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Catalytic properties of the Gas family β -(1,3)-glucanosyltransferases active in fungal cell wall biogenesis as determined by a novel fluorescent assay

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SYNOPSIS

Glucanosyltransferases (β -1,3-glucanosyl transglycosylases, EC 2.4.1.-) of the glycosyl hydrolase family GH72 are glycosyl phosphatidylinositol-anchored proteins that play an important role in the biogenesis of fungal cell walls. They randomly cleave glycosidic linkages in β -(1,3)-glucan chains and ligate the polysaccharide portions containing newly formed reducing ends to C3(OH) at non-reducing ends of other β -(1,3)-glucan molecules. We have developed a sensitive fluorescence-based method for the assay of transglycosylating activity of GH72 enzymes. In the new assay, laminarin [β -(1,3)-glucan] is used as the glucanosyl donor and laminarioligosaccharides fluorescently-labeled with sulforhodamine (SR-laminarioligosaccharides) serve as the acceptors. The new fluorescent assay was employed for partial biochemical characterization of the heterologously expressed Gas family proteins from the yeast *Saccharomyces cerevisiae*. All the Gas enzymes specifically used laminarin as the glucanosyl donor and SR-laminarioligosaccharides of DP \geq 5 as the acceptors. Gas proteins expressed in distinct stages of the yeast life cycle showed differences in their pH optima. Gas1p and Gas5p, which are expressed during vegetative growth, had the highest activity at pH 4.5 and 3.5 respectively, whereas the sporulation-specific Gas2p and Gas4p were most active between pH 5 and 6. The novel fluorescent assay provides a suitable tool for the screening of potential glucanosyl transferases or their inhibitors.

Short title: Catalytic properties of yeast β -(1,3)-glucanosyltransferases

Keywords: Gas proteins, GH72 family, transglycosylation, cell wall, yeast, cell wall

Abbreviations: BGT, β -1,3-glucanosyl transglycosylase; LamOS, laminarioligosaccharides; L6, laminarihexaose; SR, sulforhodamine; L6-SR, sulforhodamine-labeled laminarihexaose; HPAEC, high-performance anion exchange chromatography; GPI, glycosyl phosphatidylinositol; GFP, green fluorescent protein; DP, degree of polymerization; F. u., fluorescence units

INTRODUCTION

The cell wall is an external envelope which is essential for fungal viability. It consists of a matrix of cross-linked glucose polymers (β -glucans), mannoproteins and a small amount of chitin. A balance between the biosynthesis of the constituents and hydrolysis/transglycosylation reactions is crucial for cell wall homeostasis, which is essential for both cell integrity and proper morphogenesis.

Family 72 of glycoside hydrolases (GH72), annotated in the carbohydrate active enzyme (CAZy) database, include endo- β -(1,3)-glucanases that are endowed with significant transglycosylase activity [1, 2]. Due to this activity these enzymes are also referred to as β -(1,3)-glucanotransferases. To date, 157 proteins have been identified in this family [<http://www.cazy.org/>, 3]. The presence of GH72 enzymes is restricted to yeast and fungal species indicating a highly specialized role in fungal wall biogenesis.

β -(1,3)-Glucan is the most abundant glucose polymer of the yeast cell wall. It is synthesized by plasma membrane enzyme complexes - glucan synthases with Fks proteins as their catalytic components [4]. On the cytoplasmic side of the cell membrane, UDP-glucose serves as a substrate for the vectorial synthesis of the polymer. In the extracellular space, GH72 enzymes catalyze the linear elongation of the polymers. Moreover, they contribute to the formation of lateral branches by elongating branching points - β -(1,6)-linked glucose residues - which are created by branching enzymes [5]. All these reactions occur through transglycosylation due to the lack of availability of small energy-exchange molecules such as ATP in the extracellular space.

In addition to GH72 and branching enzymes active on β -(1,3)-glucan, other transglycosylases are involved in fungal cell wall formation [6]. Remarkably, in *S. cerevisiae* it has been demonstrated that Crh enzymes (Crh1, Crh2 and Crr1 of family GH16) cross-link chitin both to the β -(1,6)-glucan, which in turn tethers GPI-trimmed mannoproteins to the wall, and to the β -(1,3)-glucan [7, 8]. Crh1p and Crh2p are required to covalently link a pool of the yeast Gas1 protein molecules to the bud scars [9]. The *in vivo* role of other glycanases/transglycosylases such as Dcw1p and Dfg5p (putative mannanases of family GH5) and Scw10/Scw4 proteins (putative glucanases of family GH17) is still unclear.

Hydrolases/transglycosylases also play a role in plant cell wall growth and modification and are the object of intense biochemical investigation. An example is provided by the xyloglucan-endotransglycosylase/hydrolase (XET/XTH), ubiquitous plant enzyme involved in the loosening and reformation of xyloglucan tethers between cellulose microfibrils in the cell walls [10-13]. Thus, the creation of an interwoven texture of polymers in the cell wall shares some common principles both in fungal and plant cells.

The first clues to the activity of the GH72 enzymes came from biochemical studies on Gel1p from *A. fumigatus* [14]. When Gel1p was incubated with borohydride-reduced laminarioligosaccharides of 10 or more glucose residues ($rG_{\geq 10}$), both shorter and longer products were obtained. This was consistent with a double-inversion transglycosylating reaction mechanism of the so-called retaining glycoside hydrolases [15]. In the first step, the enzyme cleaves an internal β -(1,3)-glycosidic linkage in the donor substrate molecule, the non-reducing part is released and the newly created reducing-end portion of the donor molecule is linked *via* an ester bond to the catalytic acid residue in the enzyme, thus forming a covalent glycosyl-enzyme intermediate. In the next step, the glycan portion is transferred from the intermediate to a molecule of water (hydrolysis) or to C3(OH) of non-reducing end glucose residue in the acceptor molecule with formation of a β -(1,3)-glycosidic linkage (transglycosylation). Consequently, a disproportionation of the substrate takes place and a series of oligosaccharide products with DP both higher and lower than that of the donor are

formed. High-performance anion-exchange chromatography (HPAEC) was used to analyze the reaction mixture and to detect the transglycosylation activity [14, 16]. The GH72 enzymes are not pure transglycosylases but also possess a significant hydrolytic activity. The ratio of transferase versus the hydrolytic activity depended on the substrate concentration; for Gel1p from *Aspergillus fumigatus* it was 100% at high substrate concentration but dropped to 35% at low concentration [14]. The term β -(1,3)-glucan elongase was also coined to indicate the ability of these enzymes to elongate the substrate molecules [17].

The HPAEC method used in the previous studies to detect transglycosylation activity of the Gas proteins enables both qualitative and quantitative characterization of the reaction products, but determination of overall transglycosylation activity is complicated. Moreover, the HPAEC assay is rather cumbersome and does not permit simultaneous measurements of multiple enzyme samples. Of considerable importance may also be the fact that the HPAEC instrumentation is quite sophisticated and hence expensive. It was our aim to design a simple, rapid and cost-effective method to test the transglycosylating activity of GH72 enzymes. The new assay of β -(1,3)-glucanoyl transglycosylase activity exploits laminarin as the donor and fluorescently labeled laminarioligosaccharides as the acceptors. The assay was used to determine the basic biochemical properties of heterologous yeast Gas proteins and to map the distribution of β -(1,3)-glucanoyl transglycosylase activity in the yeast cell fractions.

EXPERIMENTAL

Substrates and chemicals

Laminarin [β (1,3)-glucan] was purchased from Sigma and pustulan [β (1,6)-glucan] from Calbiochem. Laminarioligosaccharides and N-acetyl chito-oligosaccharides were from Megazyme. Oligosaccharides derived from pustulan (DP 2-7), were prepared by partial acid hydrolysis of pustulan with 4M trifluoroacetic acid and fractionated on a Biogel P4 column (1.7 x 120 cm) eluted with water. LissamineTM rhodamine B sulfonyl chloride (LRSC) was purchased from Anaspec or from Acros Organics. All other chemicals used were of p. a. quality, mostly from Merck or Fluka.

Protein purification

Pichia pastoris strains expressing and secreting tail-less and 6x His-tagged Gas1p, Gas2p, Gas3p, Gas4p and Gas5p proteins from *Saccharomyces cerevisiae* were previously described [18, 19]. Recombinant proteins were purified from the supernatants of *P. pastoris* cultures as previously described [18, 19]. Protein concentration was determined by Quant-iTTM Protein Assay Kit (Invitrogen) using BSA as a standard. Fractions of the purified preparations were checked for purity by SDS-PAGE using 10% polyacrylamide gels. Proteins were stained with Coomassie Blue R-250 or silver nitrate.

Cell fractionation

Diploid strain *Saccharomyces cerevisiae* CCY 21-4-13 from the Culture Collection of Yeasts, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia) was grown in YPD medium containing 1% each yeast extract, bacto-peptone and glucose at 30 °C on an orbital shaker. The cells were harvested in middle logarithmic phase of

growth by centrifugation, washed twice with cold water, suspended in the homogenization buffer (0.1 M citrate, pH 5.0, containing 1 mM each EDTA, PMSF and DTT) and disintegrated with ballottini beads (0.2 mm diameter) in a rotatory disintegrator cooled from the outside with an ice-bath. All subsequent steps were carried out at 0 – 4 °C. The progress of the cell disintegration was checked microscopically. When more than 97 % cells had ruptured, the beads were decanted with the homogenization buffer and the suspension was centrifuged at 1,500 **g** for 10 min. The sediment containing the cell walls and unbroken cells was washed twice with the buffer and suspended in the 0.1 M citrate buffer, pH 5.0 (buffer A), in a volume corresponding to one tenth of the wet weight of the starting cells. The supernatant from the first centrifugation was centrifuged at 20,000 **g** for 15 min, the sediment washed twice with the homogenization buffer, suspended in a small volume of the buffer A so as to obtain thick suspension, and used as the plasma membrane-enriched fraction. The 20,000 **g** supernatant was spun at 100,000 **g** for 30 min, the sediment washed once with the buffer A, suspended in an equal volume of the same buffer and used as the microsomal fraction. The 100,000 **g** supernatant was considered as the cytosolic fraction. The prepared subcellular fractions could be stored at -70 °C for more than one week before the assays, without appreciable loss of activity.

Synthesis of sulforhodamine-labeled oligosaccharides

The oligosaccharides were labeled with sulforhodamine B according to a previously published protocol [20]. The purity of individual SR-labeled oligosaccharides derived from laminarin and from pustulan was checked by TLC on silica gel plates using the solvent system 1-butanol/ethanol/water (5/3/2, by vol.) whereas for the resolution of SR-N-acetyl-D-chito-oligosaccharides the solvent system 2-propanol/water/25% aq. ammonia (7/2/1, by vol.) was used. The chromatograms were viewed under a UV lamp. Molar concentrations of the labeled oligosaccharides were calculated on the basis of the molar absorption coefficient for LRSC, $\epsilon_{566} = 85,000 \text{ M}^{-1} \text{ cm}^{-1}$ [Certificate of Analysis for Lissamine rhodamine B sulfonyl chloride, Lot No. CM16-027 Anaspec, Inc., San José, CA].

Assay of β -(1,3)-glucanosyl transglycosylase (BGT) activity

The standard assay mixture contained 2.5 mg/ml laminarin, 30 – 45 μM of appropriate SR-labeled oligosaccharide, 50 mM citrate-phosphate buffer pH 2.5-7.5; and 0.9-3.0 μg of the respective purified Gas protein (or 0.05-0.12 mg protein in case of subcellular fractions) in the total volume of 20 μl . Incubations were carried out at 30 to 37 °C for 30-60 min. The reaction was stopped by addition of 20 μl of 40 % (v/v) formic acid. Aliquots of 5 μl were applied in quintuplicate from the stopped reaction mixture onto a chromatographic paper (Whatman 3MM) template into positions exactly fitting the positions of wells in a 96-well ELISA microtiter plate and dried. The paper was then washed with several changes of 66 % (v/v) aqueous ethanol containing 5 % (v/v) formic acid to remove the unreacted SR-oligosaccharides, and left overnight in 66 % ethanol. After drying, the paper was sandwiched between two glass plates and fluorescence was measured in a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments) at excitation wavelength 530 nm and emission 575 nm.

RESULTS

Setup of a fluorescent assay for β -(1,3)-glucanosyl transglycosylase

In designing the novel fluorescence-based approach to test the BGT activity, we took advantage of the previous demonstration that laminarioligosaccharides of 7 glucose residues and shorter were able to function as the acceptor molecules but not as the donors [14]. Therefore, laminarioligosaccharides of DP 2-7 (L2 – L7) were labeled with sulforhodamine (Fig. 1) and used as glucanosyl acceptors with laminarin as the donor substrate. To prove their transglycosylating activity, individual Gas proteins were incubated with laminarin and L6-SR and at time intervals aliquots were taken and applied onto the cellulose filter paper Whatman 3 MM and dried. Washing the paper with 66% aqueous ethanol removed the low-Mr reaction products and the unused fluorescent substrate while the high-Mr transfer products remained adsorbed to the paper. The binding of the labeled L6-SR to the polymer fraction was indicated by an increase in fluorescence adsorbed to the filter paper and resistant to washing with 66% ethanol (Fig. 2). The transfer of the label was specifically due to the enzyme activity, since the sample of L6-SR incubated under the same conditions but without enzyme showed no changes (not shown). Gas3p was not tested since it is an inactive member of the Gas family [21].

In order to analyze multiple protein samples simultaneously, the incubations were performed in 1.5 ml conical Eppendorf tubes and multiple 5 μ l aliquots from the stopped reaction mixtures were applied as dot blots onto the filter paper, dried and subsequently washed with 66% ethanol. The quantification of the transglycosylase activity was accomplished in a high-throughput mode using a fluorescent ELISA plate reader. High sensitivity of the fluorescence measurement (less than picomole amounts of sulforhodamine could be determined) enabled miniaturization of the assay and measurement of multiple parallel applications from one sample ensured high precision and reproducibility of the results. When required, a calibration curve constructed by measuring the fluorescence of a series of known quantities of the oligosaccharide acceptor was used to convert the fluorescence units into the absolute amounts of the products.

The novel fluorescent method could also be applied for the determination of BGT activity in isolated cell walls and membrane fractions prepared from vegetative yeast cells. The highest specific activity was found in the fraction of plasma membranes, although a significant BGT activity was detected also in microsomes and in the cell walls (Table 1).

Time-course of the reaction and the effect of enzyme concentration on activity

Progress curves of the reactions catalyzed by Gas proteins exhibited typical hyperbolic shapes characteristic for pseudo-first order reactions (Fig. 3). With Gas4p, the reaction rate was constant throughout the 2-h assay, presumably due to the low initial activity of the enzyme. Furthermore, the dependence of activity on the enzyme concentration was linear with all the Gas proteins (Fig. 4).

pH- and temperature optima of transglycosylase reactions

The dependence of β -(1,3)-glucanosyl transglycosylase activity of Gas proteins on pH was tested in the pH range 2.5-7.5. The amount of transfer products produced after 30 min reaction time (after 3h in the reaction with Gas4p) was determined. As shown in Fig. 5, all the Gas proteins showed approximately symmetrical bell-shaped dependences of activity on pH. There was a rather broad maximum with Gas1p. The curve showed a plateau in the pH range between 3.5 and 6 and the Gas1 enzyme still retained over 60 % of its maximal activity at pH 6. Gas5p activity was maximal at pH 3.5-4.5, decreased sharply at pH values higher than 4.5 and was <10% at pH 6. By contrast, Gas2p activity reached its maximum at pH 6 and was still over 50% at pH 7. Gas4p had a similar curve but with its maximum shifted towards pH values 5.0-6.0. In

conclusion, the pH optimum of Gas5p protein was acidic, whereas the pH optima for Gas2p and Gas4p were closer to neutral (Fig. 5). Gas1p had the broader range of pH optimum. Interestingly, these values are consistent with the functional interplay among the Gas isoforms during yeast life cycle (see the Discussion).

In the temperature assay, the incubation temperature varied between 20 and 55 °C. The temperature optima for the individual Gasps were found to be in the range between 30 and 37 °C (not shown).

Donor and acceptor substrate specificities of the Gas proteins

In order to determine the preferred donor substrate, individual Gas proteins were incubated with polysaccharides similar to or identical with those present in the yeast cell wall. Of the polysaccharides tested, only laminarin was active as the donor substrate with all the Gas enzymes. Pustulan, carboxymethyl-chitin and/or yeast α -mannan did not yield detectable amounts of transfer products.

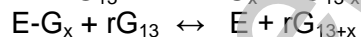
The acceptor specificity was tested using equimolar concentrations of SR-labeled hexamers derived from laminarin, pustulan and chitin representing the potential interpolymeric linkage pairs occurring in the yeast cell wall [22]. It was found that with all Gas proteins, only the glucooligosaccharides of the β -(1,3)- series were active as acceptors, whereas the formation of products with SR-pustulooligosaccharides or SR-N-acetyl-D-chitooligosaccharides was negligible.

To determine the effect of oligosaccharide chain length (expressed as degree of polymerization, DP) on the acceptor efficiency, the purified Gas proteins were incubated with a series of SR-labeled laminarioligosaccharides containing from one up to 7 glucose residues (L1-SR to L7-SR). As can be seen from Fig. 6, their capability to serve as the acceptor increased with increasing number of glucosyl units in the β -(1,3)-oligosaccharide moiety, the smallest active acceptor being laminaripentaose L5-SR with all Gasps.

DISCUSSION

The assay

In the initial studies reporting the enzymatic activity of recombinant fungal β -(1,3)-glucanosyl transferases, the activities were assayed using a reduced G_{13} laminarioligosaccharide (rG_{13}) as the substrate. A short incubation generated products compatible with the following reaction scheme:



where $4 < x < 7$ and E denotes the enzyme.

An incubation time of several hours produced longer oligosaccharides which themselves became either donors or acceptors, resulting in a wide range of products with increasing chain length (DP>30) which gradually became water-insoluble.

Monitoring the progress of the reaction and analysis of the reaction products were accomplished by high-pressure anion-exchange chromatography, HPAEC [14, 16, 19]. While the HPAEC is suitable for characterization of the reaction products, its usefulness for quantitative determinations of transglycosylase reactions is limited.

In this work we describe a new, highly sensitive fluorescent method to assay the β -(1,3)-glucanosyl-transglycosylase activity of yeast Gas proteins and of GH72 enzymes in general. The method employs principles previously applied successfully to the study of plant transglycosylases such as xyloglucan endotransglycosylase, XET [23- 25].

The fluorescent assay has several advantages over the HPAEC method previously applied to assess transglycosylating activity of GH72 proteins. Among them is the high

sensitivity, the ability to express the overall reaction rates in quantitative terms, the possibility to perform multiple assays simultaneously, and finally the simplicity which results in low price per assay. Theoretically, every catalytic event leads to a transfer of the label from the pool of acceptor molecules to the pool of donor molecules. It should be taken into account, however, that the fluorescent assay records only those labeled transfer products that are insoluble in 66% ethanol, and not the portions of donor molecules that are split off from the reducing-end and released during the first step of the transglycosylation reaction [15]. Since the solubility of SR-laminarioligosaccharides in 66% ethanol decreases with increasing oligosaccharide chain length, it is advantageous to use acceptors having their carbohydrate moiety composed of at least five glucosyl units. In such case, any lengthening of the sugar chain caused by the oligoglucosyl transfer would result in the formation of a product insoluble in 66% ethanol. However, in contrast to HPAEC, the new fluorescence-based method does not yield information about the qualitative composition of the reaction products.

Using the new fluorescence-based method, it was possible to detect β -(1,3)-glucanosyl-transglycosylase also in yeast subcellular fractions. The highest specific activity of BGT was found in the plasma membrane-enriched fraction but the presence of significant activity was detected also in microsomes and cell wall fractions (Table 1). Such distribution seems to map the typical maturation and transport pathway of GPI-proteins from the site of their synthesis in the ER to their final destination. It should be kept in mind, however, that the measured quantities may be distorted by the presence of β -1,3-glucan hydrolyzing enzymes that could degrade the product as it is being synthesized. Since for the isolation of subcellular fractions, the cells were taken from the logarithmic growth phase, the detected activity presumably belonged to Gas1 and Gas5 proteins which are expressed during vegetative growth, although we cannot exclude the contribution of other enzymes catalyzing a transglycosylase reaction in the conditions used [27].

It is perhaps noteworthy to point out to a potential versatility of the described fluorescent assay method. Namely, by selecting suitable combinations of donor/acceptor pairs, similar fluorescent assays could be devised also for other types of transglycosylases that possibly are present in fungal cell walls. As an example could serve the demonstration of transglycosylating activity of *CRH1* and *CRH2* gene products linking chitin to β -(1,3)/ β -(1,6) glucan in the yeast cell walls [8].

Enzymatic properties of Gas proteins

From the previous studies, Gas1p, Gas2p, Gas4p and Gas5p were known to be endowed with a similar type of enzymatic activity but no further analysis of their biochemical properties has been performed hitherto [14, 16, 19]. The fluorescent assay described here enabled us to ascertain the basic biochemical parameters of the Gas proteins and to establish optimal conditions for determination of their activity. All reactions catalyzed by Gas proteins exhibited attributes characteristic for an enzymatic reaction. The progression curves of the reactions were typical for pseudo first order reactions (Fig. 3), and there was a linear dependence between enzyme concentration and the transglycosylating activity (Fig. 4).

Substrate specificity

The results obtained using the fluorescent method essentially confirmed the previous findings that all Gas proteins are specific to β -(1,3)-linked glucan and β -(1,3)-oligoglucosides [14, 16, 19]. The acceptor effectivity of SR-laminarioligosaccharides increased with increasing oligoglucoside chain length, starting with DP 5. This result corroborates well the finding of Hartland et al. [14] that laminaripentaose was the smallest possible acceptor when using the purified β -(1,3)-glucanosyltransferase from

A. fumigatus. Interestingly, the recently published 3D-structure of Gas2p revealed binding of laminaripentaose in the active site both in the donor and acceptor position [26]. With regard to the donor specificity, our results are in agreement with previous data indicating that β -(1,3)-glucan is the preferred substrate for Gel proteins of *A. fumigatus* [14] and further extend this conclusion to the complete Gas protein family of *Saccharomyces cerevisiae*.

pH-optima and their biological significance

Our previous studies on the biological role of Gas proteins revealed that a Gas1/Gas5 protein pair is required for proper cell wall formation during vegetative growth, whereas Gas2 and Gas4 proteins are essential for spore wall formation [27]. In accordance with this, *GAS1-GAS5* and *GAS2-GAS4* pairs have different expression patterns. *GAS1-GAS5* is expressed during vegetative growth and repressed during sporulation, whereas the *GAS2-GAS4* pair exhibits the reverse pattern. As the Gas proteins are anchored through GPI in the outer layer of the yeast plasma membrane or spore membrane, they are exposed to changes of external pH. This probably is the reason why Gas1p and Gas5p, which are expressed in vegetative cells, are most active between pH 3.5 and 5. This is consistent with the fact that the vegetative growth-specific isoforms face the culture medium that is usually acidic due to the proton efflux occurring during yeast growth. The external pH drops from about 5.5 to almost 3 in late exponential phase. Interestingly, the defective phenotype of cells lacking *GAS1* gets worse in media buffered at pH 6.5 and pH 6.7 is a semi-lethal condition for growth of the mutant [28]. On the basis of the results described in this work, we can hypothesize that the auxiliary function of Gas5p is less effective at pH 6.5 since its activity decreases to less than 10% and therefore cell wall defects could be aggravated due to the lack of sufficient β -(1,3)-glucanoyltransferase activity. Thus, during vegetative growth the more extended optimum pH range of Gas1p compared to Gas5p could guarantee the presence of BGT activity over a wide range of extracellular pH whereas the auxiliary function of Gas5p could be effective only in a narrow range of pH's which is more typical of the initial phases of vegetative growth in unbuffered media. On the other hand, the sporulation-specific Gas2 and Gas4 proteins, which are synthesized in a narrow temporal window coincidentally with the assembly of the spore wall, face the cytoplasmic pH of the diploid cell inside which the spores develop [29]. The optima between pH 5 and 6 suggest that Gas2 and Gas4 proteins work better at pH values closer to the intracellular pH of the yeast cytoplasm, which is between 6.5 and 6.9 depending on the strain [30]. It should be also taken into account that alkalization of the medium by accumulation of bicarbonate is essential for sporulation (together with respiratory competency and high cell density) and may induce an increase of intracellular pH [31].

Although factors other than pH of the epiplasm and spore wall, such as different types of β -(1,3)-glucan chains or the presence of small molecules, may explain the requirement for a specialized pair of enzymes during sporulation, pH appears to be an important parameter that affects the activity of Gas2-Gas4 proteins. In previous studies *GAS2* and *GAS4* genes were ectopically expressed in vegetative *gas1* Δ cells by fusion to the *GAS1* promoter. *GAS4* fully complemented the defective phenotype of *gas1* Δ mutant in vegetative growth even in media buffered at pH 5.5, whereas *GAS2* could partially suppress the defects of the mutant only when ambient pH was 6.5 [32]. These findings are consistent with the pH optima for the isolated enzymes reported in this work. The character of dependence of activity on the ambient pH may help explain the presence of a specialized pair of Gas enzymes during sporulation. It should be noted that in the human pathogen *C. albicans* the *GAS1*-homologous genes, *PHR1* and *PHR2*, are differently regulated by ambient pH being *PHR1* expressed at pH values of 5.5 and higher and *PHR2* at pH values of 5.5 or lower [33, 34]. *C. albicans* colonizes

niches of the human body at very different pHs, slightly alkaline in the bloodstream (pH7.3) close to neutrality in organs like the kidney, liver and duodenum or acidic as in the stomach (pH ~ 2) or vagina (pH 4.5), Thus, we speculate that the existence of these two genes may meet the requirement to express at the cell surface glucanoyltransferase activities that work optimally in a wide range of ambient pH's. As pH affects the ionization of the aminoacid residues in the active site, the molecular models built on the basis of the recently resolved 3D-structure of Gas2p may provide further information [26].

Several studies indicate that the GH72 family of fungal enzymes plays a crucial role in fungal morphogenesis [35, 36]. The lack of these enzymes reduces β -(1,3)-glucan incorporation into the expanding cell wall and induces cell wall changes aimed to counteract the damage and prevent lysis [37]. The evoked changes cause defects in morphology and impairment in developmental processes such as sporulation or conidiogenesis [27, 35]. The fundamental role of GH72 in fungal biology is further testified by the recent finding that *GEL4* is an essential gene for *A. fumigatus* [36], a major human fungal pathogen. In addition, *GAS4⁺* and the *GAS2/GAS4* pair are essential for spore viability in *Schizosaccharomyces pombe* and *S. cerevisiae*, respectively [27, 32]. Furthermore, fungal pathogens lacking GH72 enzymes exhibit reduced virulence. An example is provided by the *PHR1* null mutant of *C. albicans* that is avirulent in a mouse model of systemic infection but uncompromised in its ability to cause vaginal infections in rats whereas the virulence phenotype of *Phr2* null mutant is the inverse, consistently with the pH-dependent expression of *PHR1* and *PHR2* genes [38]. Moreover, *PHR1* null mutant manifests defects in adhesion to and invasion of reconstituted human epithelia, two processes required for the establishment of infections [38, 39]. Despite the existence of a high degree of redundancy, it is envisaged that the active site of most GH72 members could have sufficient similarity to be targeted by inhibitors of the same type.

The armamentarium of drugs to fight fungal infections is still limited to a few compounds. The new fluorescent assay for β -(1,3)-glucanoyltransferases could provide a simple tool for the detection, identification and characterization of GH72 family enzymes in diverse fungal species as well as for screening and identification of potential inhibitors of BGT activity, which may lead to the development of new antifungal drugs and to new therapeutic strategies.

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Legends to the Figures

Figure 1 Structure of sulforhodamine-labeled laminaritriose (L3-SR)

Sulforhodamine-substituted laminaritriose is shown as a representative of the fluorescently labeled oligosaccharides used in this work.

Figure 2 Transfer of the fluorescent label from L6-SR into high-M_r fractions adherent to filter paper and insoluble in 66% ethanol

Standard reaction mixtures (see Experimental section) were incubated with individual Gas proteins under the respective optimal conditions. After indicated time intervals, 5 μ l aliquots were taken from the reaction mixtures, applied onto the chromatographic paper Whatman 3MM and dried. The paper was washed overnight in 66% ethanol and photographed under UV light. In the case of reactions catalyzed by Gas4p, the incubation times were four times longer than indicated.

Figure 3 Progress-curves of reactions catalyzed by individual Gas proteins

Standard reaction mixtures were incubated under conditions optimal for the respective Gas protein. The transglycosylation was monitored by the fluorescent method as described in the Experimental section, F. u., arbitrary fluorescence units. The error bars denote standard deviation calculated from quintuplicate dot blots.

Figure 4 Dependence of β -(1,3)-glucanosyltransferase activity on enzyme concentration

The standard incubation mixtures were incubated at 37 °C for 30 min except the reaction with Gas4p where the incubation was extended to 2 h. Conditions were as in Fig. 3, but the amounts of enzyme proteins in the reaction mixtures varied as indicated. The significance of the error bars and units is the same as in Fig. 3.

Figure 5 Dependence of β -(1,3)-glucanosyltransferase activity on pH

The standard incubation mixtures were buffered with 0.05 M McIlvaine citrate-phosphate buffers and incubated at their respective optimal temperatures for 30 to 180 min. The significance of the error bars and units is the same as in Fig. 3.

Figure 6 Effect of oligosaccharide chain length on acceptor effectivity

The reaction mixtures contained equimolar (40 μ M) concentrations of the respective SR-laminarioligosaccharides containing 1 to 7 glucosyl units, and the reactions were carried out with the individual Gas proteins under the respective optimal conditions for 1 h (5 h in the case of the reaction with Gas4p). The significance of units is the same as in Fig. 3.

Table 1 Distribution of β -1,3-glucanosyltransferase activity in subcellular fractions

The incubation mixtures contained 0.05 M citrate buffer, pH 5; 10 mg ml⁻¹ laminarin; 20 μ M L7-SR; 0.05-0.11 mg protein in a total volume of 60 μ l. The incubation was carried out at 37 °C for 3 h. The data are from a representative experiment.

Fraction	Specific activity (pkat mg protein ⁻¹)
Cell walls	0.21
Plasma membrane	0.46
Microsomes	0.22
Cytoplasm	0.04

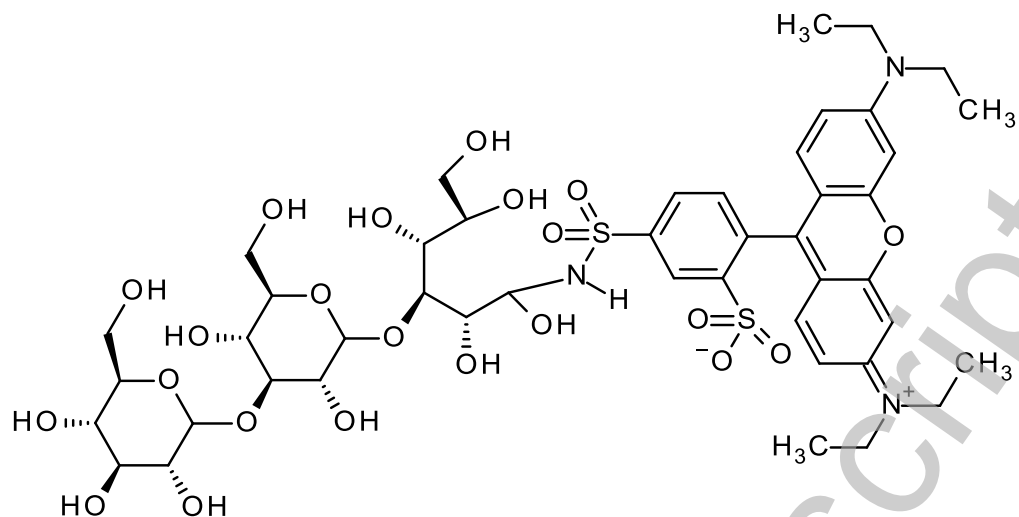


Figure 1

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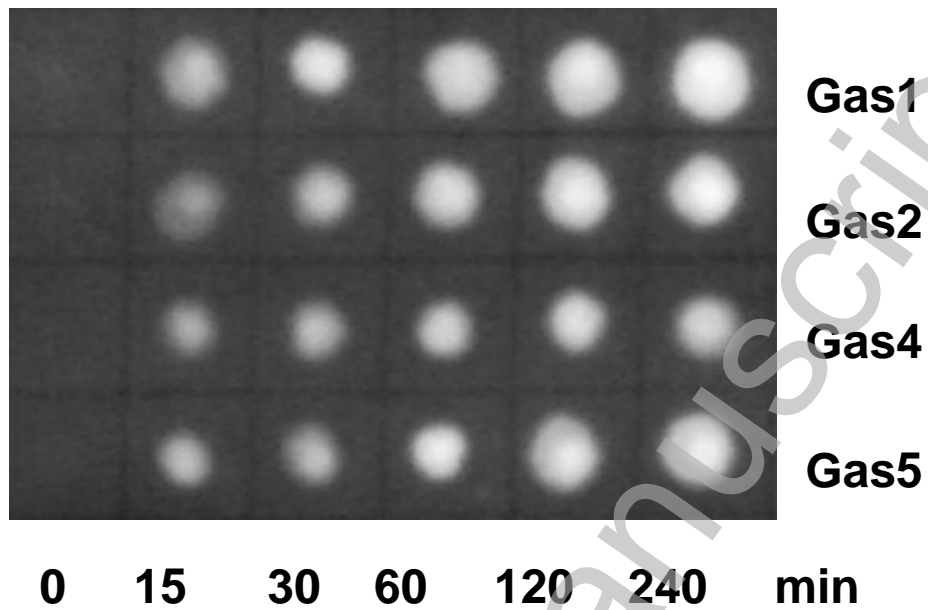


Figure 2

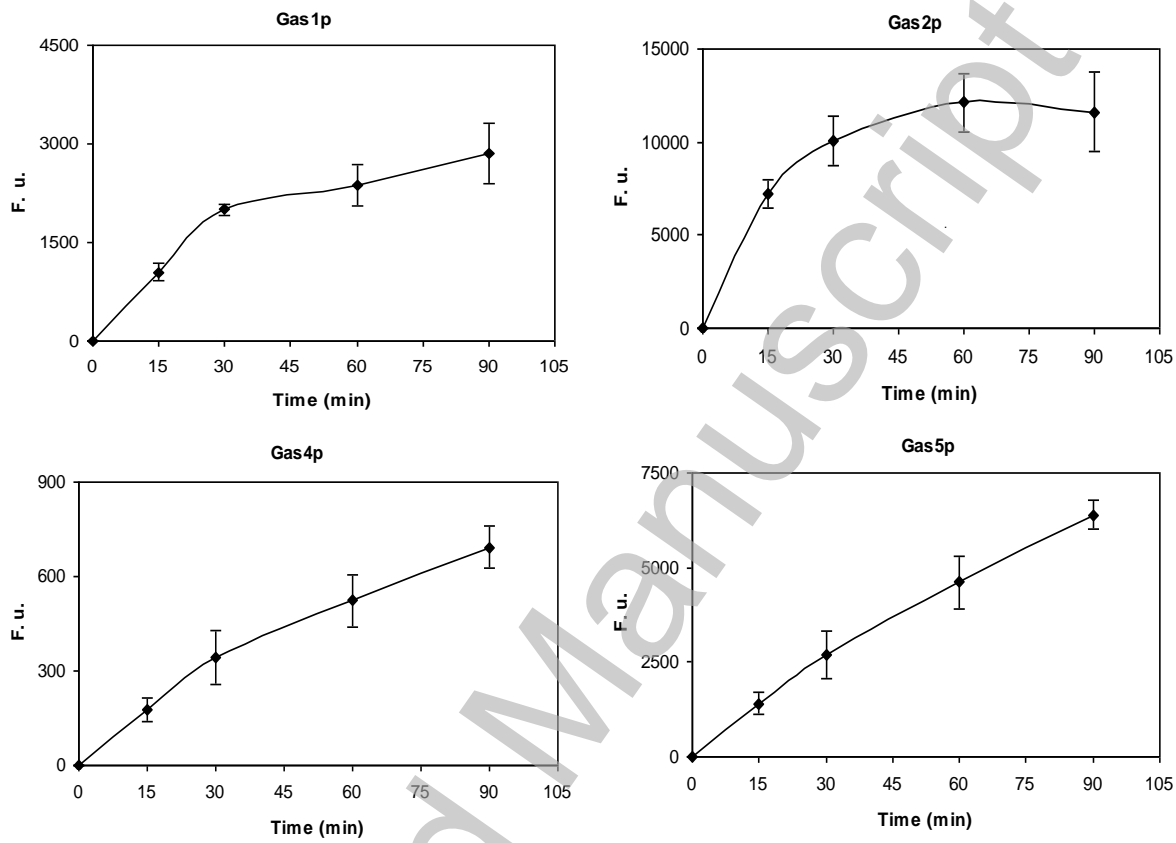


Figure 3

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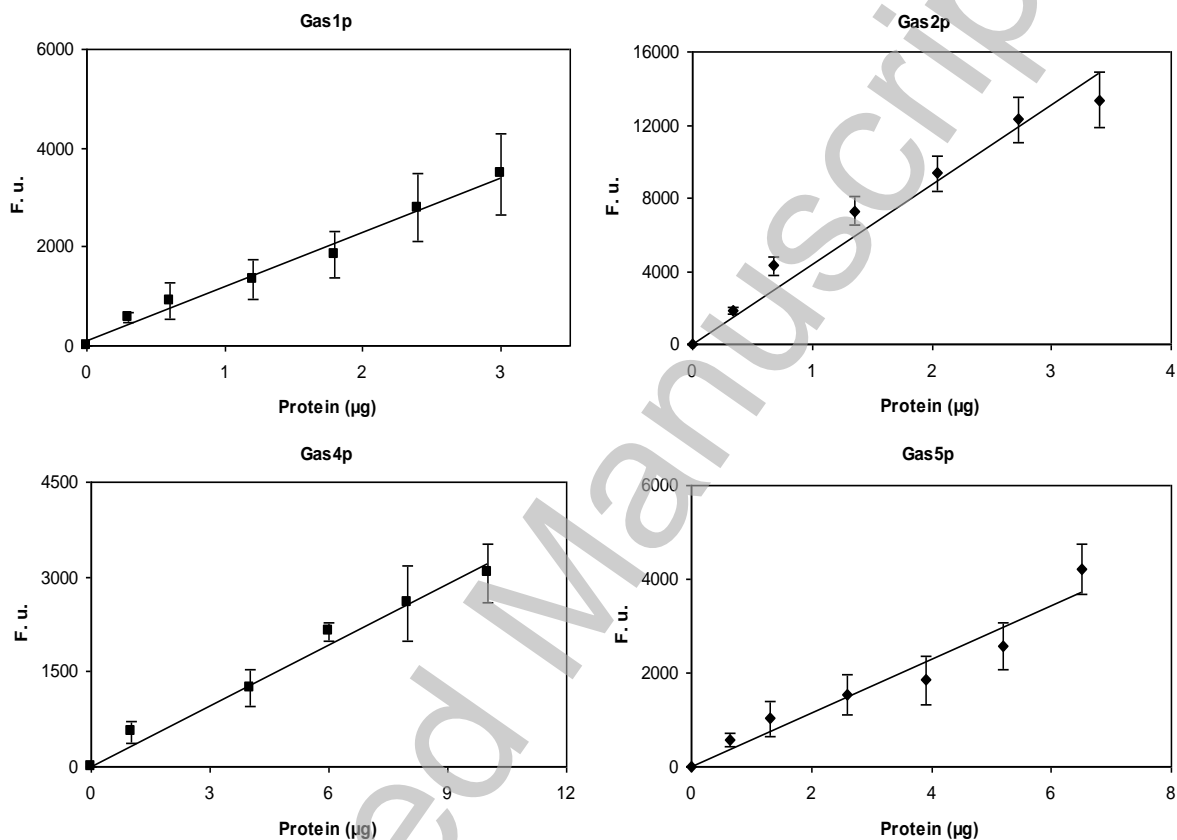


Figure 4

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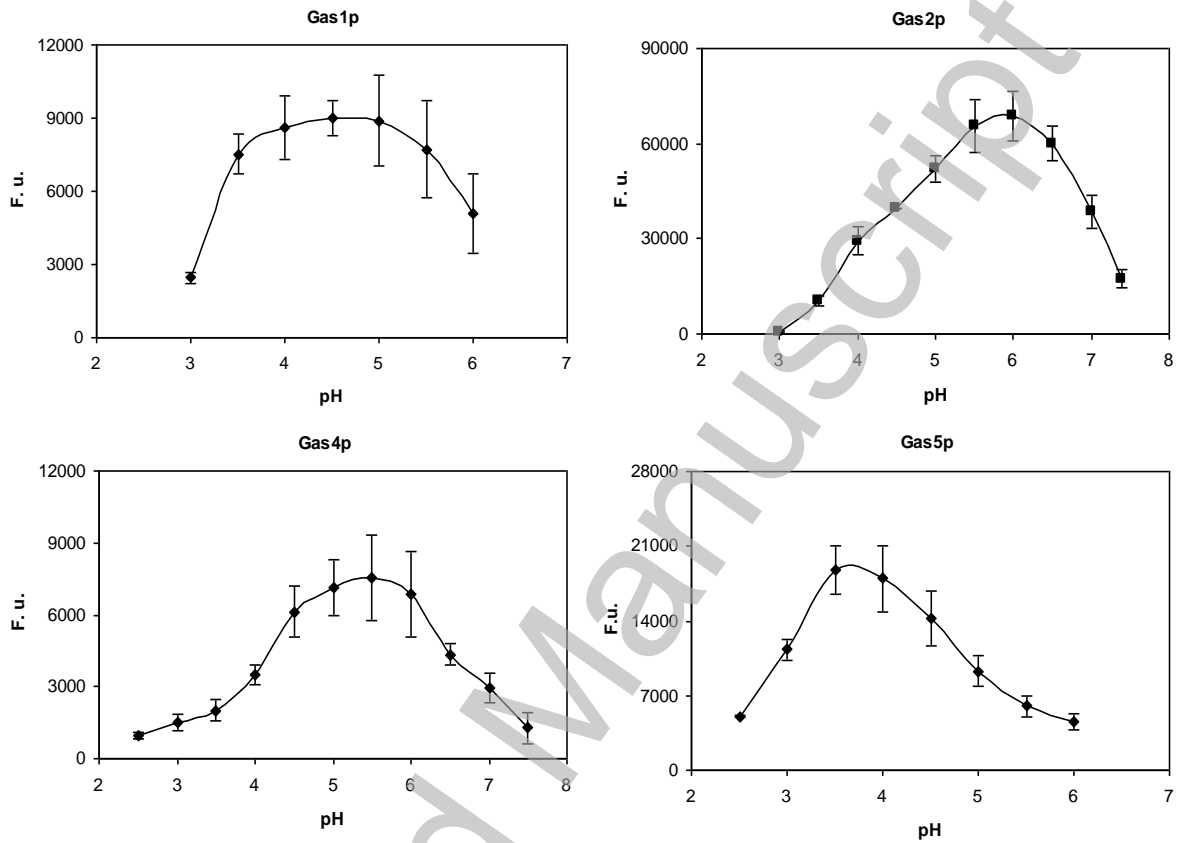


Figure 5

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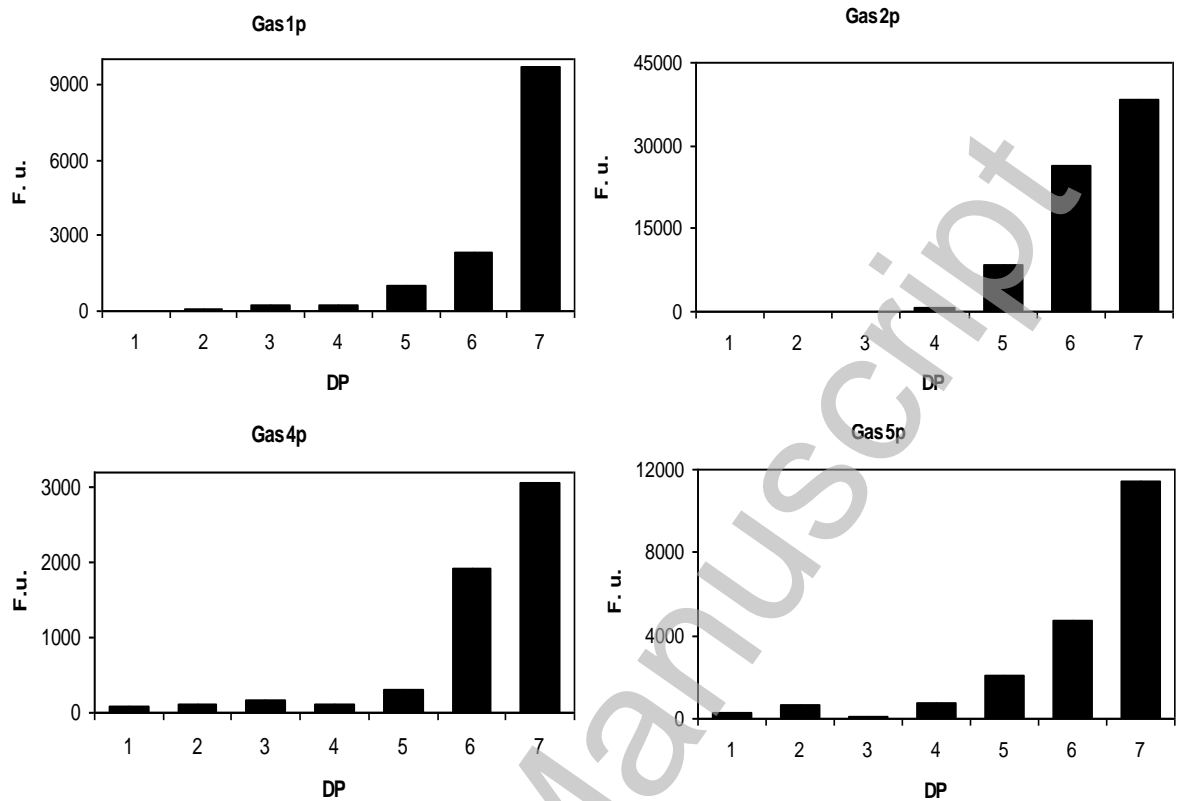


Figure 6