Proteomics-based screening and functional characterization of novel enzymes involved in the degradation of wood components

Motoyuki SHIMIZU

Faculty of Agriculture, Meijo University

Research aims

Filamentous fungi, including *Aspergillus nidulans* and *A. oryzae*, are popular model eukaryotes used in numerous genetic and biological studies. Interestingly, these fungi produce various cellulolytic and hemicellulolytic enzymes, which have recently become important natural resources for industrial biorefinery processes and the production of second-generation biofuels from plant biomass. Since hemicelluloses have complex structures, a variety of hemicellulolytic enzymes with different substrate specificities is necessary to achieve complete degradation. Based on sequence similarity, the carbohydrate-active enzymes (CAZy) database (http://www.cazy.org/) classifies these enzymes into four families: the glycoside hydrolase (GH), polysaccharide lyase (PL), carbohydrate esterase (CE), and auxiliary activity (AA) families.

Here, we analyzed the secretome of *A. nidulans* in response to various polysaccharides. Furthermore, we investigated the enzymatic function of a secreted hypothetical protein 1 (HP1). HP1 shared no significant homology with proteins of known function but displayed a rhamnogalacturonan (RG) lyase activity. Therefore, we would like to propose that HP1 is a novel RG lyase, which defines a new family of PL.

Methods

The *A. nidulans* wild-type strain A26 was grown for 48 h at 37°C in minimum media containing as the sole carbon source 1% microcrystalline cellulose (MCC), carboxymethyl cellulose (CMC), chitin, xylan, glucomannan (GlcM), or β-galactomannan (GalM). The secreted proteins from culture filtrates were then digested with trypsin, and the resulting peptides were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). MS/MS spectra were analyzed using the MASCOT search engine. The recombinant HP1 was isolated from the *Pichia pastoris* yeast strain KM71H, purified, and characterized.

Results

Using LC-MS/MS, we analyzed the secretome of *A. nidulans* grown on various single carbon sources (Fig. 1). We were able to identify 446 unique proteins (Fig. 1), including members of the GH, PL, CE, and AA families of carbohydrate-active enzymes, proteases, lipases, and proteins sharing homology with diverse proteins with known functions (Other). We also identified 82 hypothetical proteins (HPs), which have no putative conserved domains and share no sequence similarity with other known proteins (Fig. 1). Amongst these, we used *E. coli* or *P. pastoris* expression systems to express and purify HP1 to HP30.

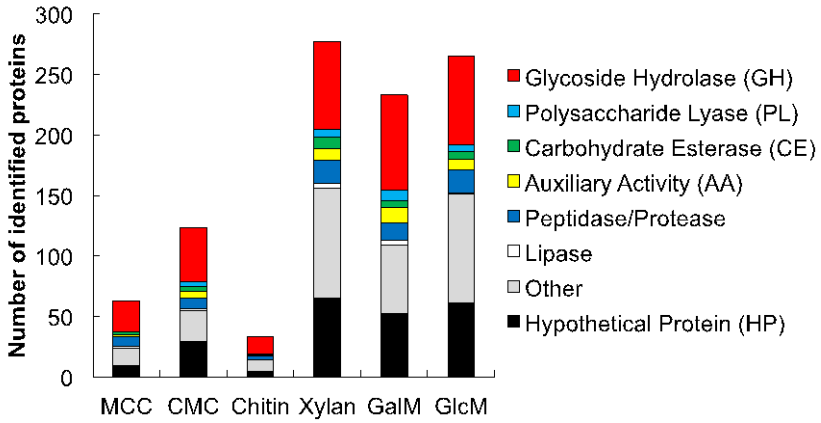

Fig. 1. Analysis of the secretome of *A. nidulans* in response to various polysaccharides.

In this study, we present our analyses of the function of HP1. On SDS-PAGE, the purified HP1 migrated as a single band with a molecular mass of 38 kDa (Fig. 2A). *In vitro*, HP1 showed significant catalytic activity towards rhamnogalacturonan I (RGI) degradation (Fig. 2B, and 2C), but had no detectable activity on MCC, CMC, chitin, xylan, GlcM, or GalM (data not shown). We next tested the catalytic activity of HP1 on α-1,5-arabinan, arabinogalactan, β-1,4-galactan, polygalacturonic acid, RGI, and linear RG (At-RG). Our thin layer chromatography (TLC) analysis of the reaction products showed that HP1 was active on β-1,4-galactan (which contained 13% RG), RGI, and At-RG (Fig. 2C). Considering that polysaccharides can be degraded by hydrolases, lyases and lytic polysaccharide monooxygenases, MS analysis of the reaction products can be used to determine the type of reaction catalyzed by HP1. Therefore, we analyzed the soluble products resulting from the degradation of At-RG by HP1 using MALDI-TOF MS (data not shown). The molecular weights of the three products detected corresponded to those of the Na^+ adducts of unsaturated galacturonosyl rhamnose (ΔGR), ΔGRGR, and ΔGRGRGR (data not shown), which suggests that HP1 has lyase activity. Moreover, we were able to confirm the lyase activity of HP1 on

At-RG by measuring the change in absorbance at 235 nm resulting from the formation of unsaturated oligosaccharides (data not shown). Together, these results show that HP1 is an RG lyase and belongs to a new family of PL in the CAZy classification (http://www.cazy.org/).

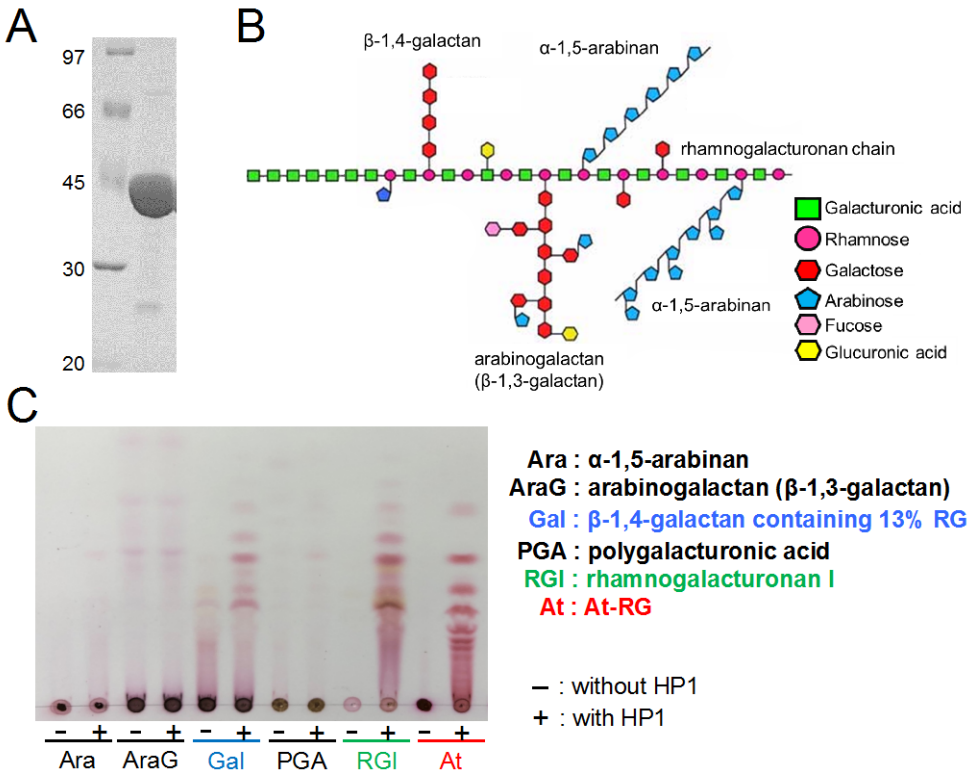

Fig. 2. Activity of purified HP1 towards various glycans. A) SDS-PAGE of HP1; B) Diagram showing the structure of RGI; C) TLC analysis of the soluble products resulting from the catalytic activity of HP1 on various glycans composing RGI.

Conclusion

We recently discovered a novel β-1,4-mannanase called Man134A, which is secreted by *A. nidulans* and shared no sequence homology with proteins of known function (including β-mannanases belonging to the GH5, GH26, and GH113 families)[1-3]. In this study, we report the identification and characterization of a novel RG lyase, which belongs to a new family of PL based on the CAZy classification. Together, these findings show that filamentous fungi remain an essential resource for the discovery and characterization of enzymes involved in the degradation of polysaccharides.

References

1) Shimizu, M. *et al.* (2015) Novel β-1,4-mannanase belonging to a new glycoside hydrolase family in *Aspergillus nidulans*. *J. Biol. Chem.* **290:** 27914-27927.
2) Sakai, K. *et al.* (2017) Biochemical characterization of thermostable β-1,4-mannanase belonging to the glycoside hydrolase family 134 from *Aspergillus oryzae*. *Appl. Microbiol. Biotechnol.* **101:** 3237-3245.
3) Sakai, K. *et al.* (2018) Characterization of pH-tolerant and thermostable GH 134 β-1,4-mannanase SsGH134 possessing carbohydrate binding module 10. *J. Biosci. Bioeng.* **125:** 287-294.