Characterization of a GH12 Endoglucanase from *Volvariella volvacea* Exhibiting Broad Substrate Specificity and Potential Synergy with Crude Cellulase

Zhen Wang, Yimei Hu, Liangkun Long, and Shaojun Ding *

Fungal glycoside hydrolase family GH12 has a single catalytic domain, exhibiting a great diversity of properties and application potentials in biomass biorefinery, feed, and textile industries. To discover new GH12 enzymes from white- and brown-rot basidiomycetes for application in the saccharification of lignocelluloses, two putative genes, *VvGH12A* and *VvGH12B*, were identified from the *Volvariella volvacea* genome and classified into basidiomycetous subfamily GH12-1 and GH12-2, respectively. One enzyme VvGH12A was successfully expressed in *Pichia pastoris*, and characterized. VvGH12A was the most active on CMC but with broad substrate specificities on polysaccharides with β-1,4 linked and β-1,3-1,4-mixed glucans. Furthermore, VvGH12A was also active on xylan and mannan. Unlike other fungal GH12 endoglucanases, VvGH12A showed a weak processivity independent of the carbohydrate-binding module (CBM) due to both "endo" and "exo" types of enzyme activity. The pH-optimum was significantly affected by the acidity and basicity of amino acid at site 98. The enzyme optimum pH was engineered to a higher neutral or alkaline pH (from pH 6.5 to pH 7.0-8.0) when Asp98 was replaced with nonpolar or neutral or amide residue. VvGH12A exhibited synergistic action with crude cellulase from *Trichoderma reesei* D-86271 (Rut C-30) in saccharification of delignified wheat straw, suggesting that VvGH12A plays a functional role in efficiently hydrolyzing plant cell wall polysaccharides.

Keywords: Volvariella volvacea; Glycoside hydrolase 12; Synergistic action; Processivity; pH-Profile engineering

Contact information: The Co-Innovation Center of Efficient Processing and Utilization of Forest Resources, Jiangsu Key Lab for the Chemistry & Utilization of Agricultural and Forest Biomass, College of Chemical Engineering, Nanjing Forestry University, Nanjing, 210037, Jiangsu, China;
** Corresponding author: dshaojun@hotmail.com*

INTRODUCTION

Plant cell wall polysaccharides, including celluloses and hemicelluloses, can be used as renewable feedstocks for the production of biofuels and biochemicals (Bhat and Bhat 1997; Lynd *et al*. 2002). Many cellulolytic bacteria and fungi produce diverse glycoside hydrolases (GHs) for efficiently hydrolyzing plant cell wall polysaccharide constituents. GHs are classified into 145 families based on amino acid sequence similarities (CAZy database, http://www.cazy.org/). The GH12 family is widely distributed in archaea, bacteria, and fungi, and displays a very broad diversity in substrate specificity (Sandgren *et al*. 2003; Picart *et al*. 2012). The GH 12 family contains β-1,4-endoglucanase (EC 3.2.1.4), β-1,3-1,4-endoglucanase (EC 3.2.1.73), and xyloglucan-specific endo-β-1,4-glucanase (EC 3.2.1.151) (http://www.cazy.org/GH12.html) capable of hydrolyzing various β-1,4-linked glucans such as cellulose, 1,3-1,4-β-glucan, and

xyloglucan in plant cell walls, respectively (Goedegebuur *et al*. 2002; Grishutin *et al*. 2006; Takeda *et al*. 2010).

Endoglucanases are key glycoside hydrolases for cellulose biodegradation and are widely used in related industrial processes (Bhat 2000; Margeot *et al*. 2009). Most known microbial endoglucanases are now classified into 14 glycoside hydrolase families in GH5, 6, 7, 8, 9, 10, 12, 26, 44, 45, 48, 51, 74, and 124 (CAZy database, http://www.cazy.org/). Endoglucanases in the GH12 family are multifunctional enzymes and have wide-range pH optima (Tishkov *et al*. 2013; Zhang *et al*. 2015). Unlike other GH family endoglucanases composed of two modules, a catalytic module and one or more carbohydrate-binding modules (CBMs), the fungal GH 12 endoglucanases lack a carbohydrate-binding module (CBM) (Goedegebuur *et al*. 2002; Zhang *et al*. 2015). The relatively small size of GH12 endoglucanases (around 30 kDa) may allow them to penetrate the plant cell wall and contribute to cellulose hydrolysis at an early stage (Cohen *et al*. 2005; Miotto *et al*. 2014). The GH 12 endoglucanases have received much attention in recent years because of the diversity of properties and application potential in biomass biorefinery, feed, and textile industries (Shimokawa *et al*. 2008; Narra *et al*. 2014).

In nature, basidiomycetes have an extensive array of cellulolytic and hemicellulolytic enzymes for efficient degradation of plant cell wall polysaccharides, including cellulose, hemicellulose, and pectin (Ohm *et al*. 2010). A few of the GH12 enzymes have been identified from white- and brown-rot basidiomycetes, including *Phanerochaete chrysosporium*, *Gloeophyllum trabeum*, *Fomitopsis palustris*, and *Lentinula edodes* (Henriksson *et al*. 1999; Cohen *et al*. 2005; Byeong-Cheol *et al*. 2008; Shimokawa *et al*. 2008; Takumi *et al*. 2013; Miotto *et al*. 2014). However, compared with the extensive studies of ascomycetous GH12 enzymes, the basidiomycetous GH12 enzymes are less understood. More biochemical and structural information about new basidiomycetous GH12 enzymes are necessary to further understand the molecular basis for substrate specificity and activity pH-profile, and their potential role in cellulose depolymerization by basidiomycetes.

Volvariella volvacea is a large-scale cultivated edible straw mushroom in East and Southeast Asia. It has complex carbohydrate-active enzymes for the depolymerization of cellulose, hemicellulose, and pectin (Zheng 2013). Herein, a novel neutral GH12 endoglucanase with broad substrate specificity was identified and characterized from *V. volvacea*. It exhibited synergistic action with crude cellulase from *Trichoderma reesei* D-86271 (Rut C-30) in the saccharification of delignified wheat straw. The distinctive role of the Asp98 residue in determining the optimum pH for enzyme activity was investigated by site-directed mutagenesis.

EXPERIMENTAL

Materials

Strains, culture conditions, vectors, and chemicals

Escherichia coli DH5α (Invitrogen, Carlsbad, CA, USA) was used as a host for vector construction and multiplication. *Pichia pastoris* KM71H and plasmid vector pPICZαA (Invitrogen) were used for the recombinant expression of VvGH12A and its mutants. Carboxymethyl cellulose (CMC, low viscosity), beechwood xylan, and chitosan were brought from Sigma (St. Louis, MO, USA). Lichenan, barley-β-glucan,

glucomannan, laminarin, and xyloglucan were purchased from Megazyme (Wichlow, Ireland). Regenerated amorphous cellulose (RAC) was produced from Avicel according to the method described by Zhang *et al*. (2006). The crude cellulase was produced from *Trichoderma reesei* D-86271 (Rut C-30) (VTTCC, Finland) by growing in modified Mandels' medium according to Long *et al.* (2016).

Expression vector construction and site-directed mutagenesis

Two putative GH12 gene sequences were identified in the genome of *V. volvacea* (Bao *et al*. 2013; Chen *et al*. 2013). The full-length cDNA of VvGH12A is 735 bp in length and encodes for a 244-amino acid peptide with a putative 18-amino acid signal sequence, while VvGH12B is 789 bp in length and encodes for a 262-amino acid peptide with a putative 19-amino acid signal sequence. The fragments encoding mature GH12A and GH12B (GenBank No. MF114116 and MF11411, respectively) were synthesized by GENEWIZ, Inc. (Suzhou, China) using *P. pastoris* biased codons. The fragments were inserted into pPICZαA at the *Eco*RI and *Xba*I sites to construct the expression vectors pPICZαA-GH12A and pPICZαA-GH12B, respectively.

The site-directed mutation for pH-profile engineering of the GH12A was carried out by PCR using the primers with selected site mutations (in Table S1) and plasmid pPICZαA-GH12A as the template. The site-directed mutagenesis led to the following amino acid substitutions in GH12A: D98A, D98T, D98H, D98Q, and D98N (numbering based on the mature sequence without signal peptide). PCR conditions were as follows: one cycle at 94 °C for 5 min, 55 °C for 30 s, and 72 °C for 4 min; 25 cycles at 94 °C for 40 s, 55 °C for 30 s, and 72 °C for 4 min followed by a final extension at 72 °C for 10 min. The PCR products were purified from gel and treated with *Dpn*I to eliminate the template plasmid. These mutated constructs were confirmed by DNA sequencing.

Methods

Expression and purification of enzymes

The transformants were inoculated in 50 mL of buffered complex glycerol medium (BMGY) (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, and 10 g L^{-1} glycerol) in a 250-mL flask at 28 °C and 250 rpm for 16 to 24 h until the cell density reached an OD_{600} value of 6, and then pelleted and resuspended in 25 mL of buffered methanol complex (BMMY) medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone) to a final OD_{600} value of 30. The recombinant proteins were induced by adding methanol every day at a final concentration of 1.5% for 6 days. After induction, cells were collected by centrifugation (6500 × g, 10 min). The supernatant was then directly loaded to the Ni-NTA affinity chromatography (Qiagen, Valencia, CA, USA) under native conditions. All purification steps were carried out at room temperature according to the manufacturer's manual. The purity and molecular weights of purified VvGH12A and the five mutants were estimated using 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Assay of enzyme activity

Enzyme activity was determined by assaying the amount of reducing sugars released from various substrates. The substrates tested were CMC, filter paper (Whatman, Little Chalfont, UK), laminarin, lichenan, glucomannan, barley β-glucan, xyloglucan, regenerated amorphous cellulose (RAC), beechwood xylan, and destarched oat and wheat bran. The assay mixtures contained 0.9 mL of potassium phosphate buffer (100 mM, pH

6.5), 0.5 mL suspension of FP, destarched oat and wheat bran (50 mg) or 0.5 mL solution of substrate (2% for CMC, 1% for RAC, and 0.5% for other substrates), and 0.1 mL of appropriately diluted enzyme sample (5 μg). The mixtures were incubated at 40 °C for 30 min. The reducing sugars released were measured at 520 nm by the Somogyi-Nelson method. Each assay was performed in triplicate. One unit of enzymatic activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent per min under the assay conditions described. Optimal pHs and temperatures were determined by using CMC as the substrate over a pH range of 2.0 to 10.0 (Universal buffer: 50 mM H_3PO_4, 50 mM CH3COOH, 50 mM H_3BO_3, pH adjusted with 0.2 M NaOH at 25 °C) and a temperature range of 30 to 80 °C, respectively. The pH and thermal stability were determined by measuring the residual activity on CMC under the standard assay conditions after pre-incubation of enzymes for different intervals at various pH and temperatures. The kinetic parameters (V_{max} and K_m) were determined at 40 °C after a 15 min reaction using CMC as a substrate at concentrations from 1 to 25 mg mL^{-1}. The V_{max} and K_m values were calculated by GraphPad Prism 5.0 software (http://www. graphpad.com/prism/) using non-linear regression.

Processivity analysis

Processivity of VvGH12A was evaluated based on the ratio of reducing sugars in soluble to insoluble fraction generated from RAC (Irwin *et al.* 1998). Enzyme reactions were carried out under standard conditions for up to 4 h. The sample was collected at set intervals and separated by centrifugation. The amounts of reducing sugars in the soluble fraction (in supernatant) and insoluble fraction (in the remaining RAC) were determined by the Somogyi-Nelson method.

Enzymatic saccharification of cellulosic biomass with crude cellulolytic enzyme.

The crude cellulolytic enzyme was prepared from *T. reesei* D-86271 (Rut C-30). The synergistic interaction between VvGH12A and crude cellulase in the hydrolysis of the delignified wheat straw was carried out at four different conditions (pH 6.5 and 40 °C or 50 °C, pH 4.8 and 40 °C or 50 °C) for 48 h with orbital shaking (200 rpm). VvGH12A (1 IU) and crude cellulase (1mg protein g^{-1} substrate) were added together to the reaction mixtures containing 0.1 g substrate in a total volume of 5 mL of potassium phosphate buffer (100 mM). Ampicillin and Zeocin (25 mg L^{-1} each) were added to prevent the microbial contamination. Samples (200 μL) were withdrawn at regular intervals and heated at boiling water bath for 10 min. The released reducing sugars in supernatant were quantified using Somogyi-Nelson method with glucose as the standard. The delignified wheat straw was prepared by treating wheat straw (1 to 2 mm, 100 g) with 200 mL of 4% NaOH (w/v) at 121 °C for 20 min, followed by 15 min washing with tap water and dried at 60 °C to constant weight. All the assays were performed in triplicate.

RESULTS AND DISCUSSION

Sequence Analysis and Expression of VvGH12A and VvGH12B

GH12 glycoside hydrolases show a wide variation in their substrate specificity, activity pH-profile, and thermal stability. The diversity of properties in the GH12 family makes it an ideal candidate for both basic and application research (Vlasenko *et al*. 2010; Zhang *et al*. 2015).

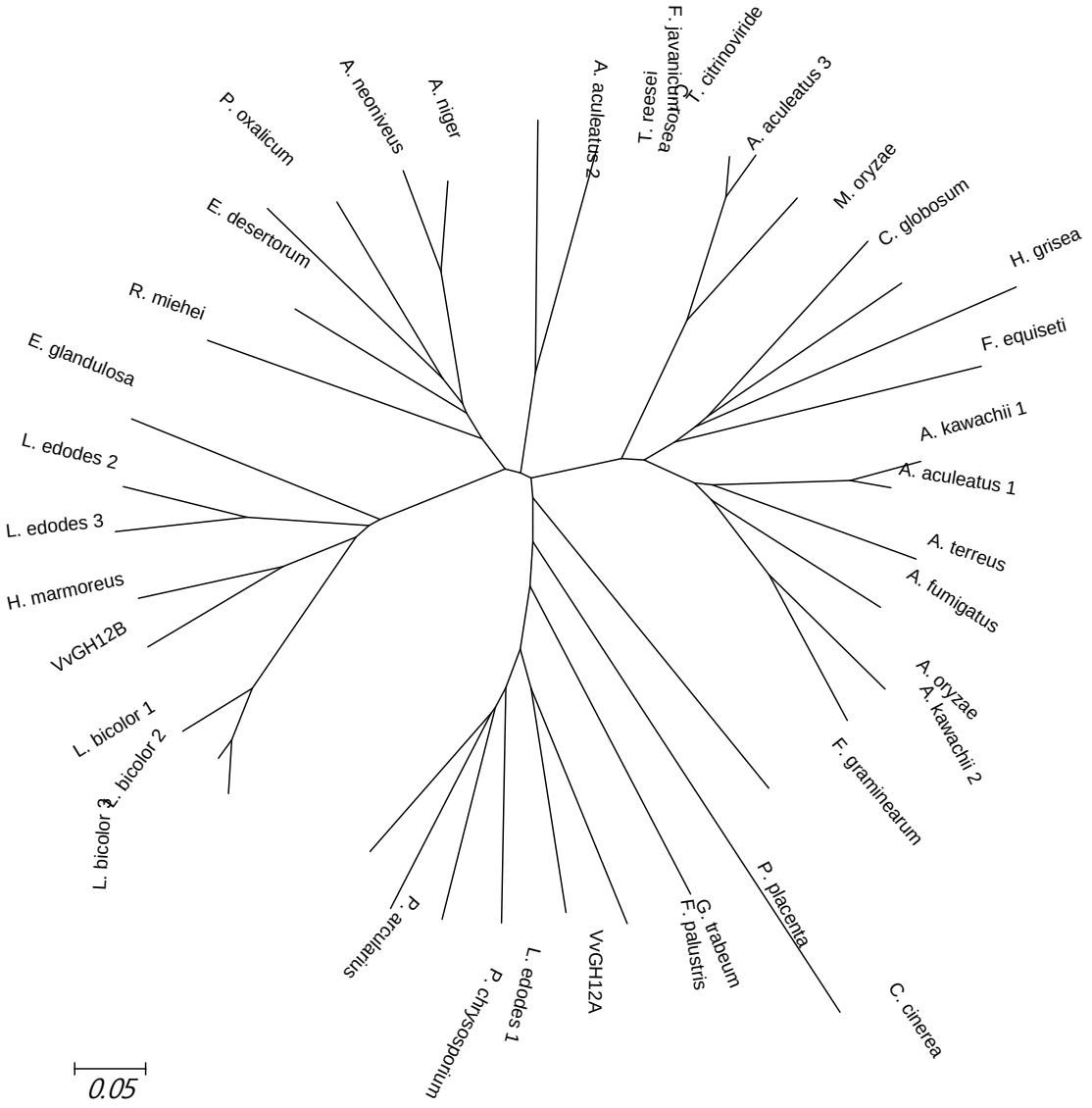

Fig. 1. Phylogenetic tree of the GH12 glycoside hydrolases from *Volvariella volvacea* and the related enzymes with fungal glycoside hydrolase family 12. Multiple-sequence alignments were done by using MEGA5.1 based on the amino acid sequences of the following enzymes: *Aspergillus aculeatus* 1 (P22669), *A. aculeatus* 2 (O94218), *A. aculeatus* 3 (AF043595), *A. kawachii* 1 (AF435072), *A. kawachii* 2 (BAA02297.1), *A. fumigatus* (EAL86857.1), *A.oryzae* (BAA22588.1), *A. terreus* (EAU30085.1), *A.niger* (ABF46829.1), *A. neoniveus* (AEV23011.1), *Chaetomium globosum* (XM_001222999), *Clonostachys rosea* (AAM77707.1), *Coprinopsis cinerea* (XM_002910519), *Emericella desertorum* (AF434181), *Exidia glandulosa* (KZV98399.1), *Fusarium javanicum* (AF434183), *Fusarium equiseti* (AF434182), *Fomitopsis palustris* (BAF49602.1), *F. graminearumPH-1*(XP_386027.1), *Gloeophyllum trabeum* (AEJ35167.1), *Hypsizygus marmoreus* (KYQ32267.1), *Humicola grisea* (AAM77714.2), *Lentinula edodes* 1(BAN51847.1), *L. edodes* 2 (BAN51848.1), *L. edodes* 3 (BAN51849.1), *Laccaria bicolor* 1 (XM_001879533), *L. bicolor* 2 (XM_001886624), *L. bicolor* 3 (XM_001890579), *Magnaporthe oryzae* (XP_368567.1), *Postia placenta* (XP_002472854.1), *Phanerochaete chrysosporium* (AAU12276.1), *Polyporus arcularius* (BAD98315.1), *Penicillium oxalicum* (AJA40324.1), *Rhizomucor miehei* (AGC24032.1), *Trichoderma citrinoviride* (AF435068), and *T. reesei* QM9414 (AAE59774.1). Calculations were performed with the neighbor-joining method.

Two sequences for putative GH family 12 glycoside hydrolases, designated *VvGH12A* and *VvGH12B*, were identified in the *V. volvacea* genome (Bao *et al*. 2013; Chen *et al*. 2013). A multiple-sequence alignment was constructed using MEGA5.1 based on the amino acid sequences of the enzymes listed in Fig. 1. The phylogenetic tree of fungal glycoside hydrolases in GH family 12 was constructed *via* neighbor-joining methods using MEGA version 5.1 on the basis of multiple sequence alignment using ClustalW software (http:// www.genome.jp/tools/clustalw/). As shown in Fig. 1, the fungal GH family 12 glycoside hydrolases were classified into 4 distinct subfamilies, according to the division of GH family 12 enzymes. The basidiomycetous GH12 enzymes were isolated from the ascomycetous ones and classified into two subfamilies: basidiomycetous GH12-1 and GH12-2, respectively (Fig. 1).

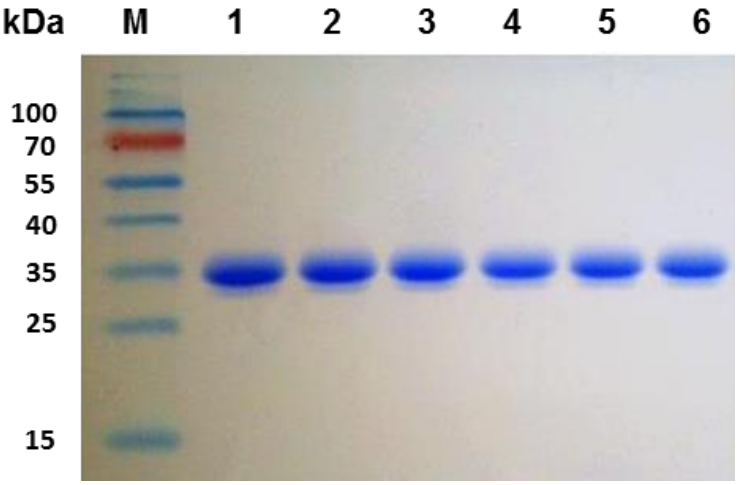

Fig. 2. SDS-PAGE of VvGH12A and mutants. Lanes: M, protein markers; lanes 1–6, VvGH12A, D98A, D98T, D98H, D98Q, and D98N, respectively.

VvGH12A and VvGH12B cluster in basidiomycetous subfamily GH12-1 and subfamily 12-2, respectively. VvGH12A and VvGH12B are most closely related to putative glycoside hydrolases from *Polyporus arcularius* (64.34% similarity) and *Hypsizygus marmoreus* (73.11% similarity), respectively. Basidiomycetous subfamily GH12-1 did not contain consensus sequences of NNLWG (Box 1), ELMIW (Box 2), and GTEPFT (Box 3), which are highly conserved in the ascomycetous subfamily GH12-1 enzymes (Goedegebuur *et al.* 2002). VvGH12A was functionally expressed in *P. pastoris*, but not succeeded for expression of VvGH12B. The recombinant VvGH12A with a C-terminal 6× His-tag was purified by Ni-NTA agarose gel affinity chromatography. SDS-PAGE analysis showed that the purified VvGH12A appeared as single band with a molecular mass of 34 kDa, slightly higher than its theoretical molecular mass (27.2 kDa) (Fig. 2).

Its optimal pH and temperature were 6.5 and 40 °C, respectively (Fig. 3A and 3B). After being treated at a different pH value for 24 h, the recombinant VvGH12A was very stable at a pH from 3.0 to 9.0, where almost 80% of the overall activity was preserved (Fig. 3C). The enzyme was stable at 40 °C; as shown in Fig. 3D, over 90% of the overall enzyme activity was preserved after incubating the VvGH12A for 90 min. However, the residue activity was reduced to about 70% of the initial activity after incubating the VvGH12A at 45 °C for 90 min (Fig. 3E).

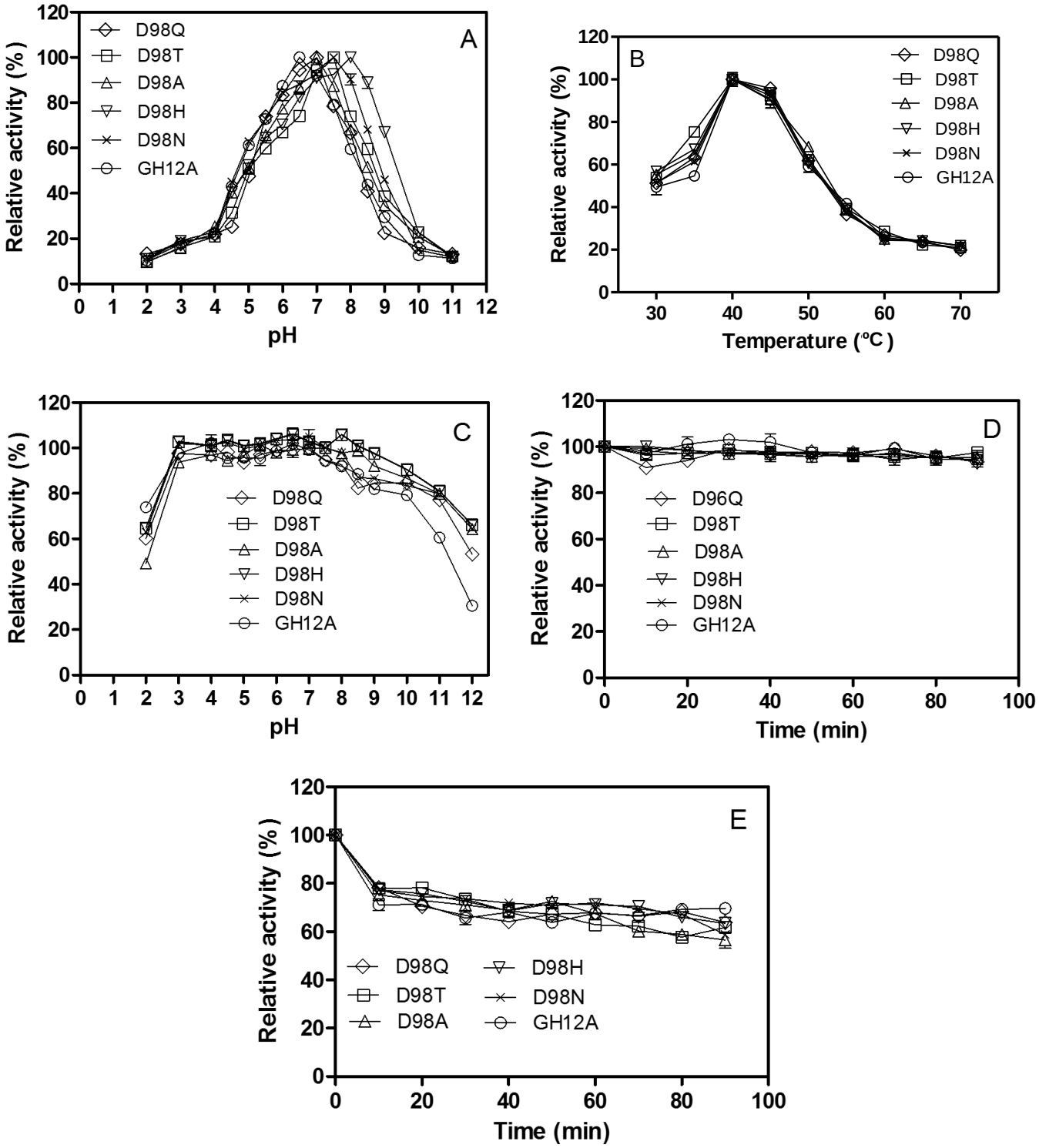

Fig. 3. Effects of pH (A) and temperature (B) on the activity of VvGH12A and mutants and effects of pH (C), and temperature at 40 °C (D) and 45 °C (E) on the stability of VvGH12A and mutants. Values shown are means of triplicate determinations ± standard error (SE).

Substrate Specificity and Mode of Action

The substrate specificity assay showed that the recombinant VvGH12A had highest activity for CMC (100%), followed by barley β-glucan (63.0%), RAC (33.3%), lichenan (29.2%), and glucomannan (18.1%). The enzyme also displayed activity towards xyloglucan (8.1%), FP (5.5%), and xylan (5.0%). Furthermore, VvGH12A was active on oat and wheat bran, natural substrates rich in β-glucan. No activity was measured with laminarin and chitosan (Table 1). This data agrees well with previous findings that subfamily GH12-1 members have broad specificity for substrates with various β-1,4-glucans activity (Kim *et al*. 2001; Grishutin *et al*. 2006; Picart *et al*. 2012). However, VvGH12A differed remarkably with most of subfamily 12-1 members, which showed preference for β-1,3-1,4-mixed glucans such as barley β-glucan and lichenan over polysaccharides with only β-1,4 linkages (Takeda *et al*. 2010; Segato *et al*. 2017). In contrast, VvGH12A displayed higher activity on polysaccharides with only β-1,4 linkages than β-1,3-1,4-mixed glucans. Furthermore, this enzyme was active on xylan and mannan. VvGH12A should be considered as a non-typical endoglucanase, differing from both the typical endoglucanases because of its broad substrate specificity and the β-1,3-1,4-glucanase because of its highest activity on CMC. This finding was very similar to that shown by a GH12 endoglucanase (EG28) from *P. chrysosporium* and EG III from *T. reesei* (Henriksson *et al*. 1999; Grishutin *et al*. 2006).

Table 1. Specific Activities of VvGH12A on Different Substrates

Substrates	Specific Activity (U μmol^{-1})	Relative Activity (%)
CMC-Na	149.94 ± 5.72	100
1,3−1,4-β-Glucan	94.47 ± 2.24	63.0
Xyloglucan	12.09 ± 0.55	8.1
Xylan	7.53 ± 0.55	5.0
Glucomannan	27.10 ± 1.23	18.1
RAC	49.88 ± 1.21	33.3
Filter paper	8.23 ± 0.58	5.5
Laminarin	ND	ND
Chitosan	ND	ND
Lichenan	43.73 ± 3.87	29.2
Destarched oat spelt bran	11.94 ± 1.04	8.0
Destarched wheat bran	13.82 ± 0.86	9.2

The recombinant VvGH12A showed a K_m value of 8.50 mg/mL and a V_{max} value of 264.52 U μmol/min of protein using CMC as the substrate. The reducing sugars in soluble and insoluble fractions were separately measured after generated by VvGH12A on RAC. The ratio of reducing sugars in soluble to insoluble fraction was 2.57 at 0.5 h, but increased to 3.90 at 4 h against RAC (Fig. 4), suggesting that it has a weak processivity due to both "endo" (on CMC) and "exo" types of enzyme activity.

Classic endoglucanases randomly cleave the interior β-1,4-glycosidic bonds in cellulose. However, several GH5 and GH9 family processive endoglucanases catalyze the hydrolysis of cellulose in both endo- and exo-mode (in processive mode) (Gilad *et al*. 2003; Li *et al*. 2007; Zheng and Ding 2013). Their processivities are commonly found to be more than 3.5. CBMs are significant for endoglucanase processivity by aiding processive movement of endoglucanases (Bommarius *et al*. 2014; Pan *et al*. 2016). Only a few cellulases were identified as processive endoglucanases independent of CBM and are reported to mediate degradation of cellulose (Sakon *et al*. 1997; Watson *et al*. 2009;

Zhang *et al*. 2014). Normally, due to lack of CBM, the hydrolysis of RAC by GH12 endo-glucanse AcCel12B from *Acidothermus cellulolyticus* 11B did not occur via processive mode at either the initial rapid phase or the later slow phase (Wang *et al*. 2015). The ratio of reducing sugars in soluble to insoluble fraction increased from 2.57 to 3.90 on RAC, as the reaction time was pro-longed from 0.5 h to 4 h, indicating VvGH12A has a weak processivity. The VvGH12A processivity was very similar to that of GH5 endoglucanase (CHU 2103) without CBM from *Cytophaga hutchinsonii* (Zhang *et al*. 2014), but much lower than EG1with CBM1 from *V. volvacea* (Zheng 2013), and other typically modular processive endoglucanases (Irwin *et al*. 1998; Li *et al*. 2007).

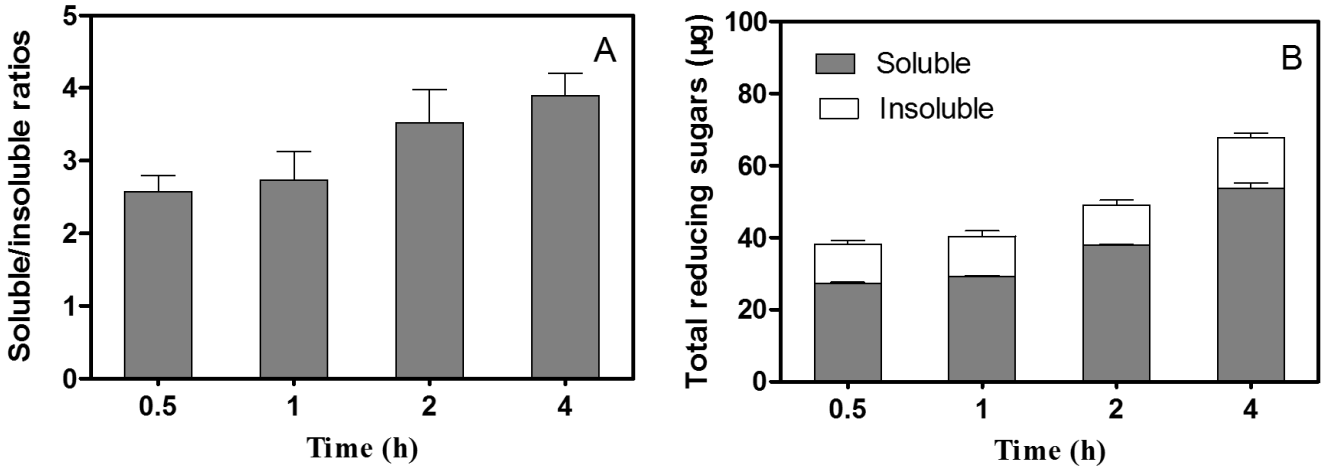

Fig. 4. The ratios of the reducing sugars in soluble fraction to insoluble fraction released from RAC (A), and total soluble and insoluble sugars released from RAC (B). Values shown are means of triplicate determinations± standard error (SE).

Engineering of the Optimum pH

The GH12 family enzymes catalyze hydrolysis through a double-displacement mechanism that retains an anomeric configuration (Sandgren *et al.* 2005). Two glutamic acid residues, E121 and E212 in VvGH12A, which are highly conserved in the sequences of GH12 enzymes from *Trichoderma reesei* (TrEGIII) and other species, might serve as the nucleophile and acid/base catalyst located in the active site. It has previously been reported that Asn95 in TrEGIII is the crucial residue affecting the enzyme activity pH-profile (Tishkov *et al.* 2013). VvGH12A has Asp98 in similar position, so the substitution was introduced at the Asp98 site by site-directed mutagenesis. The five mutants D98A, D98T, D98H, D98Q, and D98N were successfully expressed in *P. pastoris*. SDS-PAGE analysis revealed that these mutants have a similar molecular mass as wild-type VvGH12A (Fig. 2). The replacement of D98 with an amide (Gln or Asn) residue resulted in an increase in the optimum pH from 6.5 to 7.0 or 7.5. The replacement of D98 with a nonpolar (Ala) or neutral (Thr) residue resulted in an increase in the optimum pH from 6.5 to 7.5. The replacement of D98 with a basic (His) residue resulted in an increase in the optimum pH from 6.5 to 8.0. The mutants and wild-type enzymes had the same temperature optima and also displayed similar pH and thermal stability (Fig. 3). These substitutions did not affect catalytic activity, since the substrate specificity was retained at similar level for the mutants and wild-type enzyme (data not shown).

Reengineering activity pH-profiles is of importance for industrial applications of enzymes (Joshi *et al*. 2000). Protein engineering such as site-directed mutagenesis has

been used in altering the pH profiles of xylanase, α-amylase, glucoamylase, endoglucanase, and phytase (Fang and Ford 1998; Joshi *et al*. 2000; Nielsen *et al*. 2001; Turunen *et al*. 2002; Kim *et al*. 2006; Qin *et al*. 2008). The GH 12 glycoside hydrolases have a compact β-sandwich structure with the substrate binding site on the concave face of the β-sheet (Sandgren *et al*. 2005). Previous studies revealed the amino acid residue Asn95, situated at the distance of hydrogen bond formation from the Glu residue (a general acid residue in catalytic mechanism), directly affects the pH-profile of the enzyme activity of EG III from *Trichoderma reesei* (TrEGIII) (Tishkov *et al*. 2013). Using site-directed mutagenesis, the Asp98 in VvGH12A was replaced with a nonpolar (Ala) or neutral (Thr) or basic (His) or amide (Gln or Asn) residue. The single amino acid substitution did not alter the enzyme specific activities against soluble CMC and other glucans. However, the enzyme pH-optimum was shifted to neutral pH (from pH 6.5 to pH 7.0-7.5) when Asp98 was replaced with nonpolar or neutral or amide residue; the enzyme pH-optimum was shifted to more alkaline pH (from pH 6.5 to pH 8.0) when Asp98 was replaced with alkaline residue. This phenomenon indicated that the enzyme pH-optimum was significantly affected by the acidity and basicity of amino acid at this site.

Synergistic Action in Saccharification of Delignified Rice Straw between VvGH12A and Cellulolytic Enzyme

An efficient hydrolysis of lignocellulosic biomass to soluble sugars for biofuel and biochemical production necessitates the synergistic action of endoglucanases (E.C. 3.2.1.4), exoglucanases/cellobiohydrolases (E.C. 3.2.1.91 and 3.2.1.176), β-glucosidases (E.C. 3.2.1.21) belonging to different glycosyl hydrolase families as well as some auxiliary enzymes in the crude enzyme complex of cellulolytic microorganisms (Lynd *et al.* 2002). The roles of GH12 family endoglucanses in lignocellulosic biomass hydrolysis had not been fully evaluated and even overlooked compared to other GH family endoglucanases due to lack of CBM. In this study, synergistic action in enzymatic saccharification of delignified wheat straw between VvGH12A and cellulolytic enzyme was carried out using VvGH12A and crude cellulase under four different conditions considering the differences in optimal conditions for VvGH12A and crude cellulase (Fig. 5). VvGH12A alone showed little action on delignified wheat straw in 48 h. The addition of purified VvGH12A (1 U) obviously increased the saccharification efficiency of crude cellulase in 48 h to some extent under any conditions. The increase of 9.0% was achieved under optimum condition of crude cellulase (pH 4.8 and 50 °C) compared to the sum of individual GH12A and crude cellulase alone. The higher increase of approximately 16% was obtained when saccharification of delignified wheat straw was carried out at the condition with pH 6.5 and a temperature of 50 °C. This result demonstrated the synergistic action between VvGH12A and cellulolytic enzyme from *T. reesei* D-86271 (Rut C-30) in saccharification of delignified biomass.

To date, only a few GH12 enzymes have been identified from the white- and brown-rot basidiomycetes. It was proposed that the basidiomycetous GH12 enzymes function to facilitate hyphal elongation and nutrient acquisition via cleaving plant hemicellulosic polymers such as xyloglucan and 1,3-1,4-β-glucan (Takumi *et al*. 2013). The synergistic stimulatory effect with the crude cellulase demonstrated that VvGH12A played a functional role in cellulase cocktail for efficiently hydrolyzing plant cell wall polysaccharides. The similar or even higher results were previously reported for ascomycetous GH12 enzymes. Narra *et al.* (2014) reported that the addition of high

dosage GH12 endoglucanse (65 U) from *Aspergillus terreus* to crude cellulase showed 38.7% increase in saccharification efficiency of the delignified rice straw compared to the crude cellulase alone.

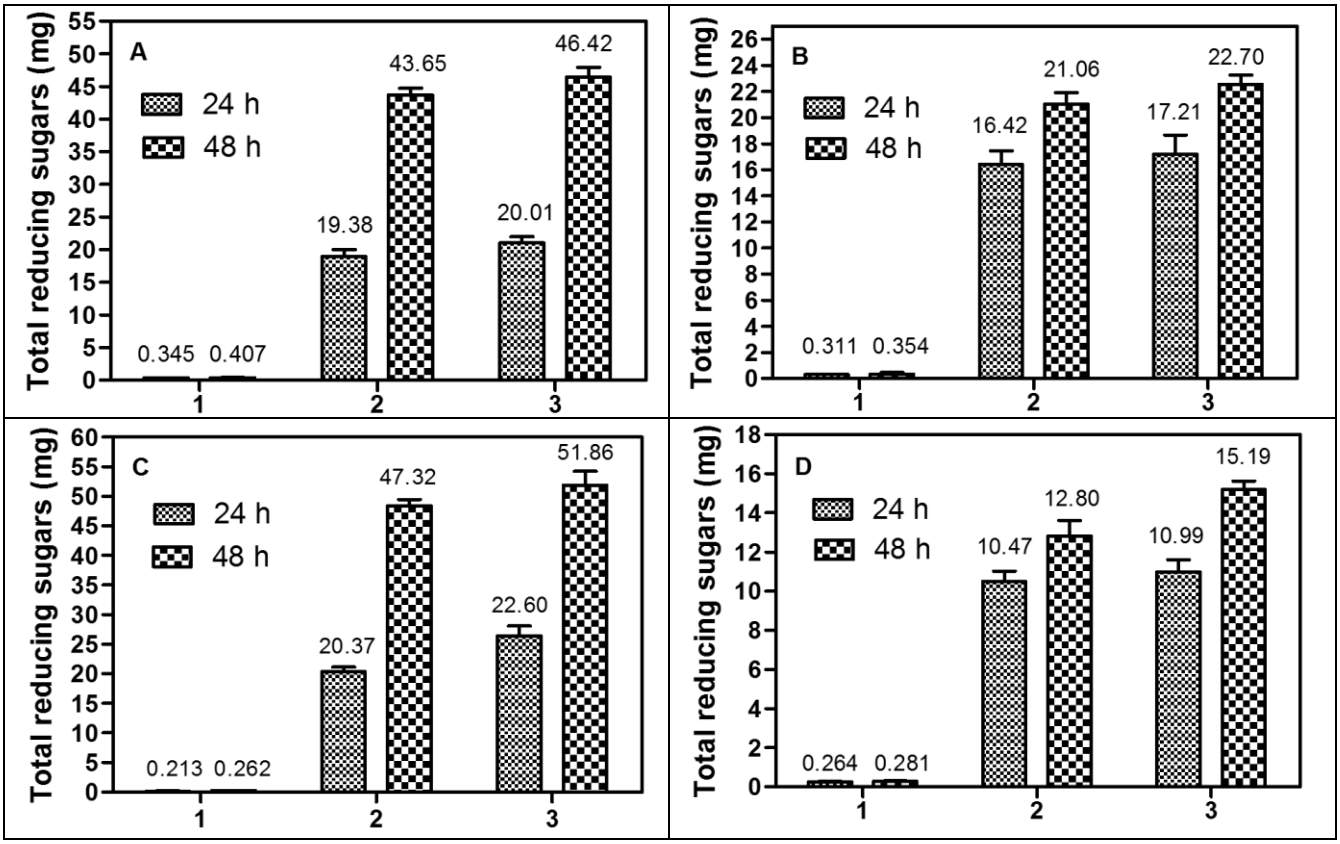

Fig. 5. Synergistic action in saccharification of delignified wheat straw between VvGH12A and crude cellulose from Trichoderma reesei D-86271 (Rut C-30) under 40 °C and pH4.8 (A), 40 °C and pH6.5 (B), 50 °C and pH4.8 (C), and 50 °C and pH6.5 (D), respectively. 1, VvGH12A only; 2, crude cellulase only; 3, VvGH12A+crude cellulase.

CONCLUSIONS

1. Two putative GH12 genes were identified from *Volvariella volvacea* genome, and one recombinant enzyme VvGH12A was characterized.
2. VvGH12A possessed processive hydrolysis mode. VvGH12A displayed higher activity on polysaccharides with only β-1,4 linkages than β-1,3-1,4-mixed glucans. Furthermore, this enzyme was also active on xylan and mannan. VvGH12A should be considered as a nontypical endoglucanase, differing from both the typical endoglucanases and the β-1,3-1,4-glucanase.
3. The pH-optimum was significantly affected by the acidity and basicity of amino acid at site 98. Replacing Asp98 with basic residues shifted the optimum pH to higher pH-optimum.

4. Synergistic action with crude cellulase in saccharification of delignified wheat straw, suggesting that VvGH12A played a functional role in the cellulase cocktail for efficiently hydrolyzing plant cell wall polysaccharides.

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