RESEARCH LETTER

Effects of xylan and starch on secretome of the basidiomycete *Phanerochaete chrysosporium* grown on cellulose

Chiaki Hori[1], Kiyohiko Igarashi[1], Akira Katayama[2] & Masahiro Samejima[1]

[1]Department of Biomaterial Sciences, Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo, Japan; and [2]Department of Biochemistry and Molecular Biology, Nippon Medical School, Tokyo, Japan

Correspondence: Masahiro Samejima, Department of Biomaterial Sciences, Graduate School of Agricultural and Life Sciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan. Tel.: +81 3 5841 5255; fax: +81 3 5841 5273; e-mail: amsam@mail.ecc.u-tokyo.ac.jp

Received 19 October 2010; revised 6 February 2011; accepted 22 April 2011.
Final version published online 2 June 2011.

DOI:10.1111/j.1574-6968.2011.02307.x

Editor: Nina Gunde-Cimerman

Keywords
Phanerochaete chrysosporium; secretome; glycoside hydrolase; biomass; cellobiose dehydrogenase.

Abstract

Lignocellulosic biomass contains cellulose and xylan as major structural components, and starch as a storage polysaccharide. In the present study, we have used comparative secretomic analysis to examine the effects of xylan and starch on the expression level of proteins secreted by the basidiomycete *Phanerochaete chrysosporium* grown on cellulose,. Forty-seven spots of extracellular proteins expressed by *P. chrysosporium* separated by two-dimensional electrophoresis were identified by liquid chromatography–tandem mass spectrometry analysis. Addition of starch to the cellulolytic culture did not affect fungal growth significantly, but did decrease the production of total extracellular enzymes, including cellulases and xylanases. In contrast, addition of xylan increased mycelial volume and the production of extracellular proteins. Xylan increased synthesis of several glycoside hydrolase (GH) family 10 putative endoxylanases and a putative glucuronoyl esterase belonging to carbohydrate esterase family 15, for which plant cell wall xylan may be a substrate. Moreover, cellobiose dehydrogenase and GH family 61 proteins, which are known to promote cellulose degradation, were also increased in the presence of xylan. These enzymes may contribute to degradation by the fungus of not only cellulose but also complex carbohydrate components of the plant cell wall.

Introduction

Most renewable organic carbon on Earth exists in the form of plant biomass, which mainly consists of cellulose, hemicellulose and lignin in the cell wall (McNeil *et al.*, 1984). Filamentous fungi belonging to *Basidiomycota* are omnipotent degraders of plant cell wall components (Eriksson *et al.*, 1990). Among them, the basidiomycete *Phanerochaete chrysosporium* is one of the best-studied fungi from the viewpoint of bioconversion of plant biomass, especially woody biomass. This fungus produces many types of extracellular glycoside hydrolases (GHs) that degrade structural polysaccharides, cellulose and hemicellulose (Broda *et al.*, 1994, 1996). In addition to GHs, the fungus produces various extracellular carbohydrate esterases (CEs) and oxidative enzymes to degrade plant cell wall components (Vanden Wymelenberg *et al.*, 2005, 2009; Kersten & Cullen, 2007; Sato *et al.*, 2007; Duranová *et al.*, 2009). Recently, the total genomic sequence of *P. chrysosporium* was disclosed (Martinez *et al.*, 2004) and many genes coding extracellular enzymes have been annotated. The results on GHs and CEs have been deposited in the carbohydrate-active enzymes database (Cantarel *et al.*, 2009) and those on oxidative enzymes in the fungal oxidative lignin enzymes database (Levasseur *et al.*, 2008). Moreover, extensive proteomic analysis of extracellular proteins, generally called the secretome, has been performed for *P. chrysosporium* (Abbas *et al.*, 2005; Vanden Wymelenberg *et al.*, 2005, 2009; Sato *et al.*, 2007; Ravalason *et al.*, 2008) in studies focused on the fungus degradation of woody biomass.

Besides woody plants, unused biomass from agricultural crops, including straw, hull, bran and leaves, is another important resource for biomass utilization (Shao *et al.*, 2010). In recent biomass projects, perennial plants belonging to *Poaceae*, such as *Erianthus*, *Miscanthus*, napier grass and switchgrass, have attracted considerable attention as feedstocks for the production of biofuel and bio-based plastics, as they grow faster than woody plants (Hames,

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2009; Keshwani & Cheng, 2009). As in the case of woody plants, biomass from *Poaceae* mainly consists of cell wall components, cellulose and xylan as the major structural polysaccharides, and often contains starch as a deposited polysaccharide (Park *et al.*, 2009; Shao *et al.*, 2010). Therefore, extracellular enzymes of basidiomycetous fungi should also be effective for the bioconversion of *Poaceae* biomass. In the present work, we have used comparative secretomic analysis to examine the effects of xylan and starch on the expression level of the proteins secreted by *P. chrysosporium* grown on cellulose.

Materials and methods

Fungal strain and cultivation conditions

Phanerochaete chrysosporium strain K-3 (Johnsrud & Eriksson, 1985) was cultivated in Kremer and Wood medium (Kremer & Wood, 1992) containing 2.0% w/v cellulose (CF11; Whatman, Fairfield, NJ), 2.0% w/v cellulose+0.2% w/v xylan from oat-spelt (Nakarai Chemicals Ltd, Kyoto, Japan) and 2.0% w/v cellulose+0.2% w/v soluble starch (Wako Pure Chemical Industries Ltd, Osaka, Japan) as carbon sources. The culture medium (400 mL) was inoculated with 10^9 spores L^{-1} in 1-L Erlenmeyer flasks, incubated at 37 °C and shaken at 150 r.p.m. for 2 days. To evaluate fungal growth, 5-mL aliquots were collected and left to stand for 30 min; the volume of fungal mycelia was then taken as representing growth. After cultivation, culture filtrates were separated from mycelia and insoluble substrate using a glass filter membrane (Advantec® GA-100; Tokyo Roshi Kaisya, Tokyo, Japan). Protein concentration of the culture filtrate was determined by means of the Bradford assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Enzyme assays

The amount of reducing sugar released by enzymatic reaction was measured using the *p*-hydroxybenzoic acid hydrazide (PHBAH; Wako Pure Chemical Industries Ltd) method (Lever, 1972), with some modifications.

For Avicelase activity, 100 μL of culture filtrate and 0.1% w/v Avicel (Funakoshi Co. Ltd, Tokyo, Japan) in 250 μL (final volume) of 50 mM sodium acetate, pH 5.0, were incubated for 300 min at 30 °C. The reaction was stopped by the addition of 250 μL of 1.0 M NaOH. The solution was mixed with 500 μL PHBAH solution (0.1 M PHBAH, 0.2 M NaK-tartrate and 0.5 M NaOH) and incubated at 96 °C for 5 min, and the absorbance of the reaction mixture at 405 nm was then measured. One unit of Avicelase was defined as the amount of enzyme required to release 1 μmol reducing sugar min^{-1} under the assay conditions using a predetermined standard curve obtained with glucose ($\varepsilon_{405} = 4.03\ mM^{-1}\ cm^{-1}$).

For xylanase activity, 100 μL of culture filtrate and 0.1% w/v xylan from oat-spelt in 250 μL (final volume) of 50 mM sodium acetate, pH 5.0, were incubated for 10 min at 30 °C. The reaction was stopped by addition of 250 μL 1.0 M NaOH and incubation was continued at 96 °C for 5 min and $A_{405\ nm}$ of the reaction mixture then measured. One unit of xylanase was defined as the amount of enzyme required to release 1 μmol reducing sugar min^{-1} under the assay conditions; xylose was used as a standard ($\varepsilon_{405} = 2.81\ mM^{-1}\ cm^{-1}$).

Glucoamylase activity was measured as described previously (Yoon *et al.*, 2006). Culture filtrates (20 μL) and 0.1% w/v amylose ($M_w = c.$ 2800, Tokyo Chemical Industry Co. Ltd, Tokyo, Japan) in 100 mM sodium acetate, pH 5.0, were incubated for 30 min at 30 °C. After incubation, the concentration of glucose was estimated with a Glucose CII-Test Wako (Wako Pure Chemical Industries Ltd) based on the glucose oxidase method. One unit of glucoamylase was defined as the amount of enzyme required to release 1 μmol glucose min^{-1} under the assay conditions.

Separation of proteins by two-dimensional electrophoresis (2DE)

Culture filtrates from medium containing cellulose (C), cellulose+xylan (CX) and cellulose+starch (CS) were centrifuged at 15 000 ***g*** for 5 min at 4 °C to remove insoluble materials. The supernatants were then concentrated using a 10 kDa Ultrafree®-0.5 Centrifugal Filter Device (Millipore, Billerica, MA) and washed with Milli-Q water three times. Samples were examined on a Multiphor system (GE Healthcare UK Ltd, Buckinghamshire, UK). Proteins (25 μg) were mixed with a rehydration buffer containing 7.5 M urea, 2 M thiourea, 4% CHAPS, 2% dithiothreitol, 0.5% IPG buffer (GE Healthcare UK Ltd) and a trace amount of bromophenol blue to a final volume of 330 μL and then loaded onto Immobiline Drystrips (18 cm, pH 3–10, nonlinear; GE Healthcare UK Ltd). After rehydration for 12 h, proteins were isoelectrically focused under the following conditions: 500 V (gradient over 1 min); 3500 V (gradient over 90 min); 3500 V (fixed for 6 h). These strips were equilibrated with buffer I [50 mM Tris–HCl pH 6.8, 6 M urea, 2% w/v sodium dodecyl sulfate (SDS), 30% w/v glycerol, 2% w/v dithiothreitol] and then buffer II (50 mM Tris–HCl pH 6.8, 6 M urea, 2% w/v SDS, 30% w/v glycerol, 2.5% w/v iodoacetamide). These strips were placed on SDS-polyacrylamide gels (ExcelGel™ SDS XL 12-14; GE Healthcare UK Ltd) and electrophoresis was conducted under the following conditions: 12 mA for 60 min, 40 mA for 5 min and finally 50 mA for 160 min. The gels were fixed in 10% v/v acetic acid and 40% v/v EtOH and then stained with SYPRO Ruby (Bio-Rad

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Laboratories) for 1 h. The staining solution was removed, and the gels were washed in 10% acetic acid and 10% v/v MeOH solution for 30 min. The stained 2DE gels were scanned with excitation at 532 nm using a Typhoon image scanner (GE Healthcare UK Ltd) and individual protein spots on different gels were matched and quantified using PROGENESIS SAMESPOTS ver. 4.0 (Nonlinear Dynamics Limited, Durham, NC).

Protein identification by liquid chromatography–tandem mass spectrometry (LC–MS/MS) and sequence analysis

The protein spots were excised, washed in 200 μL acetonitrile and then dried under vacuum. The proteins in the gel were reduced with 100 mM dithiothreitol in 0.1 M ammonium bicarbonate at 56 °C for 30 min and alkylated with 100 mM iodoacetamide in 0.1 M ammonium bicarbonate at 37 °C for 30 min in the dark. The gels were washed with 0.1 M ammonium bicarbonate, then acetonitrile and dried. These gels were reswollen with 12.5 ng μL^{-1} recombinant trypsin (proteomics grade; Roche Diagnostics Corporation, Indianapolis, IN) in 10 mM Tris–HCl buffer (pH 8.8) and then incubated at 37 °C for 12 h. After peptide extraction with extraction buffer (70% v/v acetonitrile and 5% v/v formic acid), the extracted peptide mixture was dried in a SpeedVac and dissolved in 20 μL of 0.1% trifluoroacetic acid. Peptides were subjected to HPLC separation on a MAGIC 2002 (Michrom BioResources, Auburn, CA) with a reversed-phase capillary HPLC column (C_{18}, 200 A, 0.2 × 50 mm; Michrom BioResources). As solvents, 2% v/v acetonitrile in 0.1% v/v formic acid (solvent A) and 90% v/v acetonitrile in 0.1% v/v formic acid (solvent B) were used, with a linear gradient from 5% to 65% of solvent B over 50 min. The chromatography system was coupled via an HTS-PAL (CTC Analytics, Zwingen, Switzerland) to an LCQ DECA XP ion trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). The MS/MS spectra were collected from 50 to 4500 *m*/*z* and merged into data files. In-house-licensed MASCOT search engine (Matrix Science, London, UK) identified peptides using 10 048 annotated gene models from *P. chrysosporium* v. 2.0 genome database (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html). The deduced amino acid sequences thus obtained were subjected to BLASTP search against the NCBI nonredundant database with default settings to confirm gene functions. The theoretical M_w and p*I* values were calculated using the protein parameter function calculation function on the EXPASY server (http://au.expasy.org/tools/pi_tool.html).

Results and discussion

Phanerochaete chrysosporium was cultivated in synthetic media containing C, CX and CS as carbon sources. As shown in Fig. 1a, after 2 days of cultivation, the mycelial volume in the medium containing cellulose as a carbon source reached 2.2 mL in 5 mL of culture; addition of xylan to cellulose enhanced fungal growth, and the mycelial volume reached 3.6 mL in 5 mL of culture after 2 days. In contrast, addition of starch had little effect on fungal growth. As shown in Fig. 1b, the concentration of extracellular protein produced in cellulose culture after 2 days of cultivation was 0.10 g L^{-1}. Addition of xylan to cellulose enhanced production of extracellular protein to 0.15 g L^{-1}, whereas addition of starch to cellulose decreased to the production of extracellular protein to approximately 0.04 g L^{-1}.

Cellulase (Avicelase), xylanase and glucoamylase activities in culture filtrates after 2 days of cultivation were measured and the results are shown in Fig. 2. In the cellulose culture without addition of xylan or starch, not only cellulase activity (0.66 U L^{-1}), but also xylanase and glucoamylase activities (77 and 0.38 U L^{-1}, respectively) were detected. Addition of xylan to cellulose culture resulted in a significant increase in xylanase activity, and also increased cellulase and glucoamylase activities. Addition of starch to cellulose culture enhanced glucoamylase activity, but decreased

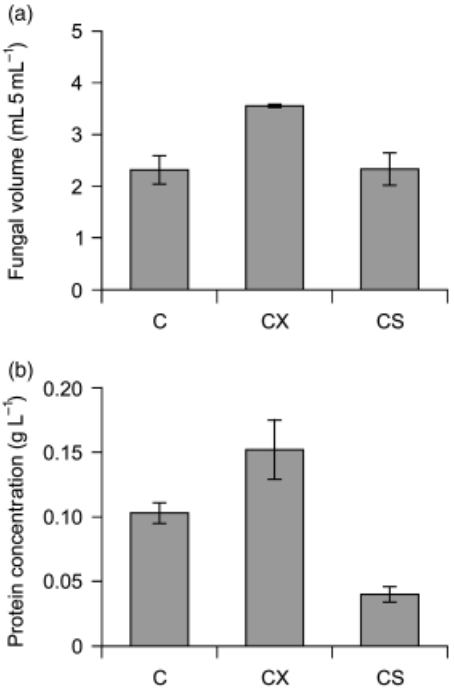

Fig. 1. Fungal growth (a) and extracellular protein production (b) of *Phanerochaete chrysosporium* cultivated for 2 days in synthetic culture containing different carbon sources. C, 2.0% w/v cellulose; CX, 2.0% w/v cellulose+0.2% w/v xylan; CS, 2.0% w/v cellulose+0.2% w/v starch. Mycelium volume per 5 mL of culture filtrate was measured as described in Materials and methods. Protein concentration of the culture filtrate was estimated by Bradford's method.

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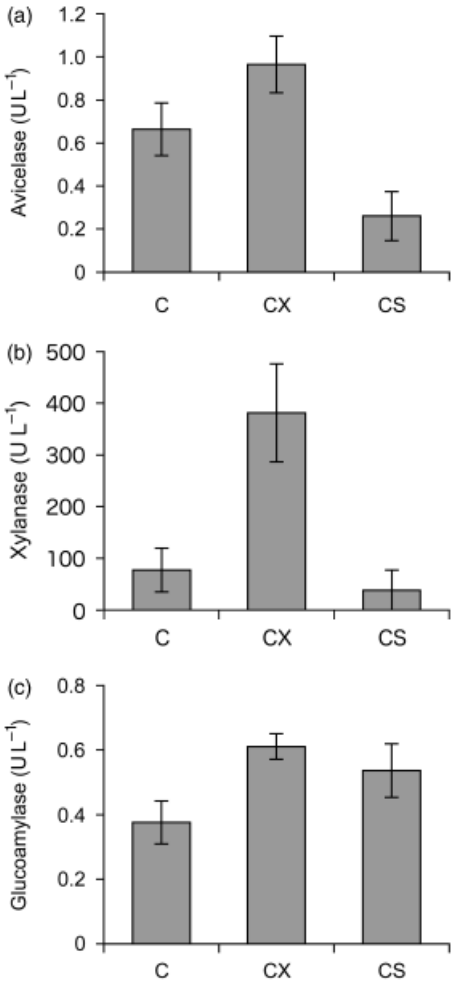

Fig. 2. Cellulase (a), xylanase (b) and glucoamylase (c) activities of 2-day cultures of *Phanerochaete chrysosporium* in C, CS and CX media. All the enzyme activities were measured in sodium acetate, pH 5.0 at 30 °C. (a, b) 0.1% w/v Avicel and oat-spelt xylan were used as substrates, respectively, and the newly formed reducing ends were estimated by means of the PHBAH method as described in Materials and methods. (c) 0.1% w/v amylose was used as a substrate, and the concentration of glucose was estimated using a Glucose CII-Test Wako as described in Materials and methods.

cellulase and xylanase activities, possibly because of carbon catabolite repression (Tempelaars *et al.*, 1994; Broda *et al.*, 1995; Suzuki *et al.*, 2009).

Extracellular proteins from *P. chrysosporium* cultivated in the synthetic media, C, CX and CS, were separated by 2DE as shown Fig. 3 and 47 spots on the gels were subjected to LC–MS/MS analysis. Among 47 spots, 41 spots, 47 spots and 39 spots were detected on the 2DE gel in C, CX and CS cultures, respectively. Table 1 presents a summary of the results; the detailed LC–MS/MS results are listed in Supporting Information, Table S1. These results revealed that most of total 47 identified proteins were classified into GHs (37 spots) and CEs (five spots), but a cellobiose dehydrogenase (CDH), a putative glutaminase and three hypothetical proteins were also included. When functionally classified, most of them were various cellobiohydrolases and endoglucanases involved in cellulose degradation, and various xylanases and accessory enzymes related to xylan degradation. They were all the same proteins with the exception of two GHs, as previously reported as secreted (Abbas *et al.*, 2005; Vanden Wymelenberg *et al.*, 2005, 2006, 2009; Sato *et al.*, 2007; Ravalason *et al.*, 2008). Major spots showing fluorescence intensity over 5.0×10^7 in all cultures were cellobiohydrolases (Cel7C, Cel7D and Cel6A: spots 5, 7 and 8, respectively), endoglucanase (Cel5B: spot 15) and endoxylanase and laminarinase (Xyn11A and lam16A: spot 24). Those three groups accounted for 39%, 45% and 37% of total extracellular proteins in the C, CS and CX media, respectively.

To investigate the effects of xylan and starch on the ratios of protein components, the fluorescence intensity of each protein spot identified in CX and CS cultures was compared with that in C culture using PROGENESIS SAMESPOTS software. In CS culture, no spot exhibiting more than a twofold increase from C culture was detected, whereas there were six spots with less than half of the intensity seen in C culture (Fig. 4). As the proteins repressed in CS culture are all minor components of total extracellular proteins, they are likely to have little impact on total protein concentration. Although the specific activity of glucoamylase was increased by the addition of starch, no spot exhibiting higher intensity was observed.

Twelve protein spots with more than a twofold increase of intensity compared with C culture were detected in CX culture (Fig. 5). Among them, five spots (spots 23, 30, 31, 32 and 42) were putative GH family 10 endoxylanases (Xyn10C), which may have contributed to the significant increase of xylanase activity in CX medium. Those spots assigned to the same gene, xyn10C, showed different p*I* and M_w values, possibly because of post-translational modification and/or fragmentation, as described in a previous report (Dobozi *et al.*, 1992). According to the total genomic sequence of *P. chrysosporium*, this fungus has six genes possibly coding GH family 10 proteins (Xyn10A-F), showing a maximum 92% identity of amino acid sequence. Although production of Xyn10A was not affected by the addition of xylan in the present study, production of Xyn10C was apparently increased by xylan, suggesting that this fungus produced xylanase isozymes differentially in response to different carbon sources. This fungus is known to have multiple genes coding GH family 7 cellulases, and they are secreted differentially in media containing different carbon sources (Vanden Wymelenberg *et al.*, 2009). Transcriptional analysis has also revealed that they are expressed differentially at the transcript level in response to various carbon sources (Broda *et al.*, 1995; Vallim *et al.*, 1998; Suzuki *et al.*,

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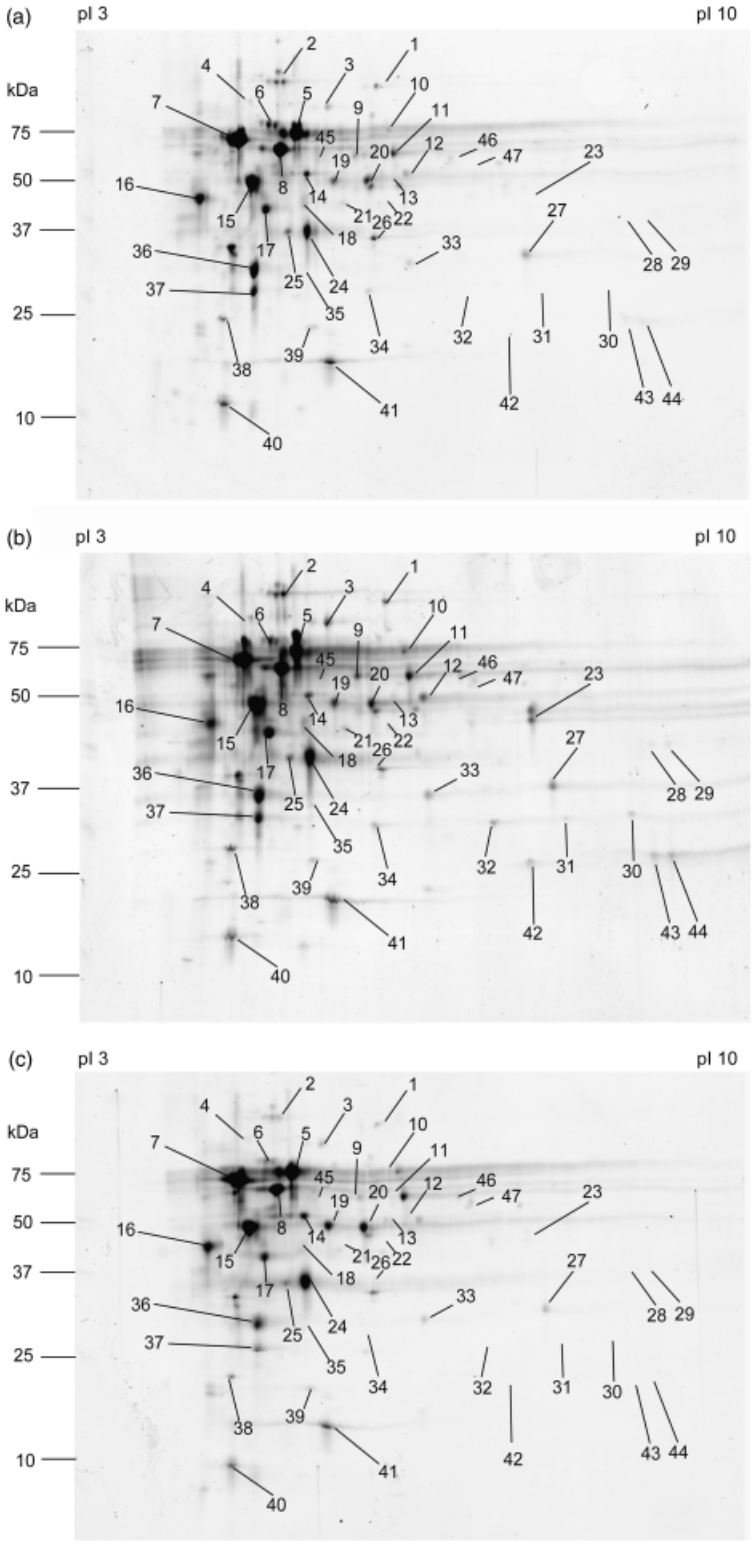

Fig. 3. 2DE of extracellular proteins stained with fluorescent dye (SYPRO Ruby). *Phanerochaete chrysosporium* was cultivated in synthetic medium containing 2.0% cellulose (a), 2.0% w/v cellulose+0.2% w/v xylan (b) or 2.0% w/v cellulose+0.2% w/v starch (c) for 2 days and an aliquot of extracellular proteins (25 μg) produced in each case was separated as described in Materials and methods. The horizontal axis of the gel is from pH 3 to 10 and the vertical axis is from 250 to 5 kDa. Spot numbers correspond to the proteins in Table 1.

2010). Similar expression studies should be performed for GH family 10 genes to clarify the role of each protein in the xylan-degrading system of this fungus.

In CX culture, a putative glucuronoyl esterase belonging to CE family 15 (spot 9) was increased almost twofold compared with C culture. This protein has been postulated

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Table 1. List of identified proteins of *Phanerochaete chrysosporium* in 2-day-incubated synthetic media containing 2.0% cellulose+0.2% xylan (CX)

Spot number	Score*	Protein ID†	Function (gene or domain)	Protein family	CBM‡	Theoretical M_w§	Theoretical pI§	Sequence cover (%)	Reference(s)¶	Protein expression‖		
										C	CX	CS
1	447	134658	Glucan β-1,3-glucosidase (bgl3)	GH3	N	85.053	5.43	16		+	+	+
4	164	129849	Putative β-glycosidase (gly3B)	GH3	–	87.136	4.72	8	1, 2, 4, 5, 6	+	+	+
15	172	4361	Endo-β-1,4-glucanase (cel5B)	GH5	N	49.721	6.21	11	2, 6	+++	+++	+++
17	257	6458	Endo-β-1,4-glucanase (cel5A)	GH5	N	39.851	5.04	32	2, 6	++	++	++
22	137	135724	β-1,3-Glucanase	GH5	–	46.864	5.55	9	6	+	+	+
47	117	5607	Putative β-glycosidase	GH5	–	49.195	5.4	5	2	+	+	+
8	484	133052	Cellobiohydrolase II (cel6A)	GH6	N	48.957	5.04	33	2, 4, 6	+++	+++	+++
5	862	127029	Cellobiohydrolase 62 (cel7C)	GH7	C	56.107	5.03	48	2, 4, 5, 6	+++	+++	+++
7	780	137372	Cellobiohydrolase 58 (cel7D)	GH7	C	59.474	4.96	39	2, 4, 5, 6	+++	+++	+++
21	105	127029	Cellobiohydrolase 62 (cel7C) (fragment)	GH7	C	56.107	5.03	4	2, 4, 5, 6	+	+	+
14	110	138345	Endo-β-1,4-xylanase (xyn10A)	GH10	N	44.065	5.21	9	2, 3, 4, 6	++	+	++
23	519	7045	Putative endo-β-1,4-xylanase (xyn10C)	GH10	(N)**	31.21	6.89	59	2, 3, 6	+	+	+
30	360	7045	Putative endo-β-1,4-xylanase (xyn10C)	GH10	(N)**	31.21	6.89	32	2, 3, 6	ND	+	ND
31	260	7045	Putative endo-β-1,4-xylanase (xyn10C)	GH10	(N)**	31.21	6.89	28	2, 3, 6	ND	+	ND
32	410	7045	Putative endo-β-1,4-xylanase (xyn10C)	GH10	(N)**	31.21	6.89	31	2, 3, 6	ND	+	ND
42	224	7045	Putative endo-β-1,4-xylanase (xyn10C)	GH10	(N)**	31.21	6.89	21	2, 3, 6	ND	+	ND
18	155	133788	Endo-β-1,4-xylanase (xyn11A)	GH11	C	30.804	5.72	11	2, 4, 6	+	+	+
24	156	133788	Endo-β-1,4-xylanase (xyn11A)	GH11	C	30.804	5.72	11	2, 4, 6	+++	+++	+++
36	87	8466	Endo-β-1,4-glucanase (cel12A)	GH12	–	26,459	4.79	8	2, 6	++	++	++
37	93	8466	Endo-β-1,4-glucanase (cel12A)	GH12	–	26,459	4.79	8	2, 6	++	++	+
38	92	7048	Putative endoxyloglucanase (cel12B)	GH12	–	27.082	4.74	10	2, 6	+	+	+
45	301	138813	Putative glucoamylase (gla15A)	GH15	C	60.991	5.24	22	2, 6	+	+	+
24	144	10833	Endo-β-1,3-glucanase (lam16A)	GH16	–	33.715	4.94	12	6	+++	+++	+++
25	187	10833	Endo-β-1,3-glucanase (lam16A)	GH16	–	33.715	4.94	16	6	+	+	++
39	90	136630	Putative lysozyme	GH25	–	24.571	5.63	15	6	+	+	+
35	272	3805	Putative endopolygalacturonase (epg28A)	GH28	–	40.207	5.02	19	2, 4, 6	+	+	+
11	431	29397	Putative rhamnogalacturonase (rgh28C)	GH28	–	44.793	5.22	35	2, 4, 6	++	++	++
46	123	29397	Putative rhamnogalacturonase (rgh28C)	GH28	–	44.793	5.22	7	2, 4, 6	+	+	+
41			Endo-β-1,4-glucanase (cel45)††	GH45	–	18.169	5.04		2	++	++	++
26	76	138710	Putative arabinogalactan endo-β-1,4-galactanase	GH53	–	36.86	5.59	11		+	+	+

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Table 1. Continued.

Spot number	Score*	Protein ID†	Function (gene or domain)	Protein family	CBM‡	Theoretical M_w§	Theoretical pI§	Sequence cover (%)	Reference(s)¶	Protein expression‖		
										C	CX	CS
34	52	138710	Putative arabinogalactan endo-β-1,4-galactanase (fragment)	GH53	–	36.86	5.59	5		+	+	+
13	122	41563	Putative GH family 61 protein (cel61B)	GH61	C	33.195	5.39	16	2, 6	+	+	+
28	160	41123	Putative GH family 61 protein (cel61C)	GH61	–	23.761	6.63	16	3, 6	ND	+	ND
29	212	41123	Putative GH family 61 protein (cel61C)	GH61	–	23.761	6.63	16	3, 6	ND	+	ND
43	238	41123	Putative GH family 61 protein (cel61C)	GH61	–	23.761	6.63	31	3, 6	+	+	ND
44	278	41123	Putative GH family 61 protein (cel61C)	GH61	–	23.761	6.63	29	3, 6	+	+	ND
6	480	138266	Putative xyloglucanase (gly74A)	GH74	–	77.79	4.72	18	2, 6	++	++	+
2	455	134556	Xyloglucanase (xgh74B)	GH74	C	89.855	5.05	18	2, 4, 5, 6	++	++	+
12	123	126075	Putative acetyl xylan esterase (axe1)	CE1	–	35.963	5.91	7	2, 4, 6	+	+	+
19	111	126075	Putative acetyl xylan esterase (axe1)	CE1	–	35.963	5.91	7	2, 4, 6	++	++	++
20	223	126075	Putative acetyl xylan esterase (axe1)	CE1	–	35.963	5.91	29	2, 4, 6	++	++	++
33	189	126075	Putative acetyl xylan esterase (axe1)	CE1	–	35.963	5.91	29	2, 4, 6	+	+	+
9	385	130517	Putative glucuronoyl esterase	CE15	N	49.731	5.55	18	6	+	+	+
3	526	11098	Cellobiose dehydrogenase (cdh1)	LO3	–	82.185	5.19	16	2, 6	+	+	+
10	448	140079	Putative glutaminase (gta1)		–	75.388	5.54	18	2, 3	+	+	+
16	129	3097	Hypothetical protein (IPR001087 lipolytic enzyme, G-D-S-L)		N	43.209	5.08	9	6	++	++	++
27	199	135606	Hypothetical protein		–	27.395	6.04	27	6	+	+	+
40	62	8221	Hypothetical protein (IPR010829 cerato-platanin)		–	14.966	4.69	10	6	++	+	++

Spot number refers to Fig. 3. All proteins identified by LC–MS/MS analysis are listed in Table S1.

*MASCOT score ($P > 0.05$).

†Protein model number ver. 2.1 (from *P. chrysosporium* genome database).

‡Deduced amino acid sequence contains carbohydrate-binding module (CBM) or not. N, N-terminal; C, C-terminal.

§Theoretical molecular weight (kDa) and p*I*.

¶Previous identification of protein in secretome analyses of *P. chrysosporium*. 1, Abbas *et al*. (2005); 2, Vanden Wymelenberg *et al*. (2005); 3, Vanden Wymelenberg *et al*. (2006); 4, Sato *et al*. (2007); 5, Ravalason *et al*. (2008); 6, Vanden Wymelenberg *et al*. (2009).

‖Spots showing fluorescence intensity over 5.0×10^7 (+++), under 5.0×10^7 and over 1.0×10^7 (++), under 1.0×10^7 (+) or not detected (ND).

**Manual examination revealed conserved CBM sequence at the N-terminus of this gene model.

††MS/MS dataset of spot 61 search on genome dataset ver. 2.0 in scaffold 6: 1798366–1797555 (Igarashi *et al.*, 2008).

Spot number	Protein family	Function (gene)	C	CS	Relative degree
1	GH3	Glucan β-1,3-glucosidase (bgl3A)			0.43
4	GH3	Putative β-glycosidase (gly3B)			0.48
37	GH12	Endo-β-1,4-glucanase (cel12A)			0.46
34	GH53	Putative arabinogalactan endo-β-1,4-galactanase			0.49
43,44	GH61	Putative GH family 61 protein (GH61C)			0 (not detected)

Fig. 4. List of the protein spots showing decrease in intensity of more than one- to twofold on the gel of CS culture comparing to C culture. Spot numbers correspond to those in Fig. 3 and Table 1, and the intensity was analyzed by PROGENESIS SAMESPOTS ver. 4.0.

Spot number	Protein family	Function (gene)	C	CX	Relative degree
23	GH10	Putative Endo-β-1,4-xylanase (xyn10C)			5.6
30	GH10	Putative Endo-β-1,4-xylanase (xyn10C)			Unique
31	GH10	Putative Endo-β-1,4-xylanase (xyn10C)			Unique
32	GH10	Putative Endo-β-1,4-xylanase (xyn10C)			Unique
42	GH10	Putative Endo-β-1,4-xylanase (xyn10C)			Unique
35	GH28	Putative endo-polygalacturonase (epg28A)			2.6
28,29	GH61	Putative GH family 61 protein (GH61C)			Unique
43,44	GH61	Putative GH family 61 protein (GH61C)			2.1
9	CE15	Putative glucuronoyl esterase			2.1
3	LO3	Cellobiose dehydrogenase			2.1

Fig. 5. List of the protein spots showing increase in intensity of more than twofold on the gel of CX culture comparing to C culture. Spot numbers refer to Fig. 3 and Table 1, and the intensity was analyzed by PROGENESIS SAMESPOTS ver. 4.0.

to hydrolyze ester linkages between the 4-*O*-methyl-D-glucuronic acid residue in xylan and the phenylpropane residue in lignin (Duranová *et al.*, 2009). Moreover, the spot assigned to the redox enzyme CDH (spot 3) was also increased twofold by addition of xylan. CDH oxidizes cellobiose and cellooligosaccharides to corresponding δ-lactones. Although many researchers have proposed various physiological functions for CDH (Henriksson *et al.*, 2000), the precise role of this enzyme in degradation of plant cell wall remains to be established. Several recent transcriptional analyses have indicated that CDH is involved in cellulose metabolism (Li *et al.*, 1996; Yoshida *et al.*, 2004). CDH may play a role in enhancing cellulase activity for cellulose degradation by relieving product inhibition (Igarashi *et al.*, 1998). Dumonceaux *et al.* (2001) reported that a CDH-deficient mutant of the wood-rotting basidiomycete *Trametes versicolor* grows poorly not only on crystalline cellulose, but also on wood, implying that CDH may have role in invasion of the plant cell wall. Further transcriptional analysis of CDH under xylanolytic conditions will be necessary for a better understanding of its physiological function.

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In addition, many protein spots of GH family 61s were enhanced by addition of xylan (Fig. 4). Recently, Harris *et al.* (2010) have reported that the protein belonging to GH family 61 enhances the activity of cellulose hydrolysis in lignocellulose, but not in pure cellulose. Quite recently, moreover, Vaaje-Kolstad and colleagues demonstrated that chitin-binding protein 21, which is structurally similar to GH family 61 protein with conserved metal ion(s) in the protein (Karkehabadi *et al.*, 2008), is an oxidative enzyme accelerating chitinase activity toward crystalline chitin (Vaaje-Kolstad *et al.*, 2010). The present finding that CDH and GH family 61 proteins are upregulated by xylan suggests that the oxidative reaction is a critical step not only for the degradation of cellulose as proposed by Eriksson and colleagues in 1970s (Eriksson *et al.*, 1974), but also for the degradation of other polysaccharides, and GH family 61 proteins may participate in the oxidation for degradation of plant polysaccharides. Although the biochemical function of GH family 61 proteins is still unclear, enhancement of production of GH family 61 proteins by xylan is consistent with the recent evidence and provides a useful clue to the function.

Conclusion

In cellulolytic culture of the basidiomycete *P. chrysosporium*, addition of starch represses production of enzymes related to degradation of cellulose and xylan. In contrast, the addition of xylan promotes the growth of the fungus and increases production of Xyn10C and a putative glucuronoyl esterase belonging to CE family 15, which may act in the degradation of the main chain and side chain of xylan, respectively. Moreover, production of CDH and GH family 61 proteins, the potential oxidative enzymes accelerating enzymatic conversion of polysaccharides, is also increased in the presence of xylan. These results indicate that xylan is not simply an inducer of xylanolytic enzymes but may promote the production of a variety of biomass-degrading enzymes by *P. chrysosporium*.

Acknowledgement

This research was supported by a Grant-in-Aid for Scientific Research to M.S. (no. 20380100) from the Japanese Ministry of Education, Culture, Sports, and Technology.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. MS results for *Phanerochaete chrysosporium* peptides.

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