion (Pir-CWPs). When isolated cell walls are digested with a crude β-1,6-glucanase and liberated proteins are separated by SDS-PAGE and analyzed by western blotting a characteristic pattern of four β-glucosylated proteins appears with relative molecular masses of approximately 60, 80, 145 and 220 kDa (Kapteyn et al., 1999). In this work, Fr I was obtained in the fist step of centrifugation after a rupture process and no enzymatic digestion was carried out. This result explains the marked differences in the electrophoretic pattern with regard to those reported by Kapteyn et al. (1999) in a previous work. Respect to Fr II sample, an important aggregation degree is observed; it is important to take into account that the membrane proteins form complexes with unsaturated polar lipids. A possible lipid oxidation during the storage of this sample could be responsible of an additional protein polymerization. This fact results evident due to the presence of a broad band in the upper part of separating gel: these species have molecular masses significantly higher than 97.4 kDa. Fr III and Fr IV are composed by proteins with a greater amount of polypeptides that penetrate to the separating gel. Fr III shows main bands at 78, 54, 48 and 37 kDa. Moreover, this sample showed an important content of small polypeptides (molecular mass < 14 kDa). On the other hand, Fr IV exhibits a main band at 50 kDa and other minor species of 78, 65, 60, 47, 41, 30, 26, 22 and 20 kDa. The presence of small polypeptides at molecular masses < 14 kDa was also evident for this sample. The effect of PMSF addition during the fractionation procedure was not clearly observed in the electrophoretic patterns of Fr III and Fr IV: no noticeable differences were observed in protein bands in absence or presence of protease inhibitor.

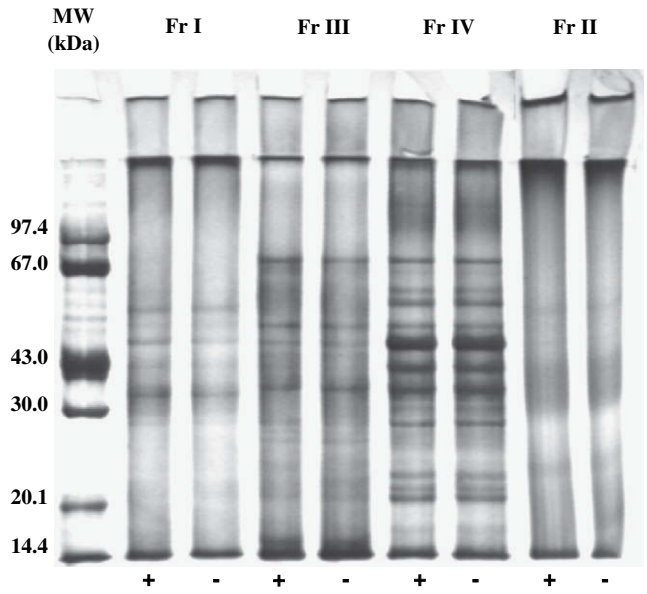

Fig. 2. SDS-PAGE profiles of yeast protein samples in reducing conditions. The symbols (+) and (−) are related with the presence and absence of protease inhibitor (phenylmethylsulfonyl fluoride, PMSF) during cell rupture. Molecular weights are expressed in kDa.

The thermal analysis of yeast and its derived protein was carried out. The corresponding thermograms are shown in Fig. 3; peak temperatures (T_p) and enthalpy values (ΔH) of endothermic peaks are shown in Table 3. Thermograms of whole cells exhibited two endothermic peaks: peak I at 66.1 and peak II at 74.4 °C (Fig. 3a); nevertheless, the process of denaturation starts to approximately 50–55 °C. Then, in a first approximation, fresh and active yeast samples are composed by two protein fractions with different thermal stability. In a previous work (Otero et al., 2000), a main peak at 66.6 °C was observed in DSC thermograms of active dry

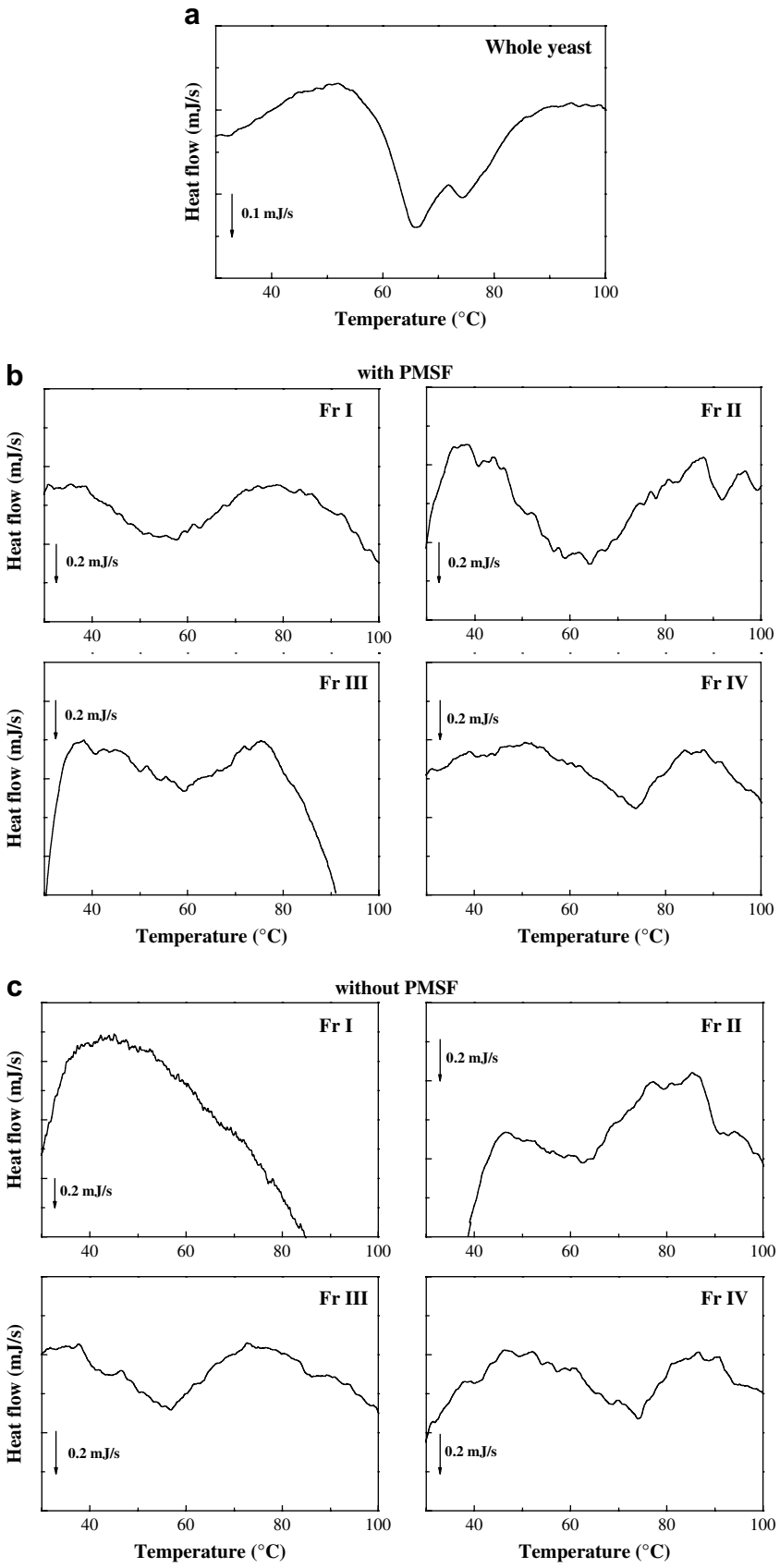

Fig. 3. Typical DSC thermograms of intact cells of *Saccharomyces cerevisiae* (baker's yeast) and protein samples obtained by fractionation of yeast homogenates. Cell rupture was performed without and with phenylmethylsulfonyl fluoride (PMSF). The experimental fractionation procedure is shown in Fig. 1.

Table 3
Peak temperatures (T_p) and denaturation enthalpies (ΔH) of whole yeast and its fractions. These samples were obtained by cell rupture and fractionation with (+) and without (−) phenylmethylsulfonyl fluoride (PMSF) (see Fig. 1).

Sample		T_p (°C)		ΔH (J/g protein)
		Peak I	Peak II	
Whole yeast		66.1 ± 0.2	74.4 ± 0.1	20.5 ± 1.6
Fraction	PMSF	Unique peak		
I	+	57.6 ± 0.4		27.1 ± 0.9
II	+	63.1 ± 0.7		46.0 ± 3.7
III	+	59.1 ± 0.2		10.7 ± 1.0
IV	+	74.1 ± 0.3		15.1 ± 0.8
I	−	No peak		0
II	−	63.4 ± 0.6		17.9 ± 0.5
III	−	57.2 ± 0.4		16.1 ± 0.2
IV	−	73.7 ± 0.1		16.1 ± 0.3

All the data were expressed as the mean and S.D. of duplicate measurements.

yeast; minor peaks at temperatures lower than 60 °C and higher than 70 °C were also observed probably due to conformational changes during the drying process. Enthalpy value of protein denaturation in fresh yeast (20.5 J/g) was higher than those obtained in dried samples (near 14–16 J/g, Otero et al., 2000, 2002), indicating a lower degree of denaturation in non-dried samples. DSC thermograms of samples Fr I–Fr IV obtained during the fractionation in presence of PMSF exhibited a unique endotherm with different T_p and ΔH values (Fig. 3b, Table 3). These results would indicate that proteins present in Fr II and Fr IV are more stable that those of Fr I and Fr III. At first, it would have a partial coincidence between fractions T_p and the T_p of peaks I and II of the fresh yeast sample. Although the native structure has not been appreciably affected, when proteins are isolated from their natural environment, a shift of denaturation peak towards smaller temperatures can occur. This result appears to be related to the protective environment of intact cells, which is lost during the rupture process (Huang & Kinsella, 1986). If we consider that protein denaturation in whole cells (peak I) starts at 50–55° C, denaturation of Fr I, Fr II and Fr III proteins would contribute with peak I, while those of Fr IV will contribute to peak II of yeast thermogram. On the other hand, the endotherms of Fr I and Fr III would correspond to the weak peak at temperatures below 60 °C in dried yeast thermogram. This fact indicates that the process of isolation and drying has increased the thermal sensitivity of proteins that constitute these fractions. According to Hromádková et al. (2003), the drying by lyophilisation affects the structure of cell wall β-glucans; thus, the same process could affect the integral structure of β-glucans and mannoproteins of Fr I.

Regarding to denaturation enthalpy, it can be seen that this value is higher in Fr I and Fr II, which are constituted by the external proteins of the cellular structure and that form complexes with polysaccharides or lipids. Fr III, composed mainly by cytoplasm proteins and nucleoproteins, exhibited T_p and ΔH values more similar to those of isolated yeast proteins informed previously (Otero et al., 2000). Nevertheless, these isolates prepared from yeast according to the described procedure (rupture and solubilization at alkaline pH followed by acid precipitation and drying) are mainly constituted by cytoplasm proteins and nucleoproteins, but contaminated with soluble proteins from cell wall and membrane. Denaturation pattern produced a unique endothermic peak with T_p below 60 °C and ΔH lower than 4 J/g (Otero et al., 2000), indicating a marked structural change by isolation process.

When fractions were obtained without the presence of protease inhibitor, Fr I did not give any DSC endotherm whereas for Fr II it is seen much reduced the denaturation enthalpy with respect to the same sample obtained with PMSF (Fig. 3c, Table 3). It suggests that, although not detectable by SDS-PAGE, some structural change in absence of PMSF must have affected the stability of cell wall proteins and, in a smaller degree the membrane proteins. The activity of enzymes non-inhibited by PMSF (β-glucanases, lipases, chitinases, etc.) could have had some relation with these changes. On the other hand, thermal stability of cytoplasm proteins and nucleoproteins exhibited no effect by the absence of protein inhibitor.

Knowing the composition and the thermal stability the obtained fractions, it was come to a study of their superficial behavior. The kinetic adsorption at the air/water interface of all fractions was followed by a dynamic method, by using a drop volume tensiometer. These assays were performed at the same soluble protein concentration (0.05 g/100 mL) and pH. In the absence of protease inhibitor, the more efficient samples for decreasing surface tension were Fr I and Fr II (Fig. 4a). These results suggest the presence of proteins with high ability to adsorb at air/water interface. For Fr I, the wall mannoproteins solubilized during the preparation of aqueous dispersions would behave as a effective tensioactive agent, due to their faster diffusion and anchorage to the interface. The high surface and emulsifying activity of mannoprotein samples, obtained in different conditions, were reported in previous works (Barriga et al., 1999; Torabizadeh et al., 1996; Vasallo et al., 2006). For Fr II, this behavior is attributed to phospholipids or protein–phospholipid complexes present in membranes. In contrast, Fr III and Fr IV, exhibited a poor and similar surface activity, as

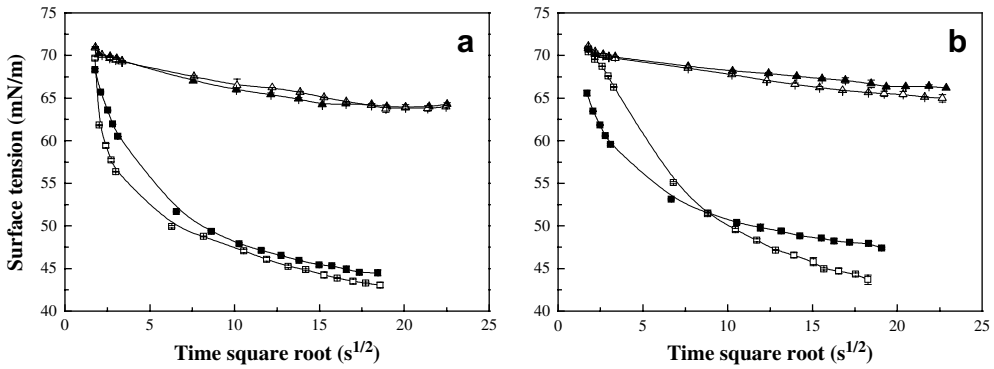

Fig. 4. Adsorption kinetics of soluble proteins of yeast protein samples at air/water interface. These samples, Fr I (■), Fr II (□), Fr III (▲) and Fr (△), were obtained by fractionation in the absence (a) and presence (b) of protease inhibitor (phenylmethylsulfonyl fluoride, PMSF). The solubilization and measurement buffer was 10 mmol/L sodium phosphate buffer (pH 7.0) and the protein concentration was 0.05 mg/mL.

a consequence of the presence of globular cytoplasm proteins and nucleoproteins that diffuse more slowly to the interface. The precipitation of these proteins in acid medium or acetone could negatively affect their surface properties. The initial slope ($\Delta\gamma/\Delta t^{1/2}$) values for Fr I, II, III and IV were 5.68, 5.71, 0.53 and 0.43 mN/m $s^{1/2}$, respectively (Fig. 5a).

Moreover, results obtained in the presence of PMSF showed that addition of protease inhibitor before cell disruption had a evident negative effect on surface activity, mainly in the first step of adsorption process (Fig. 4b). The $\Delta\gamma/\Delta t^{1/2}$ values for Fr I, Fr II, Fr III and Fr IV were 4.25, 2.67, 0.23 and 0.29 mN/m $s^{1/2}$ respectively (Fig. 5b). The presence of protease inhibitor during the fractionation avoids the generation of soluble peptides of lower molecular weight, which diffuse fastly to the air/water interface. Nevertheless, the presence of these peptides was not evident in the electrophoretic patterns (Fig. 2). The tendency observed in surface activity of samples was similar respect to those observed without PMSF. The samples obtained by differential centrifugation steps (Fr I and Fr II) showed a better surface behavior than Fr III and Fr IV.

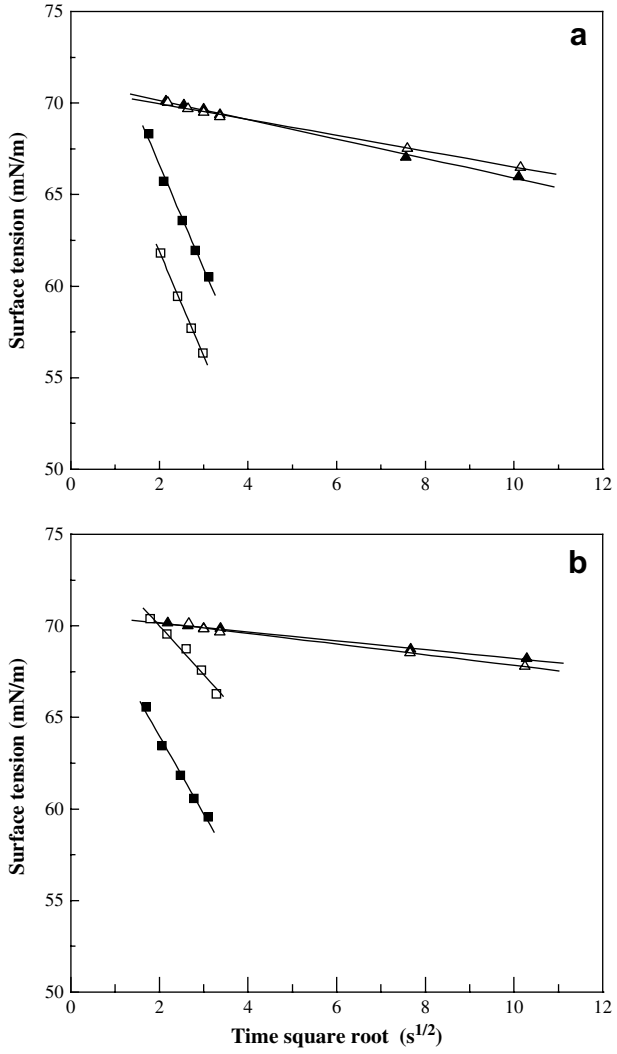

Fig. 5. Initial slope ($\Delta\gamma/\Delta t^{1/2}$) values as a function of time square root ($t^{1/2}$, in $s^{1/2}$) for yeast protein fractions obtained by cell rupture and fractionation without (a) and with (b) phenylmethylsulfonyl fluoride. Samples: Fr I (■), Fr II (□), Fr III (▲) and Fr (△). The parameter $\Delta\gamma/\Delta t^{1/2}$ is associated with the first step of adsorption process. The solubilization and measurement buffer was 10 mmol/L sodium phosphate buffer (pH 7.0) and the protein concentration was 0.05 mg/mL.

4. Conclusions

The fractionating method for yeast biomass proposed herein is easy and inexpensive and it is carried out in mild conditions. The corresponding yeast protein fractions are constituted by proteins that proceed from distinct parts of the yeast cells. Hence, they also exhibit thermal, surface properties and a chemical composition markedly different. The easiness of proposed method would make possible the scaling up in order to obtain yeast protein fractions that could have a potential application as functional ingredients.

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References

Barriga, J. A. T., Cooper, D. G., Idziak, E. S., & Cameron, D. R. (1999). Components of the bioemulsifier from *Saccharomyces Cerevisiae*. *Enzyme and Microbial Technology, 25*, 96–102.

Cameron, D. R., Cooper, D. G., & Nufeld, R. J. (1988). The mannoprotein of *Saccharomyces cerevisiae* is an effective bioemulsifier. *Applied and Environmental Microbiology, 54*, 1420–1425.

Dubois, M., Gilles, K. H., Hamilton, J., Rebers, F., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*, 349–356.

Dzeziak, J. D. (1987a). Yeast and yeast derivatives. Definitions, characteristics and processing. *Food Technology, 32*, 104–121.

Dzeziak, J. D. (1987b). Yeast and yeast derivatives. Applications. *Food Technology, 32*, 122–124.

Freimund, S., Sauter, M., Käppeli, O., & Dutler, H. (2003). A new non-degrading isolation process for 1,3-β-D-glucan of high purity from baker's yeast *Saccharomyces Cerevisiae*. *Carbohydrate Polymers, 54*, 159–171.

Gornall, A. G., Bardawill, C. S., & David, M. M. (1949). Determination of serum proteins by means of Biuret reaction. *Journal of Biological Chemistry, 177*, 751–766.

Guzmán-Juárez, M. (1982). Yeast proteins. In B. J. F. Hudson (Ed.), *Development in food proteins-2* (pp. 263–291). London: Applied Science Publishers.

Hromádková, Z., Ebringerová, A., Sasinková, V., Šandula, J., Hříbolová, V., & Omelková, J. (2003). Influence of the drying methods on the physical properties and immunomodulatory activity of the particulate (1 → 3)-β-D-glucan from *Saccharomyces cerevisiae*. *Carbohydrate Polymers, 51*, 9–15.

Huang, Y. T., & Kinsella, J. E. (1986). Functional properties of phosphorylated yeast protein: solubility, water-holding capacity and viscosity. *Journal of Agricultural and Food Chemistry, 343*, 670–674.

Jazwinski, S. M. (1990). Preparation of extracts from yeast. In D. P. Murray (Ed.), *Methods in enzymology. Guide to protein purification* (pp. 154–171). San Diego, USA: Academic Press, Inc.

Kapteyn, J. C., Van den Ende, H., & Klis, F. M. (1999). The contribution of cell wall proteins to the organization of the yeast cell wall. *Biochimica et Biophysica Acta, 1426*, 373–383.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.

Miller, R., Hoffmann, A., Hartmann, R., Schano, K. H., & Halbig, A. (1992). Measuring dynamic surface and interfacial tensions. *Advanced Materials, 4*, 370–374.

Mrsa, V., Seidl, T., Gentzshm, M., & Tanner, W. (1997). Specific labelling of cell wall proteins by biotinylation. Identification of four covalently linked O-mannosylated proteins of *Saccharomyces cerevisiae*. *Yeast, 13*, 1145–1154.

Nkonge, C., & Murray Ballance, G. (1982). A sensitive colorimetric procedure for nitrogen determination in micro Kjeldahl digest. *Journal of Agricultural and Food Chemistry, 30*, 416–420.

Otero, M. A., Vasallo, M. C., Verdecia, O., Fernández, V. M., & Betancourt, D. (1996). Whole fractionation of baker's yeast. *Journal of Chemical Technology and Biotechnology, 67*, 61–71.

Otero, M. A., Wagner, J. R., Vasallo, M. C., Añón, M. C., García, L., Jiménez, J. C., et al. (2002). Thermal denaturation kinetics of yeast proteins in whole cells of *Saccharomyces cerevisiae* and *Kluyveromyces fragilis*. *Food Science and Technology International, 8*, 163–167.

Otero, M. A., Wagner, J. R., Vasallo, M. C., García, L., & Añón, M. C. (2000). Thermal behavior and hydration properties of yeast protein from *Saccharomyces cerevisiae* and *Kluyveromyces fragilis*. *Food Chemistry, 69*, 161–165.

Pacheco, M. T. B., & Sgarbieri, V. C. (1998). Hydrophilic and rheological properties of brewer's yeast protein concentrates. *Journal of Food Science, 63*, 238–243.

Puppo, M. C., Lupano, C. E., & Añón, M. C. (1995). Gelation of soybean protein isolates in acidic conditions. Effect of pH and protein concentration. *Journal of Agricultural and Food Chemistry, 43*, 2356–2361.

Rut, M. (1973). Determination of nucleic acid on yeast and yeast-related products. *Kvasny Prumysl, 19*, 131–133.

Torabizadeh, H., Shojaosadati, S. A., & Tehrani, H. A. (1996). Preparation and characterization of bioemulsifier from *Saccharomyces cerevisiae* and its application in food products. *Lebensmittel Wissenschaft und Technologie, 29*, 734–737.

Vasallo, M. C., Puppo, M. C., Palazolo, G. G., Otero, M. A., Beress, L., & Wagner, J. R. (2006). Cell wall proteins of *Kluyveromyces fragilis*. Surface and emulsifying properties. *Lebensmittel Wissenschaft und Technologie, 39*, 729–739.

Wagner, J. R., & Añón, M. C. (1990). Influence of denaturation degree, hydrophobicity and sulfhydryl content on solubility and water absorbing capacity of soy protein isolates. *Journal of Food Science, 50*, 750–770.

Walker, G. M. (1998). *Yeast physiology and biotechnology*. Chichester: John Wiley & Sons.