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Short Communications

Amyloid-like properties of *Saccharomyces cerevisiae* cell wall glucantransferase Bgl2p

Prediction and experimental evidences

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Abbreviations: Δ*bgl2*, mutant strain without Bgl2p; Bgl2p, glucantransferase; CD, circular dichroism; CWP, covalently bound cell wall proteins; DMSO, dimethyl sulfoxide; EDTA, disodium ethylenediaminetetraacetate; EM, electron microscopy; Exg1p, glucan 1,3-β-glucosidase; kDa, kilodalton; NaCl, sodium chloride; NaN_3, sodium azide; OD, optical density; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulphonyl fluoride; SDS, sodium dodecyl sulfate; SEP, SDS-extractable proteins; ThT, thioflavin T

Key words: glucantransferase Bgl2p, cell wall amyloid-like protein

Glucantransferase Bgl2p is a major conserved cell wall constituent described for a wide range of yeast species. In the baker's yeast *Saccharomyces cerevisiae* it is the only non-covalently bound cell wall protein that cannot be released from cell walls by sequential SDS and trypsin treatment. It contains seven amyloidogenic determinants. Circular dichroism analysis and fluorescence spectroscopy with thioflavin T indicate the presence of β-sheet structures in Bgl2p isolates. Bgl2p forms fibrils, a process that is enforced in the presence of other cell wall components. Thus the data obtained is the first evidence for amyloid-like properties of yeast cell wall protein—glucantransferase Bgl2p.

Introduction

The yeast cell wall consists of mannoproteins, β-glucans and chitin forming a macromolecular complex.[1] Cell wall proteins fulfill essential functions as structural components and enzymes involved in cell wall assembly.[2-5] With respect to their biochemical characteristics two groups of yeast cell wall proteins can be discriminated. The first group includes proteins that can be removed from the cell walls by SDS/mercaptoethanol treatment at 100°C and are therefore considered to be disulphide-linked or non-covalently attached to the wall components (SEP—SDS-extractable proteins). The second group consists of proteins which are covalently linked to the glucan framework (CWP—covalently bound cell wall proteins).[1,2]

Amyloids are widely spread on cell surface of filamentous fungi and bacteria providing a functional coat for these microorganisms[6,7] and also were related to aerial growth and dispersion of spores.[8] Polypeptides forming such fibrils include unrelated proteins like hydrophobins and chaplins. A potential role has been discussed for such amyloids in context of human and animal infections by pathogenic microorganisms.[7] Yeast prions are under thorough investigation.[9] So far, amyloid-like proteins have not been described for the cell surface of yeast species. Here we demonstrated that the cell wall-residing glucantransferase Bgl2p from *Saccharomyces cerevisiae* possesses the properties of protein with amyloid-like nature.

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Results

The starting point of this investigation was the computer analysis which allowed us to predict that some yeast cell wall proteins contain potential amyloidogenic determinants. The most pronounced amyloidogenic properties were predicted for two SEPs of *S. cerevisiae* cell walls: Bgl2p and Exg1p (Table 1). It is well known that *S. cerevisiae* cell walls contain SEPs that can be extracted into hot Laemmli buffer (Fig. 1A, lane 1). We revealed that a lot of them can be extracted by treatment with 1% SDS at 37°C for an extended period of time, except two proteins which remain in the cell wall after this treatment, and then can be extracted into hot Laemmli buffer (Fig. 1A, lane 2). One of them can be removed by hot water (100°C, 5 minutes) (data not shown). It was immunologically identified as glucantransferase Bgl2p. We demonstrated that Bgl2p is hydrolyzed by proteinase K to a low extent only when attached to the cell wall. In this case Bgl2p can be extracted into hot Laemmli buffer in partly digested form (Fig. 1A, lane 3). Even after digestion under more rigid conditions an obviously proteinase K-resistant core persists (data not shown). Similarly, Bgl2p is poorly hydrolyzed by trypsin despite a high content of lysine and arginine residues (see UniProtKB/Swiss-Prot Entry No. P15703) and can be extracted into hot Laemmli buffer from cell walls treated with trypsin in non-digested form (Fig. 1A, lane 4). We used SDS and trypsin resistance of Bgl2p for its

purification (see Materials and methods). Also Bgl2p can be extracted from cell wall with dimethyl sulfoxide (DMSO) used to solubilize amyloids (Fig. 1B), other SEPs haven't been extracted with DMSO from non-treated with trypsin and SDS wild type cell walls (data not shown).

We observed that the share of isolated Bgl2p monomers decreased during incubation at room temperature (Fig. 2A lanes 1–4) if the samples were not boiled after addition of sample buffer and that boiling in sample buffer restored the prevalence of monomers (Fig. 2A lane 5). Electron microscopy inspection of Bgl2p solution after an incubation for 96 h revealed fibrils of about 12 nm width and more than 1 μm length (Fig. 2B). For control we executed parallel experiments using cell wall preparations of the Δ*bgl2* strain, no fibril-like structures was observed in such control experiments. Based on these findings we suppose that the decrease of Bgl2p monomers most likely corresponds to the formation of high molecular weight structures that cannot enter the gel under the conditions applied to the analysis. Circular dichroism analysis indicated that isolated Bgl2p was rich in β-sheet secondary structure (Fig. 2C; Table 2). Unfortunately we couldn't obtain the CD-spectrum of denaturated Bgl2p since it turns insoluble when heated at 100°C for 5 minutes, in spite of the fact that Bgl2p preserves its β-structural conformation during heating up to 90°C (see Table 2). Furthermore, in fluorescent spectroscopy, an increase of fluorescence at 490 nm was observed in Bgl2p samples treated with thioflavin T (ThT) (Fig. 2D, solid line). Boiling resulted in the disappearance of the signal (Fig. 2D, dashed line).

To minimize the probability that the isolation procedure gave rise to Bgl2p fibril formation and to obtain the additional evidence of amyloid nature of Bgl2p we analyzed ThT fluorescence of DMSO extracts obtained from wild type and Δ*bgl2* mutant strain cell walls.

The data obtained demonstrated that extract from wild type cell walls gave the fluorescence at 485 nm (Fig. 3A). In contrast DMSO-extract from Δ*bgl2* cell walls gave no peak. Bgl2p amyloid fibrils are formed more efficiently when the protein is extracted from cell walls and then incubated in the presence of cell wall preparations. We demonstrated that 6 nm wide fibrillar structures of finger print resemblance could be detected by electron microscopy after incubation of purified Bgl2p with cell wall isolates that were partially digested with glucanase (Fig. 3B). The preparation containing extracts obtained from cell walls of the Δ*bgl2* mutant strain lacked such fibrils. When Bgl2p is extracted from cell walls and then incubated in the presence of cell wall preparations it specifically binds the amyloidophilic dye Congo Red resulting in green birefringence of polarized light (Fig. 3C).

Table 1 Potential amyloidogenic determinants in *S. cerevisiae* cell wall proteins

Protein	Amyloidogenic determinants (amino acid number)	Length	UniProtKB/Swiss-Prot Entry No.
Bgl2p	83–89	7	P15703
	168–174	7	
	186–195	10	
	205–218	14	
	227–233	7	
	263–276	14	
Exglp	59–66	8	P23776
	90–96	7	
	107–113	7	
	128–140	13	
	168–174	7	
	204–214	11	
	249–257	9	
	273–280	8	
	293–300	8	
	403–412	10	
	422–429	8	

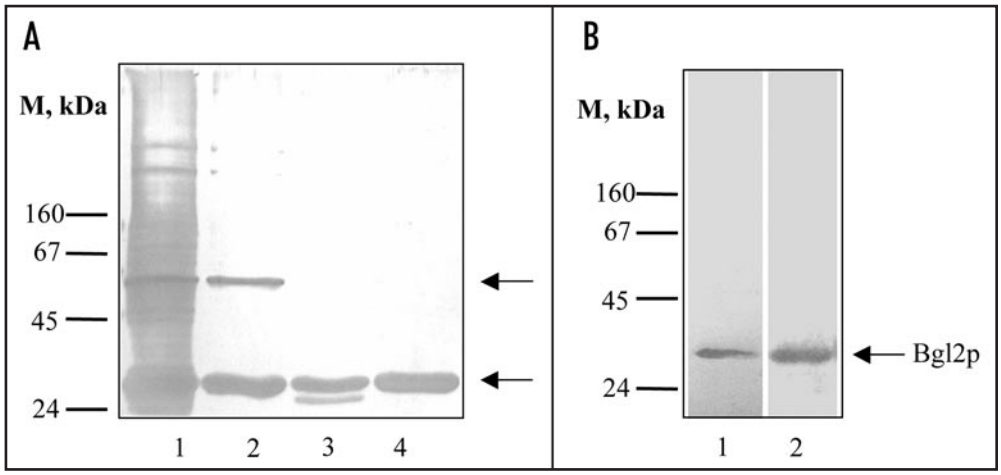

Figure 1. Analysis of non-covalently bound proteins (SEPs) from the cell wall of *S. cerevisiae*. (A) SDS-PAGE of SEPs. Lane 1, SEPs extracted from wild type cell wall with hot Laemmli sample buffer (5 min, 100°C). Lane 2, SEPs extracted from cell walls after incubation (1 hour at 37°C) with 1% SDS; lane 3, with 1% SDS and proteinase K (10 mg/ml 1 hour at 37°C) step by step; lane 4, after incubation with 1% SDS and trypsin (2.5 mg/ml 30 min at 37°C) step by step. Upper arrow, presumptive Exg1p (61 kDa); lower arrow, Bgl2p (29 kDa). M stands for molecular weight markers, masses of marker proteins indicated on the figure (kDa). Proteins were separated on two-step 10/12% resolving gel and visualized by silver staining. (B) Silver stained SDS-PAGE (lane 1) and Western blot analysis (lane 2) of DMSO extracts of wild type cell walls. Proteins were extracted from cell walls, separated on 10% SDS gels and transferred to a nitrocellulose membrane. The transferred proteins were probed with an anti-Bgl2p antibody.

Discussion

Bgl2p is a major cell wall protein of different yeast species. In pathogenic yeasts it participates in virulence[10] and a high Bgl2p antibody titre is a marker of systemic candidiasis.[11] Here we demonstrated that Bgl2p possess amyloidogenic features. Firstly—low solubility in SDS and resistance to protease digestion are considered to be

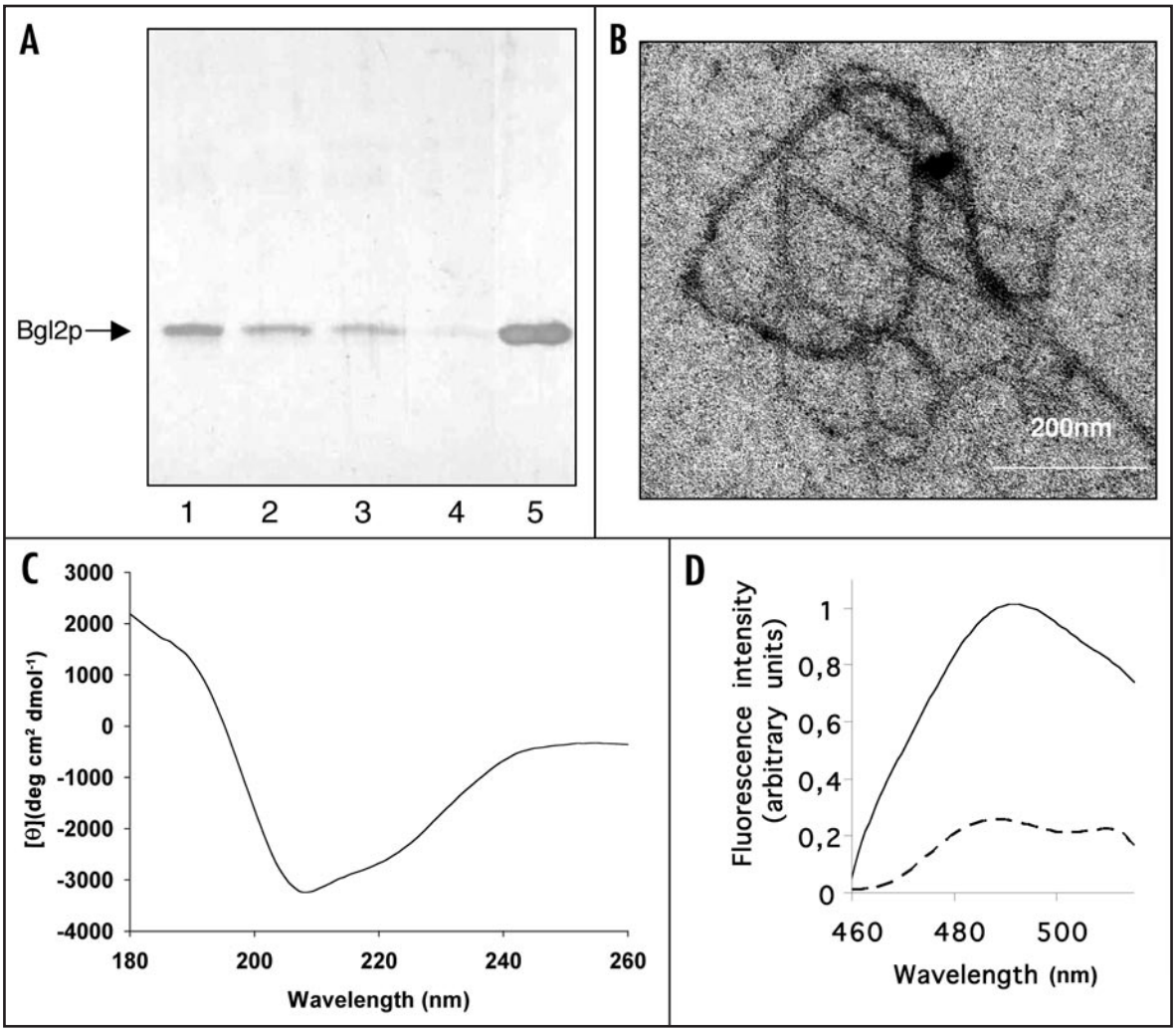

Figure 2. Amyloid-like properties of Bgl2p. (A) SDS-PAGE analysis Bgl2p. Bgl2p was extracted from cell wall preparations as described in Materials and Methods. Proteins were visualized by silver staining. After extraction Bgl2p samples were incubated at room temperature for 0 (lane 1), 1 hour (lane 2), 24 hours (lane 3) and 96 hours (lane 4) and applied to SDS-PAGE without boiling after addition of Laemmli buffer. In lane 5 the Bgl2p sample identical to that applied to lane 4 was boiled after buffer addition (5 min, 100°C). (B) Electron microscopy analysis of Bgl2p samples. Bgl2p sample were incubated at room temperature for 96 hours and inspected by electron microscopy. Bgl2p is present as high molecular weight structures forming fibrils (for control we executed parallel experiments using cell wall preparations of the Δ*bgl2* strain, no fibril-like structures were observed in such control experiments). Negative-staining EM was used. (C) Circular dichroism spectrum of Bgl2p at 20°C. Bgl2p was extracted from cell wall as described in Materials and Methods. The spectrum revealed the high β-sheet structure content in the sample. (D) Fluorescence emission spectra of Bgl2p-bound thioflavin T. Bgl2p was extracted from cell wall preparations (solid line) and the identical sample was boiled for 5 minutes before applying (dashed line). Autofluorescence of protein in the absence of ThT was negligible. Excitation wavelength is 435 nm.

indicative of amyloidogenic proteins.[12] Bgl2p is rich in β-sheet secondary structure (Fig. 2C; Table 2), a distinctive feature of amyloids,[12-14] and Bgl2p samples treated with ThT give an increase of fluorescence which is characteristic for amyloid fibril-bound ThT.

We suppose that Bgl2p is not the only amyloid-like protein in the yeast cell wall. The second SEP which cannot be removed from cell wall isolates (Fig. 1A, lanes 1 and 2) is most likely glucan 1,3-β-glucosidase Exg1p by molecular weight. And we revealed that Exg1p contains eleven, Bgl2p six short amino-acid stretches of potential amyloidogenic determinants. Such stretches were also found in some additional cell wall proteins, but were of insufficient number and length to define the proteins as amyloidogenic (data not shown). But we restricted our study to Bgl2p, the protein of a more pronounced protease resistance.

We suppose that fibril formation is not restricted to purified isolates but that Bgl2p proteins reside in the cell wall as fibrillar structures however this suggestion needs further experimental confirmation.

It is not unlikely that interaction with other cell wall components is required for fibril formation. Previously we have demonstrated that Bgl2p molecules can be detected in the growth medium when GPI anchor formation is impaired and Bgl2p presents in yeast cell wall at low level.[15] Under such conditions Bgl2p is secreted to the medium in non-fibrillar form (data not shown). This suggests a possible involvement of GPI proteins in the conversion of Bgl2p into amyloid fibrils.

The cell surface amyloids of microorganisms usually perform an important role in cell adaptation to the environment, for example SC3 hydrophobin lowers surface tension at water-air interface

allowing filamentous fungi to escape the aqueous phase to form aerial structures.[16] Although Bgl2p has been studied since 1989, its physiological role remains obscure. Strain overexpressing *BGL2* exhibit a decreased growth rate,[17] Δ*bgl2* mutant strains are characterized by increased chitin levels in the cell wall.[18] Deletion of *BGL2* gene is not lethal. Moreover viability of Δ*bgl2* mutant strains is not impaired under standard cultivation conditions and a decreased growth rate of Δ*scw4* and Δ*scw10* strains is suppressed when deleting *BGL2*.[19] Similar to the functions of amyloids in other microorganisms Bgl2p may contribute to forming of cell wall assembly, especially under extreme conditions.

Bgl2p from *Candida utilis* and *C. albicans* have a similar share of amyloidogenic determinants (0.15 for *S. cerevisiae*, 0.30 for *C. utilis*, 0.11 for *C. albicans*). Bgl2p from *C. utilis* exhibits properties similar to that of *S. cerevisiae*. Bgl2p from *Hansenula polymorpha* as well as that from *S. cerevisiae* couldn't be extracted from cell wall by incubation with SDS at 37°C (Vitaly V. Kushnirov personal communication, Institute of Experimental Cardiology). Our data provide the first evidence for a protein with amyloidogenic properties in the cell wall of yeasts. Bgl2p is a conserved protein with homologous counterparts described for a wide range of yeast species.[17,20,21]

Materials and Methods

Prediction of amyloidogenic regions in protein sequence. For prediction of amyloidogenic regions, we used the previously described method based on large number of contacts per residue in protein sequences.[22-24] For each amino acid residue within a protein sequence, an expected number of contacts per residue is defined for the various residue types (which is the average number of contacts at a distance below 8 Å for the given type of residue in 3D structures of proteins).[22,23] Then, the values are averaged with a sliding window of seven residues.[23] Sequences composed of residues above a threshold value (which is 21,4 expected contacts per residue) are predicted as amyloidogenic if the size of such a region is not smaller than the sliding window.[22-24] Thus, the predicted amyloidogenic regions are regions which have a large number of expected contacts per residue.

Yeast strains and growth conditions. The *S. cerevisiae* parent strains MAY591,[25] (MATα *leu2-3,112 lys2-801 ura3-52 his3-Δ200*) and DBY 746 (MATα *ura3-52 leu2-3,112 trp1-289 his3-Δ1*) were used; they are referred to as wild type (or wt) in the text. The mutant strains were obtained in our laboratory by disruption of chromosomal copy of *BGL2* gene by insertion of *URA3* (MATα *leu2-3,112 lys2-801 ura3-52 his3-Δ200 BGL2::URA3* and MATα *ura3-52 leu2-3,112 trp1-289 his3-Δ1BGL2::URA3* respectively); they are denoted to as Δ*bgl2*.

The *C. utilis* strain VKM Y-74 was obtained form All-Russian Collection of Microorganisms—VKM (Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow Region, Russia).

Yeast cells were grown at 30°C in YPD medium (1% yeast extract, 2% peptone, 2% glucose).

Cell wall isolation. Cell walls were obtained as previously described.[4] Cell disruption was performed with glass beads in presence of 5 mM PMSF and 5 mM EDTA. Cell walls were precipitated by centrifugation at 5000 g and then were washed sequentially with 1% sucrose, 1 M NaCl, 1% NaCl and deionized water.

Table 2 **The results of analysis of Bgl2p circular dichroism spectra for secondary structure using DICHRO WEB online server**

Algorithm and conditions used		Share of secondary structure elements[a]					
		α_R	α_D	β_R	β_D	T	U
CDSSTR[35]	20°C	-0,02	0,03	0,30	0,12	0,21	0,34
SELCON3[36]	20°C	0,049	0,191	0,275	0,149	0,065	0,260
	90°C	0,003	0,060	0,228	0,104	0,161	0,280
CONTINLL[37]	20°C	0,013[b]	0,064	0,251	0,119	0,238	0,314
	90°C	0,039	0,074	0,239	0,116	0,223	0,309
	20°C	0,004[c]	0,046	0,276	0,137	0,219	0,319
	90°C	0,005	0,048	0,290	0,141	0,210	0,307

[a]α_R, regular α-helix; α_D, distorted α-helix; β_R, regular β-strand; β_D, distorted β-strand, T, turn; U, unordered. [b]Closest matching solution with all proteins of reference set. [c]Average of all matching solutions.

Purification of Bgl2p. Bgl2p was purified from cell walls which were incubated with trypsin (1 mg/ml in 50 mM Tris/HCl pH 7.5) and with 1% SDS for 1 hour (37°C). This procedure allows removing all non-covalently attached proteins except Bgl2p from cell walls. A pellet obtained by centrifugation at 5000 g was extracted three times with cold butanol-water (0.7:1, vol:vol) for 15 min (1 volume of cell debris per three volumes of butanol-water). After each extraction the suspension was centrifuged at 10,000 g, the supernatant was discarded. The pellet obtained after the last extraction was washed twice in water. After subsequent washing with 1 M NaCl (four times), 1% NaCl (four times), 1% sucrose (twice) and water cell walls were centrifuged and then suspended in water containing 0.05% NaN_3 and 5 mM PMSF for storage. The pellet was re-suspended in water and incubated at 55°C for 15 min, centrifuged and incubated again at 70°C for 15 min and centrifuged at 10,000 g. The final extract contained the pure Bgl2p (25 μg/ml). Control extracts obtained from Δ*bgl2* mutant strain cell wall preparations with this technique contained no Bgl2p.

Protein concentration determination. Peptide bond absorption at 205 nm was measured using Varian Cary 300 Bio UV-Visible Spectrophotometer (Varian Inc., USA). Protein concentration was determined according to Scopes.[26]

Electrophoresis. Electrophoresis was performed according to Laemmli[27] in 10% or two-step 10–12% resolving polyacrylamide gels. Proteins were visualized by silver staining or immunologically detected by Western Blot analysis.

Antibodies against Bgl2p. Bgl2p antiserum was raised in male BALB/c mice (SPF status) using SDS PAGE-purified protein (40 μg per mouse).[28]

Thioflavin T fluorescence. ThT binding assay was performed according to LeVine.[29] Fluorescence measurements were carried out with a Cary Eclipse fluorescence spectrophotometer (Varian Inc., USA).

Circular dichroism analysis. Circular dichroism spectra were obtained using Chirascan Circular Dichroism Spectrometer (Applied Photophysics Ltd., UK) at 20–90°C. A cell with a 0.2 cm pathlength was used for spectra recorded between 180 and 260 nm, with sampling points every 0.5 nm. Five scans were recorded, and baseline spectra were subtracted from each spectrum. Data

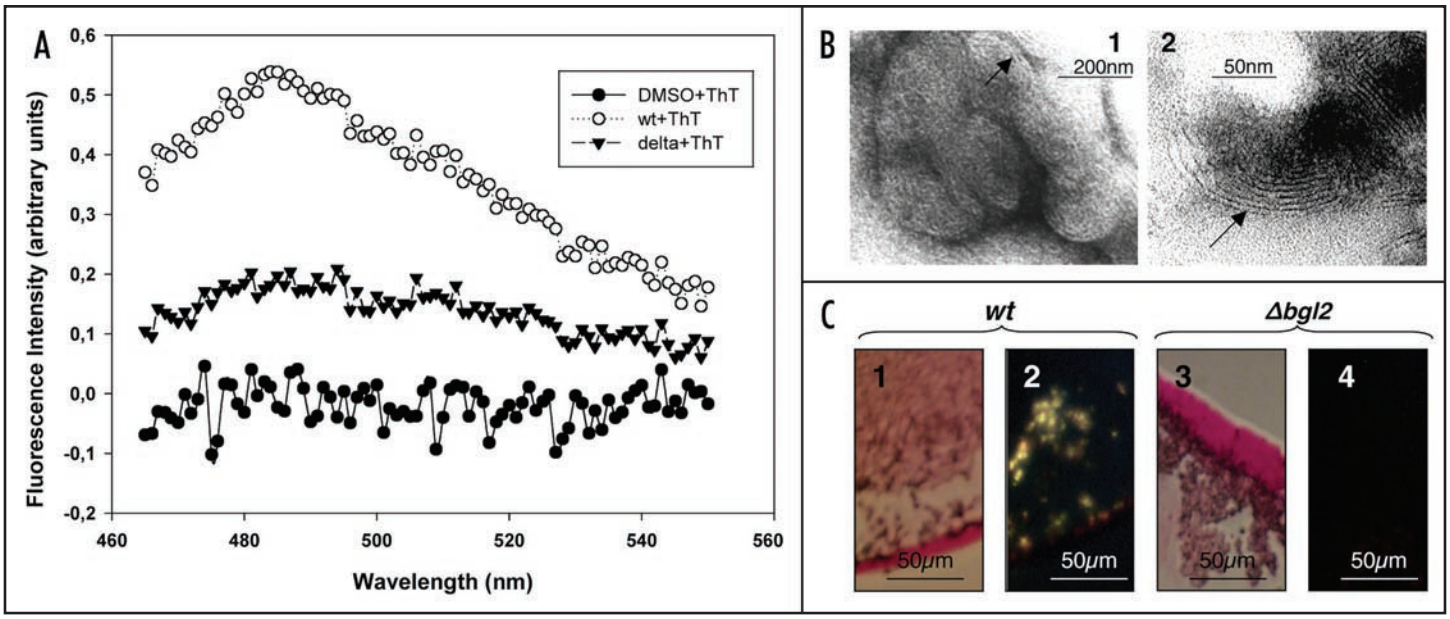

Figure 3. Analysis of amyloid-like proteins in cell wall preparations derived from wild type and Δ*bgl2* strains. (A) Fluorescence emission spectra of ThT in presence of DMSO extracts obtained from *S. cerevisiae* wild type (wt + ThT) and Δ*bgl2* (delta + ThT) cell walls and DMSO (DMSO + ThT). In all samples DMSO concentration was 2.8%. Autofluorescence of protein in absence of ThT was negligible. Excitation wavelength is 450 nm. (B) Electron microscopy analysis of Bgl2p incubated with cell walls partially digested with glucanase (1 mg/ml 30 min at 37°C). The arrows indicate "fingerprint"-like fibrillar structures. Lane 1, a group of "fingerprints"; lane 2, individual "fingerprint". Negative-staining EM was used. (C) Light microscopy analysis of extracts obtained from wild type and Δ*bgl2* cell walls incubated at room temperature for a week in the presence of corresponding cell walls partially digested with glucanase. Congo Red tinted green birefringence in polarized light. Bgl2p solution (1) and control sample obtained from Δ*bgl2* strain cell walls (3). Polarized light microscopy analysis of the same extracts of the wild type cell walls (2) and Δ*bgl2* strain cell walls (4).

were processed using Applied Photophysics Chirascan Viewer and SigmaPlot package.[12] Analysis of protein circular dichroism spectra for secondary structure was carried out using DICHROWEB online server available at www.cryst.bbk.ac.uk/cdweb/html/dw2.html.[30,31] Protein Reference Set 3 (optimized for 185–240 nm) was used.

Congo red binding assay. Samples were applied to object slides that were thoroughly washed with alcohol and dried at room temperature. Specimens were stained with 0.0002% Congo Red solution and visualized using an Opton microscope in polarized light.[12,32]

Electron microscopy (EM). Negative-staining EM was used. 2 μl samples were absorbed onto glow-discharged carbon-coated, Formvar-filmed 400-mesh copper grids and immediately dried down. 2% uranyl acetate staining solution was then absorbed for 2 min. Grids were allowed to dry in a light-protected environment and viewed in a JEM-100B (JEOL, Japan) electron microscope at the accelerating potential of 80 kV.[33]

Other methods. Yeast cell walls were partially digested with glucanase (laminarinase, Sigma, 1 mg/ml 30 min at 37°C) and with proteinase K (Serva, 10 mg/ml 1 hour at 37°C). Prior to use in further experiments the enzymes were washed out.

DMSO extraction of Bgl2p from yeast cell walls was performed as described earlier for dissolution of amyloid fibrils with some modifications.[34]

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