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SHORT COMMUNICATION

Isolation and characterization of a novel GH67 α-glucuronidase from a mixed culture

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Abstract Hemicelluloses represent a large reservoir of carbohydrates that can be utilized for renewable products. Hydrolysis of hemicellulose into simple sugars is inhibited by its various chemical substituents. The glucuronic acid substituent is removed by the enzyme α-glucuronidase. A gene (*deg75-AG*) encoding a putative α-glucuronidase enzyme was isolated from a culture of mixed compost microorganisms. The gene was subcloned into a prokaryotic vector, and the enzyme was overexpressed and biochemically characterized. The DEG75-AG enzyme had optimum activity at 45 °C. Unlike other α-glucuronidases, the DEG75-AG had a more basic pH optimum of 7–8. When birchwood xylan was used as substrate, the addition of DEG75-AG increased hydrolysis twofold relative to xylanase alone.

Keywords α-Glucuronidase · Library screening · Hemicellulose · Alkaline-active

Abbreviations

GH	Glycoside hydrolase
MeGlcA	4-*O*-methyl-D-glucuronic acid
DEG75-AG	α-Glucuronidase from compost microorganism
IPTG	Isopropyl-β-D-thiogalactopyranoside

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Introduction

One of the most viable renewable resources for the world's fuel and chemical feedstock needs is plant material. Great attention has been placed on harnessing the structural carbohydrates (lignocelluloses) of plants [25]. The primary challenge of utilizing lignocellulosic biomass is that the structural carbohydrates serve a protective function and thus have evolved to be resistant to breakdown into simple sugars. This characteristic is accomplished in part by the crosslinked matrix of three of the main components of biomass (cellulose, hemicellulose, and lignin), and each of these fractions is composed of distinct precursors and linkages [26]. One of the greatest difficulties in breaking down lignocellulose is to develop the appropriate mixture of enzymes that will hydrolyze the various bonds found in this substrate [3, 9].

Hemicellulose reinforces the lignocellulose matrix by forming hydrogen bonds with the cellulose microfibrils and covalent linkages to lignin [10, 27]. Glucuronoxylan is one of the most common forms of hemicellulose. The backbone is composed of a polymer of β-1,4-linked xylose residues. This polymer can be cleaved by endoxylanase enzymes (E.C. 3.2.1.8) to produce short xylooligosaccharides that can then be acted upon by β-xylosidase enzymes (E.C. 3.2.1.37) to release fermentable xylose monomers [21, 23]. However, the xylan backbone is often decorated with multiple chemical constituents, and these substituents can impede complete hydrolysis by the endoxylanases and β-xylosidases. For instance, the 4-*O*-methyl-D-glucuronic acid (MeGlcA) moiety can attach to xylose via an α-1,2-glycosidic bond. This modification sterically prevents enzymatic hydrolysis of the xylosidic bonds of xylan. In addition, the MeGlcA group can form covalent crosslinks with lignin, further contributing to the recalcitrance of the lignocellulose matrix [5, 6, 29].

The enzymes that release MeGlcA substituents from the xylan substrate backbone are the α-glucuronidases (E.C. 3.2.1.131) [7]. A few examples have been isolated from individual, pure cultures [8, 24]. At present, all the cloned α-glucuronidase enzymes can be classified based on their catalytic domains into either glycosyl hydrolase (GH) family 67 or family 115 [7, 12, 20]. The GH67 α-glucuronidases act only on xylooligomers, whereas the GH115 enzymes will release MeGlcA from polymeric glucuronoxylan [20].

The crystal structures of two GH67 α-glucuronidases from *Cellvibrio japonicus* and *Geobacillus stearothermophilus* have been determined [11, 18]. The central domain of the enzymes forms a $(\beta/\alpha)_8$-barrel structure that contains the catalytic region. Dimerization is mediated in the *C. japonicus* and *G. stearothermophilus* enzymes by the central/C-terminal domains or the C-terminal domain alone, respectively. The oligomerization state has been demonstrated to play an important role in the activity of α-glucuronidase [22].

In this report, we describe the discovery of a novel α-glucuronidase (DEG75-AG). The gene encoding this enzyme was isolated from a mixed culture of compost microorganisms. The recombinant enzyme was purified and biochemically characterized. It was found to have an activity pH optimum higher than that of any previously reported α-glucuronidase. It was also demonstrated to function synergistically with xylanase in the hydrolysis of xylan substrate.

Materials and methods

Bacterial strains and reagents

Escherichia coli SOLR and BL21(DE3)pLysE strains were obtained from Agilent (Santa Clara, CA, USA) and Novagen (Madison, WI, USA), respectively. DNA restriction and modification enzymes were purchased from New England Biolabs (Ipswich, MA, USA). The mixed aldouronic acid substrate (Megazyme, Bray, Ireland) was composed of a mixture of aldo-triouronic, also-tetrauronic, and aldo-pentauronic acids (approximately 2:2:1 ratio), and the MeGlcA was linked to the non-reducing xylopyranosyl. All other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Library construction and screening

Using a compost starter (Dr. Earth Company, Vacaville, CA, USA) as inoculant, a mixed population of compost microorganisms was cultured in EZ Rich liquid medium (Teknova, Hollister, CA, USA) supplemented with 0.67 % each of oat spelt, birchwood, and beechwood xylans (Sigma-Aldrich) as the primary carbon source. The cultures were shaken at 60 °C for 40 h before harvesting. Cells were disrupted and genomic DNA was isolated using the Fast-DNA kit (Qbiogene, Carlsbad, CA, USA). The cell lysis was conducted with the CLS-TC buffer, a single 0.25-in. ceramic sphere, and garnet dust according to the manufacturer's instructions.

The isolated genomic DNA was partially digested with *Apo*I restriction enzyme. The digested DNA was separated on an agarose gel by electrophoresis, and the fragments sized 4–10 kb were excised and purified using the Wizard SV Gel Clean-Up kit (Promega, WI, USA). The fragments were ligated to a lambda phage vector and packaged into lambda phage (Lambda ZAP II Vector and Gigapack III Packaging Extract, Agilent). The phage library was propagated and subjected to in vivo excision resulting in the genomic DNA fragments in the pBluescript vector backbone in the host *E. coli* SOLR strain according to the manufacturer's protocol.

Screening the library for α-glucuronidase activity was conducted as previously described [14]. Briefly, the *E. coli* SOLR cells containing the genomic DNA library were spread onto nylon membranes (Genetix, MA, USA) that had been placed on Luria–Bertani agar containing ampicillin (25 μg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM). The bacteria were grown at 37 °C overnight under these inducing conditions. The membrane was then transferred colony-side down onto a lysis/activity assay agarose medium (50 mM sodium phosphate (pH 6), 0.86 mg/ml mixed aldouronic acid (Megazyme), 4 U/ml horseradish peroxidase, 2 U/ml pyranose oxidase, 1 mM 3,3-diaminobenzidine tetrahydrochloride (DAB), 1 mg/ml lysozyme, 1 mg/ml polymyxin B sulfate, and 1.5 % low-melting-point agarose). The membrane was incubated overnight at 37 °C, and positive colonies were chosen based on the production of a dark, brown precipitate and rescreened to confirm activity. The cloned genomic DNA fragments from the positive colonies were isolated and sequenced. The DNA sequence was analyzed for open reading frames (Vector NTI, Invitrogen, CA, USA) and protein homologies (BLAST) [1].

deg75-AG cloning and protein expression and purification

The *deg75-AG* gene was subcloned into the pET29b+ prokaryotic expression vector (Novagen). The gene was amplified by PCR using the following primers, which had an *Nde*I and an *Xho*I restriction enzyme recognition sites engineered in the linker sequences (underlined) of the 5′ and 3′ ends of the gene, respectively:

75-aglu-5-c: cgaacatATGGAGGCATCGCCACCG
75-aglu-3: cggctcgagATAAATCGTGCGGCCGTGC

The PCR product and the pET29b+ vector were digested with the *Nde*I and *Xho*I restriction enzymes, purified, and ligated. The resulting plasmid contained the *deg75-AG* gene fused in-frame to a 6X-histidine tag at the 3′ end of the gene.

The expression plasmid was transformed into *E. coli* strain BL21(DE3)pLysE. Transformants were cultured in Luria–Bertani broth at 37 °C until the cells reached an optical density of 1.5 at 600 nm. At that time, protein expression was induced by adding IPTG to a final concentration of 1 mM. After 3 h, the cells were harvested and lysed with CelLytic B reagent (Stratagene) supplemented with 100 mM protease inhibitor cocktail (P8340, Sigma-Aldrich), 50 U/ml benzonase (Novagen), and 2 mg/ml chicken egg white lysozyme. After lysis, sodium chloride and imidazole were added to final concentrations of 300 and 20 mM, respectively. The lysate was centrifuged at 4 °C, and the soluble protein was applied to a HisTrap HP column (GE Healthcare, Piscataway, NJ, USA) and eluted by imidazole gradient (20–500 mM) in running buffer (50 mM sodium phosphate (pH 7.2) and 300 mM sodium chloride). The peak fractions were collected and the buffer was exchanged by applying the protein to an EconoPac 10DG column (Bio-Rad, Hercules, CA, USA) that had been equilibrated in 50 mM sodium phosphate (pH 7), 10 mM sodium chloride, and 10 % glycerol.

α-Glucuronidase activity assays

Enzymatic reactions were composed of 0.07 μM purified DEG75-AG enzyme, 6 mg/ml mixed aldouronic acid substrate (reduced form, Megazyme), 0.5 mg/ml bovine serum albumin, and 100 mM universal buffer (33.3 mM each of citric acid, monobasic potassium phosphate, and boric acid). Reactions were conducted at various pHs (4–11) and temperatures (30–60 °C). Temperature stability was determined by incubating the enzyme at various temperatures (40, 50, and 60 °C) up to 120 min at pH 7, cooling the enzymes on ice, then assaying for activity at 45 °C. To determine the impact of various cations, DEG75-AG activity was measured in the presence of 1 mM cations. One unit of activity is defined as the amount of enzyme that releases 1 μmol of MeGlcA per minute.

The extent of enzymatic digest was quantified by measuring the release of MeGlcA from the substrate using a colorimetric assay based on the Milner and Avigad method [17]. Briefly, 0.025-ml enzymatic reactions were stopped by the addition of 0.1 ml of copper solution A. The mixture was boiled for 15 min, cooled to 4 °C, and then 0.063 ml arsenomolybdate reagent was added. Absorbance of the solution was measured at 750 nm. To determine the enzyme-liberated MeGlcA by the Milner-Avigad method, calibration was done with GlcA. All assays were conducted in triplicate.

Xylan hydrolysis assays

Birchwood xylan (1 %) was used as the substrate for xylanase assays. Reactions using combinations of a GH10 endoxylanase enzyme (MANF-X10, 9.2 nM) [15] and DEG75-AG (0.35 μM) were incubated with the xylan substrate at 45 °C at pH 8 up to 24 h. The extent of enzymatic digestion was quantified by measuring the release of reducing sugars [16]. Reactions of 50 μl were stopped by the addition of 75 μl of DNSA reagent (1 % dinitrosalicylic acid and 30 % potassium sodium tartrate in 0.5 M sodium hydroxide), heated to 100 °C for 5 min, and cooled to 23 °C. The absorption at 540 nm of the resulting solution was measured on a microplate reader, and xylose standards were used to plot a calibration curve. All reactions were conducted in triplicate.

Results and discussion

deg75-AG gene cloning and structure

A genomic DNA library was generated from a mixed population of compost microorganisms. This library was screened using a high-throughput assay previously developed to detect α-glucuronidase activity [14]. One of the genomic clones we isolated and sequenced was a 3.9-kB fragment that encoded a putative α-glucuronidase enzyme (Fig. 1a) and sugar transporter (data not shown) on the same coding strand (GenBank ID: JQ282894). The α-glucuronidase gene (*deg75-AG*) encoded a predicted 84.5-kD protein. BLAST analysis identified a conserved GH67 motif from amino acids 74–748 (nucleotides +220 to +2,244). The DEG75-AG protein was highly homologous to several α-glucuronidase enzymes in the GenBank database with up to 60 % identity to the enzyme from *Paenibacillus* sp. (accession ZP_04851095). When aligned to the sequence of the two α-glucuronidases with solved structures, the DEG75-AG has much higher identity to the enzyme from *G. stearothermophilus* (57 %) [11] compared to that of *C. japonicus* (36 %) [18]. Thus, it seems possible that any multimerization of the DEG75-AG enzyme would be mediated primarily through the C-terminal domain as observed with the *G. stearothermophilus* enzyme [11, 22]. Within the DEG75-AG protein sequence are two highly conserved amino acids (D434 and E462) that have been implicated as critical catalytic residues [31]. The 5′ end of the gene contains five 29-base pair repeats (Fig. 1a, b). These repeats are not in the same reading frame, so the repeating motif is not manifested in the amino acid sequence, thus it is unclear if the repeats have any significance upon the activity of the enzyme.

When the upstream 5′ region of the gene was analyzed by a bacterial promoter search program (BPROM, Softberry, Inc., Mount Kisco, NY, USA), −35 and −10 promoter elements were identified (Fig. 1a). This further substantiates that the correct 5′ end of the gene, including the 29-base-pair repeats, was identified. The 3′ untranslated region of the gene encodes a potential stem-loop structure (nucleotides +2,307 to +2,324) with seven bases on each side of the stem and a four-base loop. This is followed by a T-rich sequence motif that may represent a transcription termination signal [30].

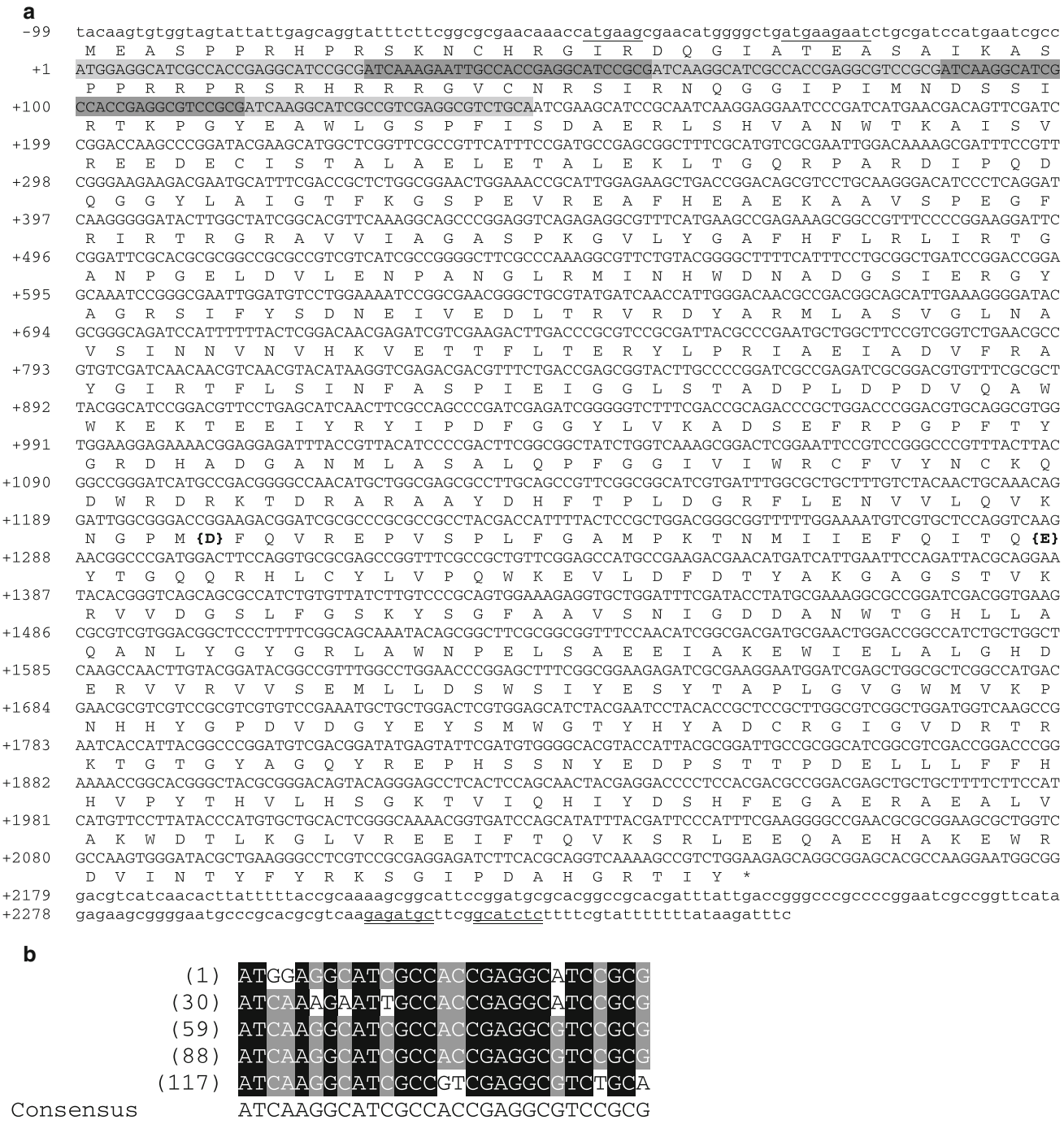

Fig. 1 *deg75-AG* gene and flanking regions. **a** α-Glucuronidase gene is encoded by nucleotides +1 to +2,244. Potential promoter regions are *underlined*. Nucleotide repeats are denoted by alternating *light gray*/*dark gray* highlighting. Catalytic residues D434 and E462 are marked by *curly brackets*. Transcription termination signal is *double-underlined*. **b** Alignment of the five 29-nucleotide repeats

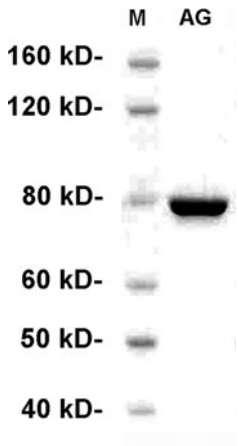

Fig. 2 Purified DEG75-AG enzyme. M, molecular weight marker; AG, α-glucuronidase enzyme

DEG75-AG biochemical characterization

The *deg75-AG* gene was subcloned into a prokaryotic plasmid and the enzyme was overexpressed and purified from a bacterial host with a yield of 18 mg/l of culture (Fig. 2). Enzyme activity assays were conducted with an aldouronic acid preparation (Megazyme) that was a mixture of aldotriouronic, aldo-tetraouronic, and aldo-pentauronic acids (2:2:1 ratio). The optimum temperature for activity of the DEG75-AG enzyme was 45 °C with a maximum of 11.7 U/mg enzyme (Fig. 3a). This temperature optimum is similar to that seen for α-glucuronidases from other microorganisms (35–50 °C) [7]. However, it is significantly lower compared to the optimal temperatures of α-glucuronidases from thermophilic microorganisms (60–85 °C) [19, 31].

The optimum pH for the DEG75-AG enzyme was determined at 45 °C. The enzyme had the greatest activity at pH 7 and pH 8 (Fig. 3b). There is a dramatic decrease in enzyme activity at pH 6 and pH 9, and there is little activity at pH 4 and pH 5. The pH profile of DEG75-AG is in contrast to the vast majority of other α-glucuronidases which have pH optimums of 4.5–6.5 [7]. Only one other enzyme that has been classified as an α-glucuronidase has been reported to have a basic pH optimum. The α-glucuronidase from *Thermotoga maritima* was found to have a pH optimum of 7.8 [28]. However, the *T. maritima* enzyme acted only on an artificial substrate (*p*-nitrophenyl α-D-glucuronopyranoside) and was unable to release glucuronic acid from native substrates. Thus, it is unlikely that the *T. maritima* enzyme would have any industrial value in biomass hydrolysis.

The thermostability of the DEG75-AG was tested by preincubating the enzyme at various temperatures before assaying for residual activity at 45 °C (Fig. 3c). After 2 h at 40 and 50 °C, the enzyme retained most of the initial activity. However, at 60 °C, half of the activity was lost at 30 min, and no activity remained after 1 h.

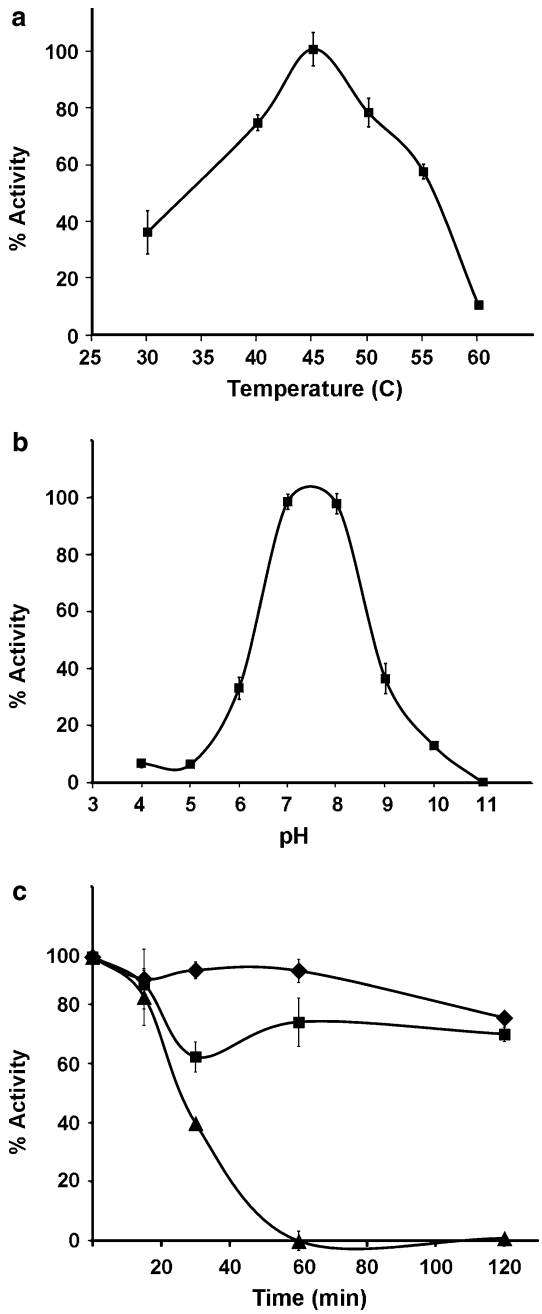

Fig. 3 Properties of DEG75-AG. **a** Activity of DEG75-AG across a range of temperatures. **b** Activity of DEG75-AG across a range of pH at 45 °C. **c** Thermostability of DEG75-AG assayed at 45 °C and pH 7 after preincubating for various times at 40 °C (*diamonds*), 50 °C (*squares*), and 60 °C (*triangles*)

The activity of the DEG75-AG enzyme was tested in the presence of various metal cations ($CaCl_2$, $CoSO_4$, $CuSO_4$, $FeCl_3$, LiCl, $MgCl_2$, $MnSO_4$, and $ZnSO_4$). Overall, the activity was not dramatically impacted when cations were used at 1 mM (data not shown). Calcium addition resulted in a slight activity decrease (86.3 %) while the presence of manganese produced higher activity (117.4 %).

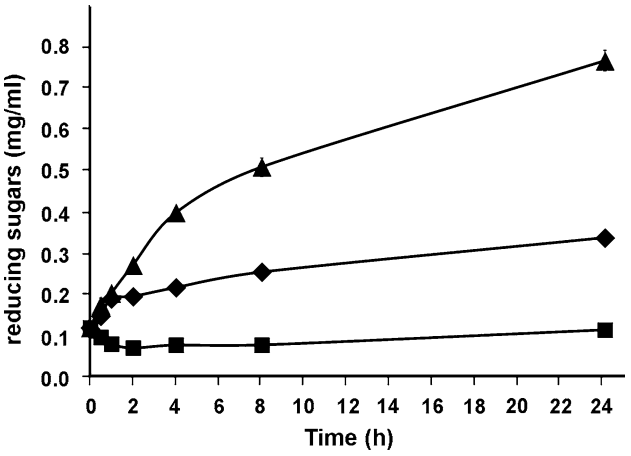

Fig. 4 DEG75-AG and xylanase activities on birchwood xylan. Time course of reaction with DEG75-AG (*squares*), xylanase (*diamonds*), and DEG75-AG/xylanase combination (*triangles*)

The ability of DEG75-AG to act synergistically with xylanase enzyme to hydrolyze xylan substrate was tested. One percent birchwood xylan, which is decorated mainly with MeGlcA groups, was incubated with different combinations of enzymes (Fig. 4). As expected, DEG75-AG alone did not result in any significant release of reducing sugar. When DEG75-AG was used in combination with xylanase enzyme, a large stimulation in activity was seen. At 24 h, there was more than a twofold increase in released reducing sugar for the DEG75-AG/xylanase combination (0.77 mg/ml) compared to the xylanase alone (0.34 mg/ml). The main products of the hydrolysis reaction were xylotriose, xylotetraose, and xylopentaose based on analysis by thin-layer chromatography (data not shown). The overall yield of each of these products was increased in the DEG75-AG/xylanase combination compared to the xylanase-alone reaction, but the relative ratios were similar.

In summary, the DEG75-AG enzyme represents a new α-glucuronidase that will hydrolyze native substrates synergistically with endoxylanase and will function optimally at a higher pH than previously reported enzymes of this family. DEG75-AG can potentially be utilized in industrial processes that occur under alkaline conditions. For instance, DEG75-AG could be combined with alkaline-active endoxylanases in biobleaching of pulp to dramatically decrease the use of harsh chemicals [2, 4, 13].

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