A novel fluorescence assay and catalytic properties of Crh1 and Crh2 yeast cell wall transglycosylases

Marian MAZÁŇ1, Noelia BLANCO†1, Kristina KOVÁČOVÁ*, Zuzana FIRÁKOVÁ*, Pavel ŘEHULKA†, Vladimír FARKAŠ*2 and Javier ARROYO†2

1Institute of Chemistry, Center for Glycomics, Department of Glycobiology, Slovak Academy of Sciences, 84538 Bratislava, Slovakia, †Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense de Madrid, IRYCIS, 28040 Madrid, Spain, and §Institute of Molecular Pathology, Faculty of Military Health Sciences, University of Defence, Táborská 1575, CZ-500 01 Hradec Králové, Czech Republic

The mechanical properties of fungal cell walls are largely determined by composition and mutual cross-linking of their macromolecular components. Previous work showed that the Crh proteins are required for the formation of cross-links between chitin and glucan at the Saccharomyces cerevisiae cell wall. In the present study, the proteins encoded by CRH1 and CRH2 were heterologously expressed in Pichia pastoris and a sensitive fluorescence in vitro soluble assay was devised for determination of their transglycosylating activities. Both proteins act as chitin transglycosylases; they use soluble chitin derivatives, such as carboxymethyl chitin, glycol-chitin and/or N-acetyl chito-oligosaccharides of DP (degree of polymerization) ≥ 5 as the oligosaccharyl donors, and oligosaccharides derived from chitin, β-(1,3)-glucan (laminarin) and β-(1,6)-glucan (pustulan), fluorescently labelled with sulforhodamine or FITC as acceptors. The minimal number of intact hexopyranose units required by Crh1 and/or Crh2 in the molecule of the acceptor oligosaccharide was two and the effectivity of the acceptor increased with the increasing length of its oligosaccharide chain. Products of the transglycosylation reactions were hybrid molecules composed of the acceptor and portions of carboxymethyl chitin attached to its non-reducing end. Both proteins exhibited a weak chitinolytic activity in different assays whereby the ratio of endo- compared with exo-chitinase activity was approximately 4-fold higher in Crh1 than in Crh2. The pH optimum of both enzymes was 3.5 and the optimum temperature was 37 °C. The results obtained in vitro with different fluorescently labelled oligosaccharides as artificial chitin acceptors corroborated well with those observed in vivo.

Key words: cell wall cross-link, chitin, Crh protein, family 16 of glycose hydrolyses (GH16), glucan, transglycosylation.

INTRODUCTION

Fungal cells are surrounded by the cell wall, an essential structure that determines the cell shape, mediates cellular interactions, protects the cells from adverse effects of the environment and provides protection against internal turgor pressure that would cause bursting of the cell [1]. The yeast cell wall consists of three principal polysaccharides: β-(1,3)-glucan, the major structural component, β-(1,6)-glucan and chitin. Although chitin is a minor component representing 1–2 % of the cell wall dry weight, this polysaccharide is essential for cell survival. Additionally, mannoproteins (approximately 25–50 %) are present as an external layer of the cell wall [2,3]. Individual polymer components of the cell wall are mutually linked by covalent bonds, thus creating large macromolecular complexes. The backbone of these complexes is formed by β-(1,3)-glucan chains, branched at random by short chains of β-(1,6)-glucan extended by chitin linked by β-(1,4) glycosidic bonds at their non-reducing ends. In addition, GPI (glycosylphosphatidylinositol)-anchored mannoproteins are linked to the complex via β-(1,6)-glucan side chains [3–5].

Despite its apparent rigidity, the cell wall structure needs to accommodate continuously to morphological changes during cell growth. Therefore a balance between the biosynthesis of the wall constituents and their breakdown and reorganization is essential for both cell integrity and proper morphogenesis [6,7]. The biosynthesis of the respective cell wall components takes place at different locations in the cell. The mannoproteins are synthesized in the endoplasmic reticulum, further glycosylated in the Golgi apparatus and transported by exocytosis to the cell wall, whereas the insoluble cell wall constituents chitin [8], β-(1,3)-glucan [9] and probably β-(1,6)-glucan [10,11] are formed at the plasma membrane and extruded into the cell wall. Moreover, the synthesis of some of these polymers, such as β-(1,3)-glucan and β-(1,6)-glucan, seems to be co-ordinated [12].

The final stage of cell wall construction, the creation of a supramolecular structure through covalent cross-links between the individual components exported by the cells into the extracytoplasmic space, must therefore take place outside the plasma membrane, catalysed by enzymes located in the cell wall. In analogy to the plant system [13], the most probable candidates for this function are the polysaccharide hydrolyses/transglycosylases capable of creating covalent bonds between individual polysaccharide molecules by transglycosylation. The transglycosylation...
mechanism consists of splitting a glycosidic bond in the polysaccharide molecule (called the donor) and linking the formed fragment by its newly created reducing end to a hydroxy group at the non-reducing end of the other polysaccharide or oligosaccharide molecule (called the acceptor) with the formation of a new glycosidic bond. The molecules of the acceptor can be of the same structural type as the donor (in such case, the reaction is called homo-transglycosylation) or they can be of a different type (hetero-transglycosylation). As there are no low-Mᵣ energy carriers such as ATP in the cell wall, the new glycosidic bond must be formed at the expense of the energy released by breaking pre-existing linkages [14]. Classic examples of plant cell wall-located transglycosylases are XET (xyloglucan endotransglycosylase)/XTH (xyloglucan hydrolase) cutting and transferring pre-existing linkages [14]. Other examples are the mixed-linkage β-glucan:xyloglucan endotransglycosylase [18] or the mannan endotransglycosylase [19].

To date, several yeast proteins presumably involved in remodeling the cell wall have been characterized as transglycosylases. To this category belong the proteins of the Gas family in vitro acting as β-(1,3)-glucosanoyl transferases [20–22] and their homologues Pfr1 and Pfr2 from Candida albicans [23]; Bgl2, which removes laminaribiose units from the non-reducing end of β-(1,3)-glucan and transfers them to the non-reducing ends of other β-(1,3)-glucan molecules with formation of β-(1,6)-glucosidic linkages [24]; and the proteins of the Crh family that have been shown to be responsible for linking chitin to β-glucans both in vivo and in vitro [25–27].

The three members of the Crh family, Crh1, Crh2 and Crr1, exhibit significant homologies with bacterial β-1,3/1,4-glucanases and XETs/XTHs from plants [28] and they have been included into the glycohydrolyase family GH16 according to the CAZY database [29]. Crr1 is involved in spore cell wall biogenesis [30], whereas Crh1 and Crh2 function in vegetative growth [28]. By using different methodologies for the analysis of polysaccharide cross-links in the cell wall in wild-type and crh mutant strains, Cabib and co-workers [25,27] demonstrated that all the chitin in a crh1Δ crh2Δ mutant is free, indicating that the formation of linkages between chitin and either β-(1,6)- or β-(1,3)-glucan in vivo depends on Crh1 and Crh2 activities. The linking reaction between chitin and glucan was explored recently in vivo and in isolated cell walls by using SR (sulfonhodamine)-labelled LamoSs (laminariligosaccharides) as acceptor substrates of β-(1,3)-glucan. In vivo, the incorporation of these acceptors was detected in bud scars and at a lower level in the lateral cell wall, being in both cases dependent on the CRH genes [26]. Furthermore, using digitonin-permeabilized cells or isolated cell walls as sources of chitin and enzymes, with SR-labelled β-(1,3)-glucan derived-oligosaccharides as acceptors, it was possible to generate new chitin-glucan links in vivo [26]. With the permeabilized cells, the transfer largely depended on continued chitin synthesis, suggesting that, in order to be attached to the β-glucan core, the chains of chitin have to be in a nascent state, i.e. still not mutually associated by hydrogen bonds before they can be tethered to β-glucans by the Crh enzymes.

In the present study, we have devised a novel fluorescence assay for determination of Crh1 and Crh2 transglycosylating activities in vitro. The assay uses soluble CM (sodium-carboxymethyl)-chitin as the glycosyl donor and SR-labelled laminari- or N-acetylated-Chitosan (chito-oligosaccharides) as acceptors. With the new assay, we have investigated the catalytic properties of recombinant Crh1 and Crh2 proteins. A perfect correlation of the results with those from the in vivo experiments was found.

### MATERIALS AND METHODS

#### Strains and growth conditions

The Saccharomyces cerevisiae strains used in the present study were: BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), GRA005 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 crh1::hphMX4), GRA006 (MATa leu2Δ0 ura3Δ0 crh2::His3) and GRA007 (MATa leu2Δ0 ura3Δ0 crh2::His3 crh1::hphMX4) [25]. Cells were grown at 24°C or 30°C in SC (synthetic complete) medium (0.67 % yeast nitrogen base, 2 % glucose plus synthetic complete mixture).

#### Plasmids

The construction of plasmids pNBC13 (YPE352-CRH2-HA) and pNB10 (YPE352-CRH1-HA) has been described previously [25]. The construction of the plasmid pHIL-CRH1MH, used for the production of the recombinant tagged Crh1 protein, has been also described previously [31]. In order to create a fusion of the CRH2 gene, lacking the putative GPI-anchoring signal, plus a His₆ tag in the C-terminal, we designed a PCR strategy using plasmid pJV40E [28] as a template. CRH2-His was amplified using the primers 5’-ACCGCTCGA-GCTACATTTTTGCAATGCAACTCAAG-3’ and 5’-ACCGGGA-TCCTCAATGTGATGATGTGTTGTCGAGCGCTGACT-GCTGAGGCGCTTCTC-3’. These primers allow amplification of a DNA fragment of 1248 bp encoding residues from Ala²⁴ to Ser⁴⁹ of Crh2. In addition to the CRH2-specific sequences, the 5’ oligonucleotide includes a Xhol restriction site, whereas the 3’ oligonucleotide includes a BamHI restriction site, a stop codon and an in-frame His₆ tag, respectively. The amplified DNA fragment was digested with Xhol and BamHI and then cloned into the Pichia pastoris expression vector pHIL-S1 (Invitrogen) previously cleaved with the same enzymes. This plasmid (pHIL-CRH2-His) includes the PHO1 secretion signal present in the vector, fused in-frame with the CRH2 sequence from nucleotide 69 to the ATG to nucleotide 1317. The correct sequence of this construction was verified by DNA sequencing.

#### Production of recombinant Crh1 and Crh2 proteins

To express the recombinant proteins Crh1 and Crh2, the P. pastoris GS115 strain (Invitrogen) was transformed with the plasmids pHIL-CRH1-MHs and pHIL-CRH2-His, previously digested with BglII. Transformants were selected for the Mut⁺ phenotype, following the manufacturer’s instructions. Production of recombinant proteins was carried out in methanol-containing medium according to the manufacturer’s instructions. Briefly, cells were cultured in BMGY (buffered glycerol-complex) medium at 30°C for 2 days, then transferred to BMMY (buffered methanol-complexed) medium at 20°C for 5 days, adding 1.25 % methanol every 24 h to induce the expression of the protein. Supernatants were recovered by centrifugation at 2300 g for 1 h and filtered through a 0.22 μm filter, followed by concentration to the desired volume using a Pellicon XL filter (Millipore). The proteins were purified by affinity chromatography using Ni-NTA (Ni²⁺-nitriotriacete) agarose columns (Qiagen). Before binding, the column was equilibrated with Buffer B containing 20 mM NaHPO₄ and 500 mM NaCl, then the sample was allowed to soak into the column and incubated at 4°C for 1 h. After the column was washed with 20 mM phosphate buffer, pH 6, containing 0.5 M NaCl, proteins were eluted with 50 mM histidine solution. Finally, the eluted fraction was dialysed against 2.5 mM histidine and stored at −80°C. To determine the purity of the recombinant proteins and the efficiency of the production, recombinant
proteins were monitored by SDS/PAGE (Supplementary Figure S1 at http://www.biochemj.org/bj/455/bj4550307add.htm) and quantified by Bradford analysis.

Substrates

Carboxymethyl chitin \([O-(6-carboxymethyl)]\) chitin, CM-chitin\], degree of substitution 0.16–0.20, was prepared from crab shell chitin (Sigma) using the procedure developed by Trujillo [32]. Gly-chitin \([O-(2-hydroxyethyl)]\) chitin, glycol-chitin\] was prepared by acetylation of commercial glycol-chitosan (Sigma). Briefly, 100 mg of glycol-chitosan was dissolved in 12 ml of 10\% \((v/v)\) acetic acid and 1.2 ml of acetic anhydride was added stepwise under continuous stirring. After 4 h stirring at room temperature (22\°C), the mixture was dialysed against several changes of distilled water at 4\°C for 2 days and freeze-dried. Oligosaccharides derived from chitin, chitosan, \(\beta-(1,3)/\beta-(1,6)-\) glucan (laminarin), \(\beta-(1,6)-\) glucan (pustulan) and from mixed-linkage \(\beta-(1,3)/\beta-(1,4)-\) glucan were in part prepared by limited hydrolysis (0.4–1 M trichloroacetic acid, 0.5–2 h, 100\°C) of parent polysaccharides or they were from commercial sources (Megazyme). The individual oligosaccharides were isolated from the respective hydrolysates by fractionation on Biogel P-6 column \((\text{Megazyme})\). The individual oligosaccharides were identified by MALDI–TOF–MS. Tamarind seed xyloglucan was a gift from Dr Mayumi Shirakawa (DSP Gokyo Food and Chemical Co. Ltd., Osaka, Japan). The yeast \(\alpha\)-mannan was prepared from exponential-phase cells of \(S.\ cerevisiae\) as described previously [33]. Fluorescent labelling of the oligosaccharides was accomplished by reacting the respective oligosaccharide glycamines with Lissamine rhodamine sulfonyl chloride (Acros Organics) or with FITC (Sigma) as described previously [34]. Glucanase-free chitinase from \(Serratia marcescens\) was a gift from Dr Enrico Cabib (Genetics of Simple Eukaryotes Section, National Institute of Diabetes and Digestive and Kidney Disease, National Institutes of Health, Bethesda, MD, U.S.A.).

Crh1 and Crh2 assays

The standard incubation mixture contained 0.1–0.2 \% CM-chitin, 20–40 \(\mu\)M SR-labelled oligosaccharides, 0.15–2.5 \(\mu\)g of the respective recombinant Crh protein and 50 mM citrate buffer, pH 3.5, in a total volume of 20 \(\mu\)l. The incubation was carried out at 37\°C for 10–60 min. The reaction was stopped by addition of 20 \(\mu\)l of 40 \%(v/v) formic acid. Aliquots (5 \(\mu\)l) from the stopped mixture were spotted in quintuplicates on to a filter paper (Whatman 3 mm) template, in size and shape corresponding to a standard 96-well microtitration plate. After drying, the paper was washed for 8–16 h with three to four changes of 66 \%(v/v) ethanol containing 5 \%(v/v) formic acid. The washing removed unreacted label, whereas CM-chitin and the high-\(M_t\) products of its reaction with the SR-labelled acceptors remained attached to the paper. The paper was dried, placed between two glass plates and the fluorescence was measured in a Synergy HT-1 (Biotek) ELISA microplate reader equipped with a fluorescent detector and filters with excitation wavelength at 530 ± 25 nm and emission wavelength at 575 ± 15 nm.

Product identification

Scaled-up incubation mixture (2 ml) contained 10 mg of CM-chitin, 50 \(\mu\)M L5 \((\text{laminaripentaose})\)–SR, 25 mM citrate buffer, pH 3.5, 2.5 \(\mu\)g of Crh1 protein and 0.02 \% \(\text{NaN}_3\). The incubation was carried out at 37\°C for 16 h. A total of 2 vol. of ice-cold ethanol were added to precipitate the polysaccharide, and the mixture was centrifuged. The sediment was washed several times with 66 \%(v/v) ice-cold ethanol to separate the unreacted L5–SR until the supernatant was clear. The sediment containing CM-chitin–L5–SR hybrid product was dissolved in 1 ml of 0.1 M citrate buffer, pH 6, and 10 \(\mu\)g of glucanase-free chitinase from \(S.\ marcescens\) was added and incubated at 37\°C for 1 h. Dilute solutions of the incubation mixture were then applied on to a preparative TLC plate with a 0.5 mm thick Silicagel 60 layer (Merck) and ascending chromatography was run twice with intermittent drying using the solvent system \(n\)-butanol/ethanol/water \((5:3:2, \text{by vol.})\). The major fluorescent zones \((a)\)–\((d)\) corresponding to L5–SR and hybrid products were located under UV light (Figure 4A), scraped from the TLC plate and eluted twice with 100 \(\mu\)l of 20 \%(v/v) ethanol. The collected eluate was centrifuged at 20000 \(g\) for 10 min to remove all silica particles and concentrated to a small volume by vacuum evaporation. The eluates were analysed by MALDI–TOF–MS using the UltraflexXtreme MALDI–TOF/TOF \((\text{tandem TOF})\) system (Bruker Daltonics). \(\alpha\)-Cyano-4-hydroxycinnamic acid \((5 \text{mg/ml in } 60\% \text{ acetonitrile in } 1 \text{mM citric acid})\) was used as MALDI matrix. An aliquot \((1 \mu l)\) of the extracted supernatant was mixed with 1 \(\mu l\) of matrix solution directly on the MALDI target plate. Measurements were done in the positive reflectron mode and a mixture of six calibration peptides was used for external calibration of the instrument.

Chitinolytic activity

For estimation of chitinolytic activity of Crh proteins both viscosimetric and colorimetric assays were used. According to Staudinger’s generalized viscosity law, the viscosity of dilute long-chain polymer molecules is proportionally related to their molecular mass [35]. The viscosimetric method is very sensitive and especially suited for measurements of linear polysaccharide degradation by random-acting endohydrolases. For endochitinases, the activity can be estimated by reduction in viscosity of solutions of CM-chitin [36]. The measurements were performed in Cannon-Manning semi-micro viscosimeters in 1 ml reaction mixtures containing 0.25 \% CM-chitin in 50 mM citrate buffer, pH 3.5, at 37\°C. After equilibrating the temperature and stabilizing the initial efflux time of the mixture, the reaction was initiated by addition of 10 \(\mu\)l of enzyme solution containing 3–32 \(\mu\)g of protein and efflux times were recorded in 10 min intervals. The relative viscosity was calculated according to formula \(\eta/V = (t_c/t_0)\times 100\%\), where \(t_0\) is the efflux time of the reaction mixture at the beginning of the reaction and \(t_c\) is the efflux time after \(x\) min of the assay. Controls were run without enzyme. In the colorimetric assays of chitinase activity we used the soluble chitin polymer CM-chitin–RBV (CM-chitin–Remazol Brilliant Violet) (Loewe Biochemica) as the substrate. A total of 100 \(\mu\)l of a 2 mg/ml CM-chitin–RBV suspension was incubated with 200 \(\mu\)l of 0.1 M citrate buffer, pH 3.6, and 20 \(\mu\)g of protein at 37\°C. After 1 h incubation, the reaction was stopped by the addition of 100 \(\mu\)l of 0.5 M HCl, pH 2.2, followed by cooling on ice for at least 10 min. After cooling, the samples were centrifuged for 1 h at 21000 \(g\) at 4\°C to precipitate non-degraded substrate. The supernatants were transferred to a 96-well plate and absorbance at 550 nm was measured. A control sample without protein was used to determine the background fluorescence, which was subtracted from the experimental values of each reaction sample. Specific activities were expressed as \(\Delta A_{550}/\text{min per nmol of protein}\).
Exochitinase and endochitinase activities were quantified using 4-MU (4-methylumbelliferyl) ChitOS derivatives. 4-MU-N-acetyl-β-D-glucosamine, 4-MU-N-acetyl-β-D-N,N′,N″-diacetylchitobiose and 4-MU-N-acetyl-β-D-N,N′,N″-triacetylchitriose (all from Sigma) were used as substrates to detect N-acetyl-β-glucosaminidase, chitobiosidase and endochitinase activities respectively [37]. Reaction mixtures containing 1 μl of 20 mg/ml of the respective 4-MU derivative and 4 μg of protein in 0.1 M citrate buffer, pH 3.6, in a final volume of 100 μl were incubated for 1 h at 37°C. The reactions were stopped with 200 μl of 400 mM sodium carbonate. Samples were centrifuged at maximum speed for 15 min. The concentration of the released 4-methylumbelliferone in the supernatant was measured in a fluorescence reader (Bio-Tek FL600) using an excitation wavelength of 365 nm and an emission wavelength of 460 nm. Net values of each reaction were calculated by subtracting the fluorescence obtained in a parallel reaction using the corresponding heat-inactivated protein. A standard curve for free 4-methylumbelliferone was used to determine the amount of the products formed. Enzyme activity was expressed as nmol of 4-methylumbelliferone released/h per μg of protein.

**Incorporation of fluorescent oligosaccharides in living yeast cells**

To incorporate fluorescent oligosaccharides, a procedure described previously [26] was followed, although with some modifications to adjust conditions to smaller volumes. The labelling was carried out in a final volume of 200 μl of culture in a vial adjusting the number of cells to have the culture in early stationary phase after overnight incubation at 30°C. Either 5 μl of a 300 μM SR–oligosaccharide mixture [DP (degree of polymerization) 2–7] or 5 μl of a 300 μM individual SR–oligosaccharide (DP4) were added to the cell culture for a final concentration of 7.5 μM. In some *in vivo* experiments, oligosaccharides labelled with FITC instead of SR were used. The culture was then incubated at 30°C in the dark. After overnight growth, the cultures were centrifuged for 5 min at 1500 g. Cells were then washed twice with 400 μl of 20% (v/v) ethanol and twice with 400 μl of water. Finally, the cells were resuspended in 25–40 μl of water for analysis by fluorescence microscopy, using a Nikon TE2000 fluorescence inverted microscope equipped with a CCD (charge-coupled-device). Digital images were acquired with an Orca C4742-95-12ER camera (Hamamatsu Photonics) and processed with the Hamamatsu HCImage Imaging systems software.

**RESULTS**

**In vivo cell-wall labelling with fluorescent oligosaccharides**

In a previous study, we analysed the cross-linking reaction between chitin and β-(1,3)-glucan *in vivo* by using SR-linked oligosaccharides derived from laminarin [β-(1,3)-glucan] as artificial chitin acceptors [26]. The labelled SR–LamOSs were incorporated into bud scars, and at lower levels also into the lateral walls, suggesting that they were functioning as acceptors in place of β-glucan [26]. In agreement with our previous observations [26], the fluorescent β-(1,3)-linked glucose oligosaccharides were mainly detected as bright patches corresponding to bud scars (Figure 1A). The growth of cells in the presence of SR–β-(1,6)-linked glucose oligosaccharides resulted in a similar fluorescence localization pattern, suggesting that they were also functioning as acceptors of chitin (Figure 1A). The finding that SR–β-(1,6)-linked pustulo-oligosaccharides (SR–PustOS) did incorporate into the living cells is in apparent contrast with our previous observation [26] where they were inactive. The explanation of this apparent discrepancy is that the mixture of SR–PustOS used in the present study contained a higher proportion of long-chain oligosaccharides (DP⩾5) than the mixture used previously and, as will be shown below, the acceptor efficiency of the labelled oligosaccharides increases with their length.

Since we had previously observed that N-acetyl ChitOSs were acting as effective inhibitors of β-(1,3)-linked oligosaccharide incorporation into the cell walls [26], we explored their ability to serve as acceptors as well. This assumption proved to be correct; SR–ChitOSs were found to incorporate with high efficiency into the growing yeast cells with the same pattern of fluorescence localization as the one found for β-(1,3)-linked and β-(1,6)-linked glucose oligosaccharides (Figure 1A). Quantification of the fluorescence incorporated into the cells by analysis of the corresponding microscope images revealed different efficiencies for each type of the labelled-oligosaccharides: SR–ChitOSs were incorporated with the highest efficiency, then the β-(1,3)-linked glucose oligosaccharides (SR–LamOS) and finally the β-(1,6)-linked oligosaccharides (SR–PustOS) (Figure 1B). These results were further confirmed by using FITC-labelled oligosaccharides. The pattern of fluorescence localization for FITC–LamOS, FITC–PustOS and FITC–ChitOS was identical with one of cells labelled with SR–oligosaccharides (Supplementary Figure S2 at http://www.biochemj.org/bj/455/bj4550307add.htm). Moreover,
Catalytic properties of yeast Crh1 and Crh2 transglycosylases

Figure 2  Incorporation of different oligosaccharides in \textit{crh}Δ mutant strains

WT, \textit{crh}1Δ, \textit{crh}2Δ and \textit{crh}1Δ \textit{crh}2Δ cells were labelled overnight with SR–\(\beta\)-1,3 oligosaccharides (DP2–7) (A); SR–\(\beta\)-1,6 oligosaccharides (DP2–7) (B) and SR–chitotetraose (DP4) (C) and incorporation was followed by fluorescence microscopy. Lower panels show images in the rhodamine fluorescence channel, whereas upper panels show the same cells observed under Nomarski. For each oligosaccharide, the same exposure was used for WT and mutants. Oligosaccharide incorporation in the bud scars was quantified by using HCImage Imaging software and is shown in the histograms. The signals, expressed as arbitrary fluorescence units, correspond to the median \(\pm\) S.D. for three independent experiments. The number of cells measured for each oligosaccharide was \(2 \times 10^3\). Scale bars, 10 \(\mu\)m. F.U., arbitrary fluorescence units.

The quantification of fluorescence signals of cells labelled with the FITC–oligosaccharides by flow cytometry confirmed the different efficiencies for each type of oligosaccharide (Supplementary Figure S2).

To characterize further the involvement of Crh1 and Crh2 in the cross-linking of different acceptors to chitin in the cell wall, similar experiments were carried out with single \textit{crh}1Δ and \textit{crh}2Δ strains as well as with the double \textit{crh}1Δ \textit{crh}2Δ mutant strain. With the three types of oligosaccharides, there was almost no fluorescence incorporation in strains deleted in both \textit{CRH1} and \textit{CRH2} genes (Figure 2), indicating that there is no transglycosylation in the absence of both proteins. Comparison of the amount of fluorescence incorporated into the single mutants with respect to the WT (wild-type) strain revealed that for SR–\(\beta\)-(1,6)-linked oligosaccharides and SR–\(N\)-tetra-acetyl chitotetraose, the cross-linking was mainly dependent on Crh2 and to a lesser extent on the Crh1 activity (Figure 2). However, for LamOS–SR, the fluorescence incorporation showed a small decrease in both \textit{crh}1Δ and \textit{crh}2Δ strains in respect to the WT strain (Figure 2). Apart from the small differences, both mutants incorporated these oligosaccharides with a comparable efficiency, suggesting that the transglycosylating activity depended, in this case, on both enzymes to a similar extent.

Setup of a fluorescence assay for Crh1/Crh2-mediated transglycosylation activity

To enable the study of the catalytic properties of the Crh proteins, we designed an \textit{in vitro} assay of their transglycosylation activity. The natural donor, nascent chitin, was replaced by the soluble CM-chitin and the fluorescent SR-labelled oligosaccharides derived from \(\beta\)-(1,3)-glucan, \(\beta\)-(1,6)-glucan and/or from chitin served as acceptors. The products of Crh1- and Crh2-catalysed
transglycosylation were hybrid polymer molecules composed of the respective SR-labelled oligosaccharide acceptor and a portion of the donor polysaccharide attached to its non-reducing end (see below) and could be separated by adsorption on filter paper. The unused SR-labelled oligosaccharides were removed from the paper by washing in 66% (v/v) ethanol whereby the hybrid polymeric product remained attached to the paper. The application of 5 μl aliquots of the reaction mixture as discrete spots on the filter paper in a pattern corresponding exactly to the 96-well microtitre plate format allowed high-throughput determination of the fluorescence incorporated into the polymer with a fluorescence ELISA plate reader. When required, the absolute quantification of the products was done by employing a calibration curve constructed with known amounts of the respective labelled acceptor oligosaccharide.

Enzymatic properties and substrate specificity of Crh proteins

As potential oligosaccharyl donors, CM-chitin, Gly-chitin, chitosan, CMC (carboxymethyl cellulose), HEC (hydroxyethyl cellulose), β-(1,3)-glucan (laminarin), β-(1,6)-glucan (pustulan), barley mixed-linkage β-(1,3)/β-(1,6)-glucan, TXG (tamarind seed xyloliglucan) and yeast α-mannan were tested in the in vitro assay using L5–SR as the acceptor. From these, only the soluble chitin derivatives CM-chitin, glycol chitin (Figure 3) and N-acetyl ChitOSs of DP≥5 (Figure 7) proved to be able to serve as the oligosaccharide donors with both enzymes. The donor efficiency of 0.25% Gly-chitin was 25–40% of that obtained with the same concentration of CM-chitin (Figure 3). The enzymatic character of the transglycosylation was confirmed by the fact that the rate of Crh1/Crh2-catalysed incorporation of the labelled SR-oligosaccharides into the 66% ethanol-insoluble fraction was directly proportional to the amount of enzyme present in the incubation mixture and that the reaction progressed linearly with time (e.g. Figure 5). Both Crh1 and Crh2 exhibited maximum activity at pH 3.5 and the optimum temperature was 37°C (Supplementary Figure S3 at http://www.biochemj.org/bj/455/bj4550307add.htm). Divalent cations as well as 1 mM EDTA did not significantly influence the reaction. Both Crh1 and Crh2 lost approximately 50% activity upon heating at 95°C for 15 min (Supplementary Figure S4 at http://www.biochemj.org/bj/455/bj4550307add.htm).

The product obtained by Crh1-catalysed reaction with CM-chitin as the donor and L5–SR as the acceptor was a high-M, polysaccharide containing incorporated fluorescent label. MALDI–TOF-MS of the principal fluorescent fragments obtained after treating the product with purified glucanase-free Serratia chitinase and their separation by TLC (Figure 4A) identified the hydrolysis products migrating in zones (a)–(d) as L5–SR with attached portions of CM-chitin of various lengths (Figure 4B and Supplementary Figure S5 at http://www.biochemj.org/bj/455/bj4550307add.htm). Zone (a) on the chromatogram corresponded to liberated L5–SR. The hydrolysis product HP1 migrating on the TLC plate in zone (b) consisted of the acceptor L5–SR with one unit of GlcNac attached to it. Zone (c) contained fragment HP2 consisting of L5–SR with two GlcNac units attached and a minor fraction HP2-CM, where one of the two GlcNac molecules was carboxymethylated. Zone (d) contained a single compound HP3-CM identified as L5–SR with three GlcNac units, one of them carboxymethylated. A detailed characterization of the fluorescent fragments released from the hybrid product with chitinase is shown in Supplementary Table S1 (at http://www.biochemj.org/bj/455/bj4550307add.htm).

Although the donor specificity of both enzymes was restricted to soluble chitin derivatives, both Crh1 and Crh2 exhibited considerable flexibility as concerns the nature of the acceptor. As expected on the basis of our previous findings in vivo demonstrating that both enzymes are responsible for the formation of the cross-links between chitin and β-(1,3)-glucan as well as between chitin and β-(1,6)-glucan [25,27], both enzymes were active with SR-labelled oligosaccharides derived from laminarin and pustulan, albeit with different efficiencies. Moreover, SR-labelled oligosaccharides derived from chitin also served as acceptors in the in vitro reaction, in agreement with their incorporation into the cell wall of living yeast in a Crh1- and Crh2-dependent manner (Figures 1 and 2). From among the labelled tetrasaccharides, the most efficient acceptor was CH4–SR (N-tetra-acetyl chitotetraose–SR), followed by β-(1,3)-linked L3–SR (laminaritetraose–SR) with both enzymes. In addition, Crh2 was active also with β-(1,6)-linked P4 (pustulturitetraose)–SR (Figure 5A). Surprisingly, both enzymes exhibited considerable activity also with SR–cellotetraose, but no activity was observed when SR–maltohexaose or SR–chitohexaose (deacetylated) were used in the place of acceptors.

As previously observed in vivo [25], the individual native N-acetyl-ChitOSs inhibited the transglycosylation between CM-chitin and LamOS–SR also in vitro whereby the extent of the inhibition was proportional to the concentration of the ChitOS. The Dixon graph in Figure 5(B) indicates that inhibition of the transferase reaction with CH3 (N-triacetyl chitotriose) was competitive in relation to the acceptor L5–SR.

Michaelis constants (Km values) obtained with L4–SR and CH4–SR as the respective acceptors (Table 1) show that with both enzymes, the chito-derivative binds to the enzyme more readily than the LamOS. This may be interpreted as that the acetamide group at C-2 facilitates the binding of the substrate to the enzyme, but is not necessary for the reaction to proceed. Interestingly, the SR–oligosaccharides derived from chitosan were inactive as acceptors.

To identify the minimal length of oligosaccharides capable of acting as the acceptors, the relative rates of the transfer reactions were measured using individual LamOS–SR and SR–ChitOS of different lengths. Significant transfer rates were observed with oligosaccharides of DP3 and larger, indicating that the minimal requirement for the acceptor was two intact hexopyranosyl units

**Figure 3 Donor specificity**

The efficiency of selected soluble polysaccharides to serve as oligoglycosyl donors in the reactions catalysed by Crh1 and Crh2 was evaluated. Standard 20 μl reaction mixtures contained 1 mg·ml⁻¹ of the respective polysaccharide, 0.15 μg of Crh1 or 2.5 μg of Crh2 and 30 μM L5–SR as the acceptor. F.U., arbitrary fluorescence units.

TheAuthors Journal compilation © 2013 Biochemical Society

© The Authors Journal compilation © 2013 Biochemical Society
Catalytic properties of yeast Crh1 and Crh2 transglycosylases

Figure 4  Analysis of the hybrid polysaccharide product formed from CM-chitin and L5–SR by the Crh1-catalysed transglycosylation reaction

(A) The transglycosylation product was hydrolysed with purified Serratia chitinase and, after the indicated time interval, the incubation mixture was resolved by TLC. (B) MALDI–TOF-MS analysis of the individual zones from the TLC chromatogram shown in (A). Detected peaks are labelled with their measured m/z values and ion composition.

at the non-reducing end of the SR–oligosaccharide, applicable both for LamOS– and for ChitoOS–SR. In general, the acceptor efficiency of all types of labelled oligosaccharides increased with their increasing chain length throughout the whole range of DPs (2–7) of the respective oligosaccharide series (Figures 6A and 6B). It should be noted that a similar increase in transfer efficiency with oligosaccharide length was observed also in vivo [26].

Besides CM-chitin and Gly-chitin, the soluble fragments of chitin represented by N-acetyl ChitOSs of DP\(\geq 5\) also served as glycosyl donors. The transferase reaction with both CH4–SR and L4–SR as the respective acceptors led to the formation of a series of labelled products of different size (Figures 7A and 7B), indicating stepwise elongation of the acceptor molecule by transfer of sugar units from the oligosaccharide donor to the labelled acceptor. Interestingly, in contrast with the reaction with Crh1, the transfer to L4–SR was rather slow with Crh2. Prolonged incubations yielded high-

M

r fluorescent products remaining on the starting line during TLC (Figure 7).

Chitinase activity of Crh1 and Crh2

The reaction mechanism of transglycosylases includes the formation of a transient covalent glycosyl–enzyme intermediate. The intermediate decomposes by transferring the glycosyl residue to an OH-group of a sugar acceptor or, under conditions when the concentration of suitable acceptor is low, to a molecule of water [38]. To verify this possibility, we used different assays to determine the chitinolytic activity of Crh1 and Crh2. Both enzymes caused a slow reduction in viscosity of CM-chitin solutions in a time-dependent manner. Addition of ChitOS, or to a lesser extent also of LamOS, markedly stimulated the rate of CM-chitin decomposition by Crh1, but had very little effect on the activity of Crh2 (Figure 8).

The chitin-depolymerizing activity of Crh1 and Crh2 was also measured by alternative colorimetric and fluorimetric methods. The soluble chitin polymer CM-chitin–RBV was used as a substrate to detect chitinase activity of these proteins. Both Crh1 and Crh2 exhibited chitinolytic activity against this substrate (Table 2). To distinguish between exochitinase and endochitinase activities, 4-MU-N-acetyl-\(\beta\)-D-glucosamine, 4-MU-N-acetyl-\(\beta\)-D-N,N′-diacetylchitobiose and 4-MU-N-acetyl-\(\beta\)-D-N,N′,N″-triacetylchitotriose were used as substrates to detect N-acetyl-\(\beta\)-glucosaminidase, chitobiosidase and endochitinase activities respectively [37]. The rates of hydrolysis of these compounds by Crh1 and Crh2 are shown in Table 2. No N-acetyl-\(\beta\)-D-glucosaminidase activity was detected for Crh1, whereas Crh2 exhibited a very low, but measurable, activity with the same
Figure 5  Acceptor efficiency of different types of oligosaccharides

(A) Comparison of the rates of transglycosylation catalysed by Crh1 and Crh2 using equimolar concentrations (25 μM) of the respective SR-labelled tetrasaccharide and CM-chitin as a donor. The reactions were carried out under standard conditions. (B) Dixon graph illustrating the inhibitory effect of CH3 on the transglycosylation reaction between CM-chitin and L4–SR catalysed by Crh1. Standard incubation mixtures contained 1 mg·ml⁻¹ CM-chitin, the indicated concentrations of L4–SR and 0.15 μg of Crh1 in a total volume of 20 μl. F.U., arbitrary fluorescence units.

Table 1  Kinetic parameters of Crh1 and Crh2 with L4–SR and CH4–SR as the respective acceptors

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Acceptor</th>
<th>Kₘ (μM)</th>
<th>Vₘₐₓ (pmol·s⁻¹·ml⁻¹)</th>
<th>kₐₜ (s⁻¹) (×10⁻³)</th>
<th>kₐₜ/Kₘ (s⁻¹·µM⁻¹) (×10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crh1</td>
<td>L4–SR</td>
<td>61.0 ± 11.2</td>
<td>1.880 ± 0.25</td>
<td>26.642 ± 4.14</td>
<td>0.426 ± 0.092</td>
</tr>
<tr>
<td></td>
<td>CH4–SR</td>
<td>27.9 ± 3.1</td>
<td>0.366 ± 0.07</td>
<td>5.105 ± 0.96</td>
<td>0.182 ± 0.011</td>
</tr>
<tr>
<td>Crh2</td>
<td>L4–SR</td>
<td>65.1 ± 5.5</td>
<td>0.296 ± 0.16</td>
<td>1.038 ± 0.213</td>
<td>0.016 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>CH4–SR</td>
<td>8.5 ± 1.7</td>
<td>0.161 ± 0.03</td>
<td>0.282 ± 0.05</td>
<td>0.034 ± 0.008</td>
</tr>
</tbody>
</table>

DISCUSSION

Cell wall remodelling is an essential process continuously taking place during fungal morphogenesis and growth. Our previous work demonstrated that both Crh1 and Crh2 are responsible for the attachment of chitin to β-(1,3)-glucan and to β-(1,6)-glucan at the yeast cell wall in vivo [25–27]. In accordance with this, all the chitin in a crh1Δ crh2Δ strain is free (i.e. not covalently bound to glucan) [27]. Moreover, earlier experiments also showed that SR-labelled β-(1,3)-oligosaccharides were abundantly incorporated in live cells at bud scars in a Crh1/Crh2-dependent manner, presumably functioning in vivo as artificial substrates for the transglycosylation reaction [26].

Devising a simple, sensitive and inexpensive assay method for determining the activity of Crh enzymes represents an important step towards elucidating the mechanisms of cell wall formation in fungi. Experiments in vitro described in the present study confirmed some of the earlier in vivo observations [25–27] as concerns the nature of the donor and acceptor molecules. Both proteins use chitin derivatives (CM-chitin, Gly-chitin and higher N-acetyl ChitOSs, but not chitosan) as glycosyl donors. In contrast with the strict donor specificity, the range of acceptors effective in vivo and in vitro included, besides β-(1,3)-linked LamOSs and β-(1,6)-linked gluco-oligosaccharides derived from pustulan, the SR-N-acetyl ChitOSs. Comparison of the relative rates of transglycosylation indicates that, in vitro, N-acetyl ChitOSs are the preferred acceptor substrates for these enzymes followed by β-(1,3)-linked LamOSs, whereas the transfer to β-(1,6)-linked PustOSs, although significant with Crh2, was clearly slower (Figure 5A). Supporting this, the values of Michaelis constants (Kₘ) show that CH4–SR interacts with the enzymes more readily than L4–SR (Table 1). Interestingly, the results of the in vitro assays in respect to the preference of the different acceptors correlate well with the incorporation of the corresponding labelled-oligosaccharides in vivo (Figure 1).

The transglycosylation of CM-chitin to β-(1,6)-linked PustOSs carried out by Crh1 in vitro was rather inefficient. However, the analysis of the chitin cross-linked to β-(1,6)-glucan in a crh1Δ strain and in a crh1Δ crh2Δ mutant expressing Crh1 from a
Catalytic properties of yeast Crh1 and Crh2 transglycosylases

Figure 6 Effect of the sugar chain length of the acceptor SR-oligosaccharide on transglycosylation activity of Crh1 and Crh2

The standard 20 μl reaction mixtures contained 0.25 % CM-chitin as the donor and 66–70 μM SR-LamOSs (A) or 40–43 μM ChitoOS-SR (B) of different DPs as acceptors. F.U., arbitrary fluorescence units.

multi-copy plasmid [25], together with the incorporation of SR-PustOSs into these cells overexpressing Crh1 (N. Blanco, R. Hurtado, A.B. Sanz, J.M. Rodriguez-Peña, C. Nombela, V. Farkas and J. Arroyo, unpublished work) provide evidence that, in vivo, Crh1 is also able to react with β-(1,6)-linked oligoglucosides.

Such a broad substrate specificity of carbohydrate-active enzymes is quite unusual, nevertheless, there are few examples of promiscuous behaviour of glycoside hydrolases. For example, for bacterial levansucrases belonging to the family 68 of glycoside hydrolases [29], a large variety of non-conventional fructosyl acceptors has been defined [39,40]. Moreover, among the glycoside phosphorylases, sucrose phosphorylases also show acceptor promiscuity by transferring a fructose moiety to a wide variety of mono-, di- and tri-saccharides [41]. Additionally, purified endotransglycosylase HvXET5 from barley, belonging to the glycoside hydrolase family GH16, similar to Crh proteins, covalently link the different donor polysaccharides TXG, HEC and (1,3/1,4)-β-D-glucans with a range of acceptors, including oligo-xyloglucosides and cellodextrins, albeit with different efficiencies [42].

The strict specificity for chitin and its derivatives in the donor site and the absence of activity with chitosan or the hexasaccharide derived from it used as the donors suggest that the acetyl group at C-2 in the N-acetyl glucosamine units is critical for the interaction of the donor with the enzyme. On the other hand, this requirement does not seem to be strictly valid for the acceptor site, since not only oligosaccharides derived from chitin but also β-(1,3)-, β-(1,6)- and even β-(1,4)-linked gluco-oligosaccharides (but not the deacetylated oligosaccharides derived from chitosan) could act as the acceptors. The loose specificity of the acceptor site is difficult to explain unless we get more information about the tertiary structure of Crh enzymes.

Figure 7 Native N-acetyl ChitOSs of DP ≥ 5 can serve as glycosyl donors in Crh1- and Crh2-mediated transglycosylation

Reaction mixtures contained 30 μM SR-labelled N-tetra-acetyl chitotetraose CH4–SR (A) or 30 μM SR-labelled L4–SR (B) as the respective acceptors and 1 mg·ml⁻¹ of individual N-acetyl ChitOSs (GlcNAc)n of different lengths (DP2–6) as the respective donors, 0.15 μg of Crh1 or 0.6 μg of Crh2 respectively in 20 μl of 25 mM citrate buffer, pH 3.5. The incubations were carried out at 37°C for 16 h. Aliquots of the reaction mixtures were resolved by TLC on silica gel in the solvent system propan-2-ol/water/25 % ammonia (7:2:1, by vol.) run twice with intermittent drying. Photographed under UV light.

On the basis of their structural characteristics, Crh1 and Crh2 belong in family 16 of glycoside hydrolases of the Carbohydrate Active Enzymes database (CAZy) [29]. All members of this family, including bacterial endo-1,3-1,4-β-D-glucan hydrolases and plant XETs, share a common molecular double-displacement reaction mechanism leading to overall retention of configuration of the anomeric carbon of the sugar ring undergoing catalysis (retaining glycosidase hydrolases) [38]. Its characteristic feature is the participation of two catalytic carboxylic amino acids located at the catalytic site. One of the carboxylic groups functions as a nucleophile attacking the C-1 carbon of a sugar unit of the cleaved polysaccharide chain in the first step, whereas the second carboxylic group provides general acid catalysis. A proton from this carboxylate attacks the glycosidic oxygen, thus facilitating the fission of the glycosidic bond yielding a covalent glycosyl–enzyme intermediate [38,43,44]. In the following step, the second carboxylate residue functions as a general base to activate the incoming nucleophile, an alcohol in the case of transglycosylation, which hydrolyses the glycosyl–enzyme, transferring the sugar residue to another carbohydrate acceptor molecule. Additionally, several retaining glycoside hydrolases hydrolysing β-N-acetyl glucosaminic bonds use a double-displacement mechanism in which the nucleophile is donated.
not by the enzyme, but by the 2-acetamido group of the substrate, forming an oxazolinium ion intermediate through a ‘substrate-assisted catalysis’ mechanism [45]. Our previous observations showed that a double Crh1-D136N/E138Q mutant is unable to complement the Congo Red sensitivity of a crh1Δ crh2Δ strain [28]. Moreover, Crh1 and Crh2 proteins harbouring mutations in the putative glutamyl catalytic nucleophile (Crh1-E134Q and Crh2-E166Q respectively) do not complement the morphogenetic defect of a cla4Δ crh1Δ crh2Δ strain [46], suggesting that these proteins utilize a classical retaining mechanism with an enzyme nucleophile.

The transglycosylase reaction catalysed by Crh1 and Crh2 proteins involves the cleavage of the β-(1,4) linkages of the chitin and subsequent attachment of the fragment of the donor molecule to the molecules of the donor substrate CM-chitin from their non-reducing end, transferring them to a suitable acceptor without the presence of suitable oligoglycoside acceptors [44,47]. The endochitinases cleave chitin chains randomly generating disperse molecular mass GlcNAc multimers, whereas exochitinases hydrolyse chitin from the non-reducing end releasing monomeric (N-acetyl-β-D-glucosaminidases) or dimeric (chitosidases) units of N-acetylgalactosamine. Although both Crh1 and Crh2 degraded the endochitinase substrate MU-Ch3 (4-MU-N-acetyl-β-D-N,N′′-diacetylchitotriosiose) more efficiently than the exochitinase substrate MU-Ch2 (4-MU-N-acetyl-β-D-N,N′′-diacetylchitobioside), the ratios of their activities with these two substrates were different (Table 2). Whereas with Crh1, the ratio of MU-Ch3 activity compared with MU-Ch2 was 15, i.e. the endo-mechanism of transglycosylation clearly prevailed, with Crh2 this ratio was 3.8, indicating that the enzyme possesses a substantial exo-activity. It can be imagined that Crh2 preferentially attacks the molecules of the donor substrate CM-chitin from their non-reducing end, transferring them to a suitable acceptor without resulting in an appreciable reduction in their size and therefore causing only negligible loss of viscosity. The different modes of action of both enzymes on chitin molecules and the dominant endochitinase activity of Crh1 are the probable reason why in vivo the exogenous ChitOSs caused morphological aberrations in the cla4Δ crh2Δ strain, but not in the cla4Δ crh1Δ strain [46].

The observed multiple specificities of Crh1 and Crh2 are tempting to speculate that in vivo they could act both as hetero-transglycosylases attaching nascent chains of chitin to β-(1,3)- and β-(1,6)-glucan as well as homo-transglycosylases mutually joining nascent chains of chitin. The hetero-transglycosylase activity of these proteins cross-linking chitin to β-(1,3)-glucan and to β-(1,6)-glucan, previously demonstrated in vivo [25–27], was corroborated in the present study by the in vitro data. It should be noted that the possibility of Crh1 and Crh2 acting also as homo-transglycosylases in vivo linking together nascent chains of chitin among themselves is deduced from the findings in vitro and from experiments showing incorporation of SR–ChitOSs into cell walls of living cells, but is merely speculative since the formation of chitin-to-chitin linkages in the cell wall is difficult, if not impossible, to prove. Incorporation of SR-oligosaccharides in crh1Δ crh2Δ strains revealed that both Crh1 and Crh2 are required for the in vivo transglycosylation reaction at the motherbud neck and bud scars, in accordance with the localization of both proteins in these sites [25,28]. Moreover, experiments with the single crh1Δ and crh2Δ mutants suggested that Crh2 activity

---

**Table 2** Chitinolytic activities of Crh1 and Crh2

<table>
<thead>
<tr>
<th>Protein</th>
<th>CM-chitin–RBV</th>
<th>4-MU-(GlcNAc)</th>
<th>4-MU-(GlcNAc)₂</th>
<th>4-MU-(GlcNAc)₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crh1</td>
<td>17.76 ± 3.2</td>
<td>0.0002 ± 0.0001</td>
<td>0.0194 ± 0.0009</td>
<td>0.2896 ± 0.0108</td>
</tr>
<tr>
<td>Crh2</td>
<td>38.53 ± 6.9</td>
<td>0.0080 ± 0.0004</td>
<td>0.0224 ± 0.0014</td>
<td>0.0842 ± 0.0055</td>
</tr>
</tbody>
</table>

---

**Figure 8** Stimulatory effect of ChitOSs and LamOSs on the rate of viscosity reduction of CM-chitin catalysed by Crh1

Reaction mixtures (1 ml) contained 50 mM citrate buffer, pH 3.5, 0.25 % CM-chitin, the indicated concentrations of oligosaccharides and 9 μg of Crh1 or 32 μg of Crh2 protein. Control incubation was performed without the oligosaccharides added. The assay was carried out at 37°C. CH4 and L4 were used as the representatives of the respective types of oligosaccharides.
is more important, particularly for transglycosylation of chitin to β-1,6-linked oligosaccharides. These results corroborate well with previous chemical determinations of the chitin cross-linked to glucan in vivo, showing that the amount of chitin attached to β-(1,3)-glucan is equally reduced in both crh1Δ and crh2Δ mutants and completely eliminated in a crh1Δ crh2Δ strain, whereas the amount of chitin bound to β-(1,6)-glucan is more reduced in a crh2Δ strain [25,27].

AUTHOR CONTRIBUTION
Marian Mazďí, Kristína Kočárová and Zuzana Fišáková prepared the labelled substrates, carried out the enzyme expression and isolation and performed the biochemical experiments. Noelia Blanco isolated plasma membranes, carried out the enzyme expression and isolation and performed the biochemical experiments. Pavel Rehulka carried out the MALDI-TOF measurements and interpreted the results. Vladimír Farkaš and Javier Arroyo directed the research and wrote the paper with the participation of the other authors.

ACKNOWLEDGEMENTS
We thank Enrico Cabib for all his interest and support, for his bright and inspiring ideas and especially for the years of fruitful scientific collaboration, now ending due to his retirement. Critical reading of the paper by Ramón Hurtado and Enrico Cabib prior to submission is acknowledged. We also thank Raul Garcia for help with the Figures.

FUNDING
This work was supported by the Agency for Science VEGA (Slovak Grant Agency for Science, Slovakia) [grant number 2/0020/12 (to VF)], Ministerio de Ciencia e Innovación (MICINN) [grant number BIO2010-22146 (to J.A.)], Universidad Complutense de Madrid (UCM) [grant number GR68/08 (to J.A.)] and the Comunidad de Madrid [grant number S2010/BDM-2414 (to J.A.). This contribution is the result of the project implementation: Centre of excellence for Glycomics, ITMS 26240120031, supported by number S2010/BDM443 2414 (to J.A.). This contribution is the result of the project implementation: Centre of excellence for Glycomics, ITMS 26240120031, supported by number S2010/BDM443 2414 (to J.A.)]. This contribution is the result of the project implementation: Centre of excellence for Glycomics, ITMS 26240120031, supported by number S2010/BDM443 2414 (to J.A.)]. This contribution is the result of the project implementation: Centre of excellence for Glycomics, ITMS 26240120031, supported by number S2010/BDM443 2414 (to J.A.)]. This contribution is the result of the project implementation: Centre of excellence for Glycomics, ITMS 26240120031, supported by number S2010/BDM443 2414 (to J.A.)]. This contribution is the result of the project implementation: Centre of excellence for Glycomics, ITMS 26240120031, supported by number S2010/BDM443 2414 (to J.A.)].

REFERENCES
SUPPLEMENTARY ONLINE DATA

A novel fluorescence assay and catalytic properties of Crh1 and Crh2 yeast cell wall transglycosylases

Marian MAZÁN*1, Noelia BLANCO†1, Kristína KOVÁČOVÁ*, Zuzana FIRÁKOVÁ*, Pavel ŘEHULKA, Vladimír FARKAŠ*2 and Javier ARROYO†2

*Institute of Chemistry, Center for Glycomics, Slovak Academy of Sciences, 84538 Bratislava, Slovakia, †Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense de Madrid, IRYCIS, 28040 Madrid, Spain, and ‡Institute of Molecular Pathology, Faculty of Military Health Sciences, University of Defence, Třeběšská 1575, CZ-500 01 Hradec Králové, Czech Republic

Figure S1 SDS/PAGE of purified recombinant Crh1 and Crh2 proteins (Coomassie Blue staining)

Molecular masses are indicated in kDa.

Table S1 An overview of detected products obtained from TLC separation after chitinase digestion of carboxymethyl-chitin modified with the L5–SR acceptor attached by treatment with Crh1 (Figure 4 of the main text)

For every compound, the name of the corresponding TLC zone and summary formula of molecular species identified in MALDI–TOF–MS analysis with theoretical and experimental m/z values are shown.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Zone</th>
<th>Composition of detected species</th>
<th>Theoretical m/z</th>
<th>Experimental m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>L5–SR (acceptor)</td>
<td>(a)</td>
<td>[C57H83N3O31S2 + Na]⁺</td>
<td>1392.4344</td>
<td>1392.482</td>
</tr>
<tr>
<td>HP1</td>
<td>(b)</td>
<td>[C65H96N4O36S2 + Na]⁺</td>
<td>1595.5138</td>
<td>1595.568</td>
</tr>
<tr>
<td>HP2</td>
<td>(c)</td>
<td>[C73H109N5O41S2 + Na]⁺</td>
<td>1798.5932</td>
<td>1798.622</td>
</tr>
<tr>
<td>HP2-CM</td>
<td>(c)</td>
<td>[C75H111N5O43S2 + Na]⁺</td>
<td>1856.5986</td>
<td>1856.625</td>
</tr>
<tr>
<td>HP3-CM</td>
<td>(d)</td>
<td>[C83H124N6O48S2 + Na]⁺</td>
<td>2059.6780</td>
<td>2059.762</td>
</tr>
</tbody>
</table>

1 These authors contributed equally to this work.
2 Correspondence may be addressed to either of these authors (email chemvfar@savba.sk or jarroyo@farm.ucm.es).
Figure S2  Incorporation of different FITC–oligosaccharides in the WT strain

BY4741 cells were labelled with FITC–β-(1,3) oligosaccharides (FITC-β,1-3; DP 2–7), FITC–β-(1–6) oligosaccharides (FITC-β,1-6; DP 2–7) or FITC–N-tetra-acetyl-chitotetraose (FITC-CH4) and analysed by fluorescence microscopy (A and B) and flow cytometry (C). (A) Lower panels show images of the cells in the FITC fluorescence channel, whereas upper panels show the same cells observed under Nomarski. All images were obtained with the same exposure. Scale bars, 10 μm. (B) Oligosaccharide incorporation in the bud scars was quantified in the microscopy images by using HCimage Imaging software and is shown in the histograms. The signals, expressed as arbitrary fluorescence units (F.U.), correspond to the mean±S.D. for three independent experiments. The number of cells measured for each oligosaccharide was 2×10^3. (C) Analysis of WT cells labelled with the different FITC–OS by flow cytometry. Control corresponds to the background fluorescence of non-labelled cells.
Catalytic properties of yeast Crh1 and Crh2 transglycosylases

**Figure S3** pH and temperature optima of Crh1 and Crh2

Standard 20 μl incubation mixtures contained 0.5 mg·ml⁻¹ CM-chitin, 0.1 M of the appropriate citrate buffer, 30 μM L5-SR and 0.15 or 2.5 μg of Crh1 and Crh2 respectively. The incubations were carried out for 1 h. F.U., arbitrary fluorescence units.

**Figure S4** Time course of thermal inactivation of Crh1 and Crh2

The enzymes were dissolved in 0.1 M citrate buffer, pH 3.5, and heated at 98 °C in a tightly closed tube. At the indicated time intervals samples were taken and their transglycosylating activities were determined using the standard assay with L5–SR as the acceptor.
Figure S5  Deduced structures of the fluorescent fragments released by chitinase treatment of the high-\(M\)\(_\text{r}\) product of the reaction of CM-chitin with L5–SR catalysed by Crh1 and resolved by TLC as depicted in Figure 4 of the main text.

Received 7 March 2013/18 July 2013; accepted 6 August 2013
Published as BJ Immediate Publication 6 August 2013, doi:10.1042/BJ20130354