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Revealing of *Saccharomyces cerevisiae* Yeast Cell Wall Proteins Capable of Binding Thioflavin T, a Fluorescent Dye Specifically Interacting with Amyloid Fibrils

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Abstract—Proteins binding thioflavin T leading to its specific fluorescence were discovered in a fraction of noncovalently bound *Saccharomyces cerevisiae* yeast cell wall mannoproteins. Thioflavin-binding proteins display high resistance to trypsin digestion in solution. These data are the first experimental evidence for the presence of proteins whose properties are characteristic of amyloids in yeast cell wall, except for data on glucanotransferase Bgl2p that has amyloid properties. Our data suggest the anchoring of these proteins in the cell wall by a trypsin-sensitive part of the protein molecule. Experiments with a mutant strain devoid of the *BGL2* gene suggest the compensation of absent amyloid-like protein Bgl2p by increase in contents of thioflavin-binding proteins in the cell wall.

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The binding of thioflavin T (ThT) dye with induction of its fluorescence at 480-490 nm (excitation wavelength 440-450 nm) is widely used as a test for the presence of amyloid fibrils — both in solutions of isolated proteins and in microscopy of entire cells [1-6]. The latter is possible due to selectivity and high specificity of the dye, which is very useful in identification of amyloids, particularly on tissue sections. Thioflavin T is distinguished from other amyloid-specific dyes, such as Congo red, by its ability to detect fibrils during their assembly from precursor protein, since it does not influence this process [7].

Amyloids are protein aggregates characterized by filamentous morphology and high contents of β-sheets with β-strands directed perpendicularly to the fibril axis (cross-β-structure) [8]. Their characteristic feature is a relatively high resistance to the action of proteinases and denaturing agents such as SDS. More than 20 human diseases are associated with amyloid accumulation in cells [9].

Recently, data has been reported in literature on the presence of amyloid proteins on the cell surface of both eu- and prokaryotic microorganisms [10-12]. The role of these proteins is possibly associated with virulence [13]. One isolated and characterized protein from the cell wall (CW) of the yeast *Saccharomyces cerevisiae*, Bgl2p, was found to have amyloid properties [1]. This protein cannot be removed from CW by consecutive treatment with trypsin and SDS. The Bgl2p protein, according to the data of analysis by an earlier described method [14], contains seven amyloidogenic determinants. The circular dichroism spectrum of Bgl2p shows high β-sheet content in its structure. It also has been shown that Bgl2p interacts with a fluorescent dye, ThT, with elevation of fluorescence at 490 nm. Protein sequence analysis suggests a high level of amyloids in the *S. cerevisiae* CW, which contains no less than 20 proteins of various glycosylation degrees [15]. However, no experimental data supporting this hypothesis are available to date.

The goal of the present work was to investigate the ability of yeast CW proteins (belonging to two groups differing in type of anchoring in CW — covalently or noncovalently attached to CW polysaccharide backbone) to

Abbreviations: CW, cell wall; DMSO, dimethyl sulfoxide; SEP, SDS/β-mercaptoethanol extractable proteins; ThT, thioflavin T; wt, wild type.

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bind ThT with induction of specific fluorescence at 490 nm.

MATERIALS AND METHODS

***Saccharomyces cerevisiae* strains and growth conditions.** The parental *S. cerevisiae* strains were MAY591 (MATα *leu2-3,112 lys2-801 ura3-52 his3-200*) [16] and DBY 746 (MATα *ura3-52 leu2-3,112 trp1-289 his3-Δ1*); they are designated below in the text as wild type (wt). The mutant strains with deletion of *BGL2* gene were obtained in our laboratory by homologous recombination (MATα *leu2-3,112 lys2-801 ura3-52 his3-Δ200 BGL2::URA3* and MATα *ura3-52 leu2-3,112 trp1-289 his3-Δ1 BGL2::URA3*, respectively); they are designated below in the text as Δ*bgl2*. Cells were grown at 30°C on YPD medium (1% yeast extract, 2% peptone, and 2% glucose). The solid medium contained 2% agar.

Biotinylation of yeast cell wall proteins. A method using sulfo-NHS-LC-biotin labels CW proteins only [17]. The yeast cells were centrifuged, washed twice with 50 mM K^+-phosphate buffer, pH 8.0, resuspended in a small volume of the same buffer in which 0.5 mg/ml of sulfo-NHS-LC-biotin (Pierce, USA) was then added, and incubated at 4°C for 1 h. Then the cells were washed thrice with the phosphate buffer followed by isolation of CW as described below.

Isolation of cell walls. Cell walls were isolated from biotinylated and non-biotinylated cells according to earlier developed method [18] with slight modification. The wt and Δ*bgl2* yeast cells were disrupted with glass beads in the presence of 0.005 M phenylmethylsulfonyl fluoride and 0.005 M EDTA and centrifuged at 1500*g*. The CW pellet was consecutively washed with 1% sucrose, 1 M NaCl, 1% NaCl, and deionized water. Following each wash the suspension was centrifuged at 1500*g* with removal of the supernatant.

Pretreatment of cell walls for experiments on induction of ThT fluorescence. Portions of CW prepared from non-biotinylated cells were subjected to extraction with the following reagents: 1% SDS for 1 h at 37°C; trypsin (0.5 mg/ml in 50 mM Tris-HCl, pH 7.5) for 1 h at 37°C; consecutively – trypsin and SDS; or consecutively – SDS and dimethyl sulfoxide (DMSO).

Extracts prepared by treatment of equal amounts (10 mg) of wt- and Δ*bgl2*-CW with trypsin (500 μl) were used in following experiments (here and below – tryptic extracts). The prepared cell walls were additionally treated for removal of extractants. For removal of SDS, CW were washed five times with 0.2 M Na^+-acetate buffer, pH 5.5, three times for 15 min with *n*-butanol–water mixture (0.7 : 1 v/v; one volume of CW per three volumes of the mixture), and with water – until the smell of butanol disappeared. For removal of trypsin, cell walls were washed four times with 1 M NaCl and two times with water, and for removal of DMSO – four times with water. Following each wash, the suspension was centrifuged at 1500*g* with removal of the supernatant. The pellet containing CW was resuspended in 2 ml of water, and sodium azide to the final concentration of 0.02% was added if extended storage was required. The amount of CW was determined by spectrophotometry (absorbance at 540 nm (A_{540}) of 1 ml of 500 μg/ml CW suspension equaled 1.0).

Extraction of proteins from biotinylated yeast CW for PAGE and Western-blotting [17]. Fractions of proteins that are not covalently bound to the yeast CW polysaccharide backbone (SEP) were prepared by heating of CW samples (25 mg) for 5 min at 100°C in 500 μl of Laemmli buffer (0.1 M Tris-HCl, pH 7.6, containing 3% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.01 M EDTA, and 0.0025% bromophenol blue). The extracted proteins were separated from the CW by centrifugation for 3-5 min at 8500*g*. Supernatants were stored at –20°C.

Fluorescence of thioflavin T. ThT was purchased from Sigma (USA). Its fluorescence in the presence of CW or CW extracts was determined according to the earlier described method [2] with slight modifications. A Varian Cary Eclipse fluorescence spectrophotometer (USA) was used for measurements. Diluted suspensions of wt- and Δ*bgl2*-CW (final absorbance A_{540} = 0.45) treated as described above were used in the experiments. Samples of soluble β-1,3-glucan (laminarin from *Laminaria digitata* (Sigma)) and high molecular weight polyphosphates with average chain length of 150 (kindly provided by Prof. Herbert Grunze, Institut fur Anorganische Chemie der Deutschen Akademie der Wissenschaften zu Berlin) similar to compounds comprising yeast CW [19] were used as controls. These compounds did not induce specific ThT fluorescence.

In experiments with tryptic extracts of wt- and Δ*bgl2*-CW, 20, 40, 60, 110, and 160 μl of the extracts were added to 3 ml of 10 μM ThT. Trypsin concentration was 500 μg/ml in initial extracts and 3.3, 6.6, 9.8, 17.7, and 25.3 μg/ml, respectively, in the final samples. The initial trypsin solution was used as a control. Spectra of all samples were recorded in the absence of ThT (used as baselines) and in its presence. The data were processed with Cary Eclipse Scan Application v.1.1 and SigmaPlot 10 software.

Electrophoresis of proteins under denaturing conditions. Proteins were analyzed by electrophoresis in 10% polyacrylamide gel under denaturing conditions [20]. Gels were stained with either silver nitrate or Coomassie G-250.

Western-blotting. Blots were stained using either antibodies or standard test with streptavidin. Following PAGE under denaturing conditions, the gel was washed with water, and then transfer was performed (if required) onto a nitrocellulose membrane with pore size of 0.45 μm (Schleicher & Schuell, USA) in 100 mM Tris-HCl buffer,

pH 8.0, containing 192 mM glycine and 20% methanol for 1 h at 5 mA/cm^2 [21]. The membrane was washed with water followed by incubation in TBS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) additionally containing 0.05% Tween 80 and 0.4% casein for 1 h at room temperature.

Immunostaining was carried out as follows. The membrane was incubated with mouse antibodies raised against Bgl2p protein (working dilution 1 : 1000) in the same buffer for 1 h, washed with TBS buffer containing Tween 80 – three times for 5 min, and incubated with rabbit anti-mouse serum (1 : 300) for 1 h with subsequent washing with TBS containing Tween 80. Then the membrane was incubated with mouse antibodies conjugated with peroxidase complex (1 : 200) for 1 h and washed with TBS containing Tween 80. The Bgl2p protein was visualized by incubation of the membrane in TBS buffer containing 0.3 mg/ml of diaminobenzidine and 0.005% hydrogen peroxide.

Membranes carrying biotinylated proteins were preincubated in TBS buffer containing 0.05% Tween 80 and 0.4% casein for 1 h at room temperature. Then the membrane was incubated with streptavidin (Sigma) (1 : 2000) for 1 h in the same buffer, washed, and visualized by treatment with 100 mM Tris-HCl buffer, pH 9.0, containing 10 mM $MgCl_2$, 0.692 mM 5-bromo-4-chloro-3-indolyl phosphate, and 0.734 mM nitroblue tetrazolium.

RESULTS

Induction of specific ThT fluorescence by cell wall proteins from wild-type *S. cerevisiae* and a strain with deletion of the *BGL2* gene. The wt and Δ*bgl2 S. cerevisiae* CW were treated with various extractants. ThT fluorescence levels (at 490 nm) in presence of intact and partially deproteinized CW were measured (Fig. 1a). The initial CW cause an increase in ThT fluorescence that is characteristic of ThT bound to amyloid fibrils (*1*). Incubation with trypsin led to significant (by 80%) decrease in CW-induced specific ThT fluorescence (*2*). The same result was achieved by consecutive treatment of CW with trypsin and SDS (*3*). Virtually complete absence of the induction (decrease in the initial ThT fluorescence by 95%) was observed when the CW specimen was taken after consecutive extraction with SDS and DMSO (*4*). Initial Δ*bgl2*-CW also elevates ThT fluorescence with maximum at 490 nm that is characteristic of ThT bound to amyloid fibrils (*5*). However, preincubation of Δ*bgl2*-CW with trypsin resulted in still more pronounced decrease (by 95%) in their ability (compared with that of wt-CW) to induce specific ThT fluorescence (*6*). Consecutive treatment of Δ*bgl2*-CW with trypsin and SDS made it unable to induce specific ThT fluorescence (*7*). Virtually complete absence of induction of ThT fluorescence (decrease by 98%) was also observed when the sample of Δ*bgl2*-CW consecutively treated with SDS and DMSO was taken in experiment (*8*). The spectra of ThT fluorescence in the presence of partially deproteinized CW are shown in Fig. 1, b-d.

Induction of specific ThT fluorescence by proteins of tryptic extract from wild-type CW. We tested extracts prepared by treatment of wt-CW with trypsin. These extracts, when interacting with ThT, induce its characteristic fluorescence (Fig. 2a). The fluorescence of ThT at 490 nm depends linearly (data not shown) on amounts of added extracts over a wide range of amounts of wt-CW extracted. Trypsin added to ThT at the same concentration that was used for the extraction did not cause increase in specific fluorescence.

Induction of specific ThT fluorescence by proteins of tryptic extract from Δ*bgl2*-CW. We tested extracts prepared by treatment with trypsin of CW from the strain devoid of glucanotransferase Bgl2p. These extracts, when interacting with ThT, induce its characteristic fluorescence (Fig. 2b). Fluorescence of ThT at 490 nm depends linearly (data not shown) on amounts of added extracts over a wide range of amounts of Δ*bgl2*-CW extracted.

Electrophoretic analysis of yeast CW proteins. Figure 3a shows data of electrophoresis of the SEP fraction isolated from wt (*1*) and Δ*bgl2* (*2*) CW. Lane *2* does not contain the band (29 kDa) corresponding to glucanotransferase Bgl2p, but exhibits two additional minor bands, W1 and W2 (wall proteins 1 and 2), as well as increased intensity of W3 (wall protein 3) band. No other difference in protein sets between wt and Δ*bgl2* SEP fractions is observed. Figure 3b shows the data of Western-blotting of SEP fraction isolated from wt (*1*) and Δ*bgl2* (*2*) CW (staining according to a standard test with streptavidin). This method revealed eight SEP proteins in wt-CW and seven in CW of the yeast strain devoid of glucanotransferase Bgl2p. Lane *2* compared to lane *1* exhibits increased intensity of one band (W3 on Fig. 3b) that is hardly detectable using the given method. The molecular mass of this protein is close to that of W3 identified by electrophoresis. Figure 3c shows the protein bands corresponding to glucanotransferase Bgl2p as visualized by different methods: *1*) Western-blotting, standard test with streptavidin; *2*) staining of polyacrylamide gel with Coomassie G-250; *3*) Western-blotting, immunostaining using antibodies against Bgl2p. Examination of wt-CW SEP following the treatment of CW with trypsin and SDS revealed a single protein, glucanotransferase Bgl2p [1]. Bgl2p, which is clearly detected by staining with Coomassie G-250 and antibodies and silver as well (Fig. 3a, lane *1*), is faintly visible when stained with streptavidin, i.e., is not labeled well during biotinylation of cells.

DISCUSSION

In present work the supposition that CW of *S. cerevisiae* yeasts contain proteins capable of formation of

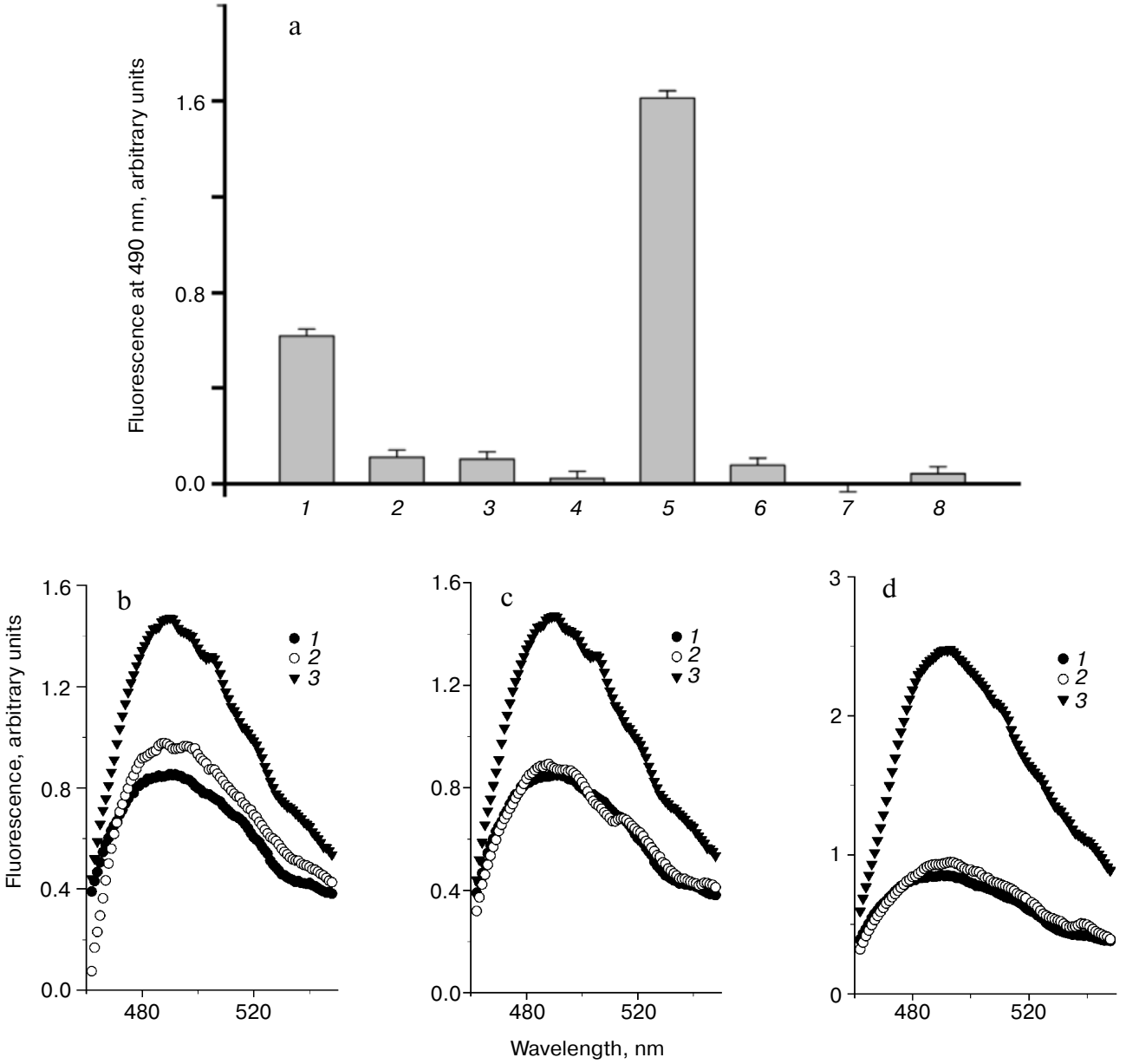

Fig. 1. Fluorescence of ThT in the presence of *S. cerevisiae* wt- and Δ*bgl2*-CW treated with various extractants, as well as intact CW. a) Fluorescence of ThT at 490 nm: *1*) untreated wt-CW; *2*) wt-CW treated with trypsin; *3*) wt-CW treated with trypsin and SDS; *4*) wt-CW treated with SDS and DMSO; *5*) untreated Δ*bgl2*-CW; *6*) Δ*bgl2*-CW treated with trypsin; *7*) Δ*bgl2*-CW treated with trypsin and SDS; *8*) Δ*bgl2*-CW treated with SDS and DMSO. b) Spectra of ThT fluorescence: *1*) in absence of CW; *2*) in presence of wt-CW treated with trypsin; *3*) in presence of intact wt-CW. c) Spectra of ThT fluorescence: *1*) in absence of CW; *2*) in presence of wt-CW treated with SDS and DMSO; *3*) in presence of intact wt-CW. d) Spectra of ThT fluorescence: *1*) in absence of CW; *2*) in presence of Δ*bgl2*-CW treated with trypsin; *3*) in presence of intact Δ*bgl2*-CW. Excitation wavelength, 450 nm.

amyloid-like fibrils other than glucanotransferase Bgl2p [15] gained its first confirmation. This conclusion is deduced from the following facts.

First, several proteins (or their fragments) inducing ThT fluorescence are present in extracts of CW pretreated with trypsin, whereas glucanotransferase Bgl2p remains associated with CW. When extracted from CW, these polypeptides demonstrate high resistance to trypsin in solution because they are not hydrolyzed by this protease (enzyme/substrate ratio 1 : 40 w/w) during more than 1 h at 37°C.

Second, thioflavin-binding proteins are found in tryptic extracts from CW devoid of glucanotransferase Bgl2p. It is very likely that anchoring of these proteins occurs by a possibly non-amyloid peptide fragment that is accessible to the proteinase.

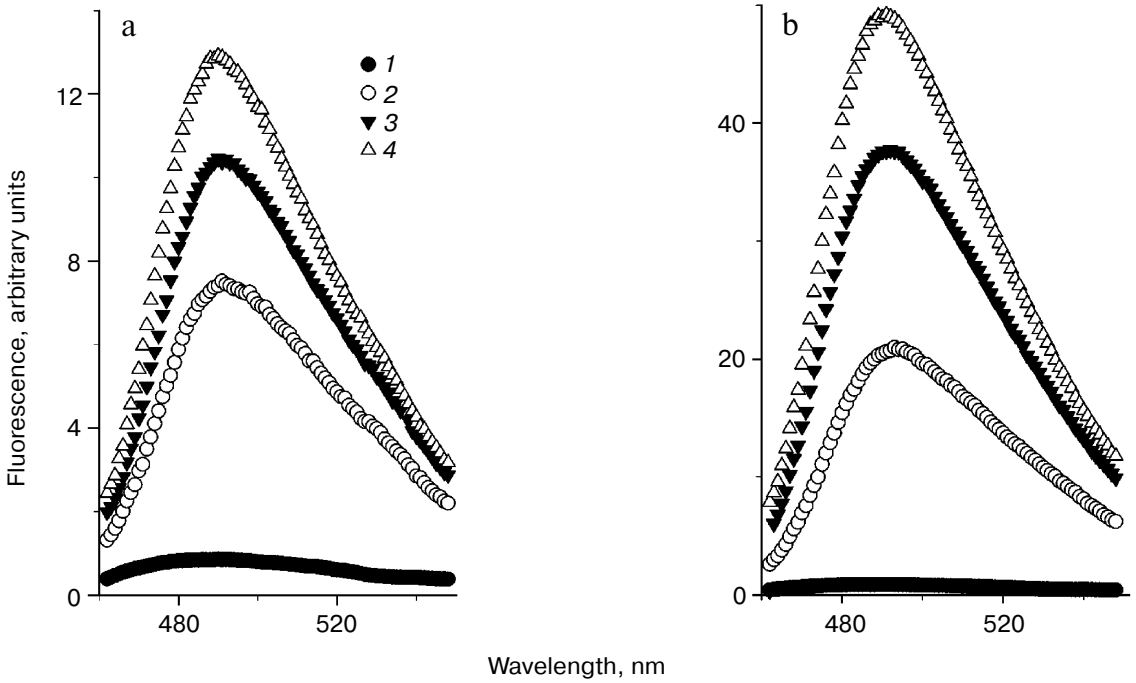

Fig. 2. Spectra of ThT fluorescence in the presence of extracts from wild type (a) and Δ*bgl2* (b) *S. cerevisiae* CW prepared by treatment with trypsin: *1*) ThT; *2-4*) ThT in the presence of tryptic extracts from 0.5, 1.5, and 8.0 mg CW, respectively. Excitation wavelength, 450 nm.

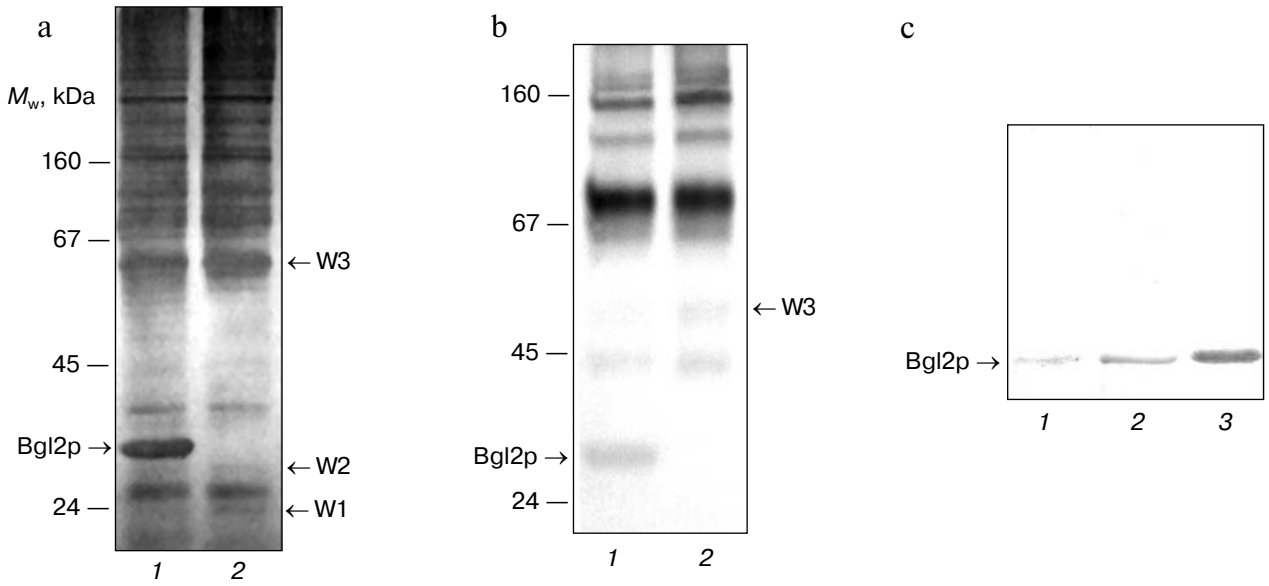

Fig. 3. Western blotting and electrophoresis of SEP (SDS/β-mercaptoethanol-extractable proteins) fraction isolated from CW of wild-type yeast *S. cerevisiae* and its strain with deletion of the *BGL2* gene. See explanations in the text.

It is important that we could not find any significant amounts of thioflavin-binding proteins among covalently attached mannoproteins of *S. cerevisiae* CW under the given growth conditions. The ability of CW consecutively treated with extractants that do not disrupt covalent bonds to induce specific ThT fluorescence decreased virtually to zero. It is worth noting that none of the chemicals used for treatment of CW (trypsin, SDS, and DMSO) affect amyloid properties of proteins [1, 8, 22]. These facts suggest that the proteins remaining in CW

treated with SDS and DMSO do not have amyloid properties. Note that a recent publication describes amyloid properties of adhesin Asl5p that is covalently bound to CW of another yeast (*Candida albicans*) [23], a pathogenic microorganism causing candidoses in animals including humans. It may be that amyloids can also be found among this type of proteins of *S. cerevisiae* under other growth conditions. The set of covalently-bound proteins varies in yeasts depending on conditions and stage of growth.

It is worth noting that the intensity of ThT fluorescence induced in the presence of Δ*bgl2*-CW is 2.5 times greater than that in the presence of the same amount of wt-CW. This increase is very likely due to compensation of absent amyloid-like protein Bgl2p in Δ*bgl2* yeast CW by increase in contents of other proteins that can form amyloid type fibrils. These properties are probably characteristic of just the proteins that are present in the yeast strain devoid of *BGL2* gene and absent in the wild-type strain, as well as proteins whose amount is elevated in the Δ*bgl2* strain. Polypeptides W1 and W2 detected by electrophoresis in polyacrylamide gel followed by staining with silver match the first criterion. The W3 protein that is detected in the molecular mass range of 50-60 kDa by electrophoresis and Western-blotting of Δ*bgl2*-CW extracts matches the second criterion. The latter protein, like Bgl2p, is not stained well when the biotinylating compound sulfo-NHS-LC-biotin that hardly penetrates plasmatic membrane is used, which can be associated with a conformational peculiarity of the CW-associated form of this protein, including formation of amyloid structure. This supposition is supported by our previously reported data that W3 protein is not extracted with SDS from wt-CW, but it is completely removed by treatment with trypsin [1]. We supposed earlier that the given polypeptide is exoglucanase Exg1p, for which a computer-aided analysis of amino acid sequence determined putative amyloidogenic sites [1].

Thus, not only glucanotransferase Bgl2p, but also other proteins having amyloid properties are present in CW of *S. cerevisiae*; these are non-covalently attached to the CW polysaccharide backbone. Since *S. cerevisiae* is widely used in food and drug industry, the properties and physiological effect on animals including humans of amyloid-like proteins from CW emphasize the importance for further studies.

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