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An α-Glucuronidase Enzyme Activity Assay Adaptable for Solid Phase Screening

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Abstract Glucuronic acid is a common chemical moiety that decorates the xylan polymer of hemicellulose. This chemical substituent impairs both enzymatic and acidic hydrolysis of xylosidic bonds. The α-glucuronidase enzyme hydrolyzes the 1,2-linked glucuronic acid from the terminal, non-reducing xylose of xylo-oligosaccharides. There are relatively few α-glucuronidase genes in the public databases. We have developed an assay with commercially available reagents that can be used to search DNA libraries for α-glucuronidase genes in a high-throughput, solid phase activity screen.

Keywords Glucuronidase · Glucuronic acid · Hemicellulase · Activity assay

Introduction

The continued usage of fossil fuels to support the world economy has significant negative environmental consequences. Thus, there is great interest in utilizing renewable sources to supply our fuel and chemical feedstock needs. Lignocellulosic biomass is a plentiful renewable substrate and, therefore, has potential to displace a large fraction of products currently derived from fossil fuels. The majority of lignocellulose is cellulose, a polymer of β-1,4-linked glucose monomers that can be hydrolyzed by cellulase enzymes to produce sugar that can then be fermented into fuel such as ethanol [1, 2]. The cellulose is enmeshed in a matrix of hemicellulose and lignin [3]. The hemicellulose serves as a crosslinker between the cellulose and lignin by forming hydrogen bonds with the former and covalent bonds with the latter. This dense network occludes the cellulase enzymes from the cellulose substrate. Much effort has been focused on techniques to disrupt the hemicellulose in order to expose the cellulose to enzymatic hydrolysis.

Hemicellulose, the second most common component of lignocellulose, is composed primarily of xylan, a polymer consisting largely of β-1,4-linked xylose residues which can

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be hydrolyzed by xylanase enzymes [4, 5]. Unlike cellulose, hemicellulose has many different chemical moieties that branch from the main xylan chain [6–8]. The exact nature and ratios of these chemical entities depend on the source of the hemicellulose. Hardwood hemicellulose is decorated with acetyl and glucuronic acid [9]. Hemicellulose from softwood and grasses is substituted with arabinofuranosides and glucuronic acid [10, 11]. The arabinofuranosides are often modified with ferulic acid that can form covalent bonds to lignin [12].

The ubiquitous glucuronic acid moiety attaches to the xylose via a 1,2-glycosidic bond [5]. The glucuronic acid is often substituted with a methyl group at the 4-hydroxyl position. The presence of the glucuronic acid moiety inhibits xylanase access to the xylose polymer and also stabilizes neighboring xylose bonds against acid hydrolysis [13]. In addition, covalent bonds form between glucuronic acid and lignin, further decreasing enzyme access to the lignocellulose substrate [14–16].

Like the other xylan decorations, the glucuronic acid is removed by a specific accessory enzyme [17]. The α-glucuronidase enzymes (E.C. 3.2.1.131) from several microorganisms have been purified and characterized. Most of the α-glucuronidases will release glucuronic acid only if the chemical moiety is located on the terminal xylose on the non-reducing end of xylo-oligosaccharides [18–21]. However, there have been reports of α-glucuronidase enzymes that will hydrolyze internal glucuronic acid residues from glucuronoxylan [22–24]. α-Glucuronidases have been demonstrated to work synergistically with xylanase enzymes to hydrolyze hemicellulose substrates [25–27].

Despite the important nature of the glucuronic acid substituent, there are very few known α-glucuronidase-encoding genes (<40). This situation is caused in part by the lack of a high-throughput activity assay. In this report, we describe an activity assay for α-glucuronidase enzyme that uses a native substrate. Our new assay can be used in a high-throughput, solid phase format to screen for genes encoding α-glucuronidase activity. In addition, the activity assay can potentially be adapted to liquid format to allow for continuous kinetic assays.

Materials and Methods

Gene Cloning and Protein Expression

The gene encoding the *Geobacillus stearothermophilus* T6 α-glucuronidase (Genbank accession number DQ868502) was amplified by PCR using genomic DNA as substrate and the following two primers:

T6-ag-5: GCGGGCCATGGCGGCGGGATACGAACCTT
T6-ag-3: GCGGGCTCGAGCCGATAAATTTTCCGCCCGTATTG

The 5′ and 3′ primers contained terminal linker regions that encoded recognition sites for restrictions enzymes *Nco*I and *Xho*I, respectively. The PCR product and pET29b(+) vector (Novagen, Madison, WI, USA) were each digested with both *Nco*I and *Xho*I and ligated to obtain the pET29-Gste-AG-his-S expression construct.

The expression construct was introduced into *Escherichia coli* BL21(DE3)pLysE (Novagen) and a transformant was used to inoculate Luria–Bertani (LB) broth supplemented with kanamycin (25 μg/ml) at 37 °C. When the culture density reached an optical density of 0.5 at 600 nm wavelength, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce expression. After 3 h, the bacterial

cells were harvested, lysed, and centrifuged to collect the soluble recombinant α-glucuronidase. The enzyme was purified by applying the clarified lysate to a HisTrap HP column (GE Healthcare, Piscataway, NJ, USA) and eluted with an imidazole gradient (4 mM to 2 M) in buffer (50 mM sodium phosphate (pH 8) and 300 mM sodium chloride).

Phage Construction and Single Clone Excision

The gene encoding α-glucuronidase was amplified by PCR using the pET29-Gste-AG-his-S plasmid as substrate and the following primers:

T6-ag-5-RI: CGAGAATTCATGACGGCGGGATACGAACCTTG
T6-ag-3-RI: GAGGAATTCTCACCGATAAATTTTCCGCCCGTATT

Both primers had an *Eco*RI recognition site in the terminal linker region. The PCR product was digested with the *Eco*RI restriction enzyme and ligated to the λZAPII predigested vector DNA (Stratagene, La Jolla, CA, USA). The ligation and resulting phage were manipulated according to manufacturer's instructions. In brief, the ligation reaction was packaged into empty viral heads, and the resulting phage were amplified in *E. coli* XL1 cells (Stratagene). Phage from individual plaques were isolated and subjected to single clone excision to obtain the gene in a pBluescript (pBS) vector background in *E. coli* SOLR cells (Stratagene).

α-Glucuronidase Activity Assay

For solid phase assay, SOLR cells carrying appropriate pBS expression vector were spotted or spread onto positively charged nylon membranes (Genetix, Boston, MA, USA) that were overlaid on LB culture plates containing ampicillin (25 μg/ml) and IPTG (0.05 mM). After overnight growth at 37 °C, the membranes were removed and placed colony-side down onto detection medium (50 mM sodium phosphate (pH 6), 0.86 mg/ml mixed aldouronic acid (Megazyme, Ireland), 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) and incubated at 37 °C.

For liquid phase assay, purified α-glucuronidase was incubated with the same components as in the detection medium described above except no agarose, lysozyme, or polymyxin B sulfate was used. The reactions proceeded at 37 °C in 100-μl volumes. Reaction progress was monitored by periodically measuring absorbance at 400 nm using a Spectramax M2 plate reader (Molecular Devices Corporation, Sunnyvale, CA, USA).

Results

The overall strategy of the project was to develop an α-glucuronidase activity assay that could be adapted to screen genomic DNA libraries. Since bacteriophage are frequently used to create and maintain high density DNA libraries, we created bacteriophage stocks that had the *G. stearothermophilus* α-glucuronidase gene non-directionally cloned into the vector. The phage were then subjected to single clone excision resulting in *E. coli* SOLR cells that carried pBS expression vectors encoding the α-glucuronidase gene in one of two directions. Clones 1A and 9A represent pBS expression vector with the α-glucuronidase gene in the same or opposite direction as the IPTG-inducible P*lac* promoter, respectively. The various SOLR strains were spotted onto a nylon membrane resting on top of an LB agar plate, and

protein expression was induced with IPTG. Once the colonies had grown, the membrane was removed and placed face down on detection agarose that contained aldouronic acid, the natural substrate for α-glucuronidase enzyme. Aldouronic acid is composed of a glucuronic acid group attached via a 1,2-linkage to xylose or the non-reducing terminal residue of xylo-oligosaccharides. In this experiment, a commercial preparation of mixed aldouronic acid was used. This substrate contained aldobiouronic, aldotriouronic, aldotetraouronic, and aldopentaouronic acid. Although all four species served as substrates for α-glucuronidase, only the hydrolysis of aldobiouronic acid substrate was predicted to release xylose, the detection of which the assay relied upon. Pyranose oxidase, horse radish peroxidase, and DAB were then used to produce an insoluble brown precipitate from the resulting xylose as previously described [28]. The SOLR strain carrying the α-glucuronidase gene in the same direction as the P*lac* promoter (1A) produced a clear signal (Fig. 1). The SOLR strains carrying only pBS vector control or the α-glucuronidase in the opposite direction of the P*lac* promoter (9A) did not yield any significant precipitation. This result demonstrated the lack of background activity when conducting the assay using *E. coli* SOLR cells. The colonies growing on the membrane were lysed when placed face down on the detection medium. However, prior to placing the membrane onto the detection medium, it was possible to make a reference replica plate by performing a colony lift with another membrane (data not shown).

To determine the practicality of screening a large number of clones, approximately 22,000 SOLR cells carrying pBS vector were spread on a nylon membrane on inducing medium in a 10-cm petri dish. This density of cells is equivalent to 50,000 clones on a 15-cm petri dish, which is the concentration of bacteriophage plaque forming units (pfu) that is used in a typical high-throughput solid phase screening project. Approximately ten SOLR cells carrying the α-glucuronidase gene (1A) were mixed with the pBS-only SOLR cells before plating. After the cells were grown and induced at 37 °C, the membrane was placed face down on agarose containing the native substrate and detection enzymes as described above. Even at very high cell densities, the few clones that had the α-glucuronidase gene produced very clear signal (Fig. 2).

The activity assay was also evaluated in liquid format. Purified α-glucuronidase enzyme was added to the mixed aldouronic acid native substrate and detection enzymes in a small volume (100 μl). The reaction was carried out at 37 °C, and the extent of hydrolysis was

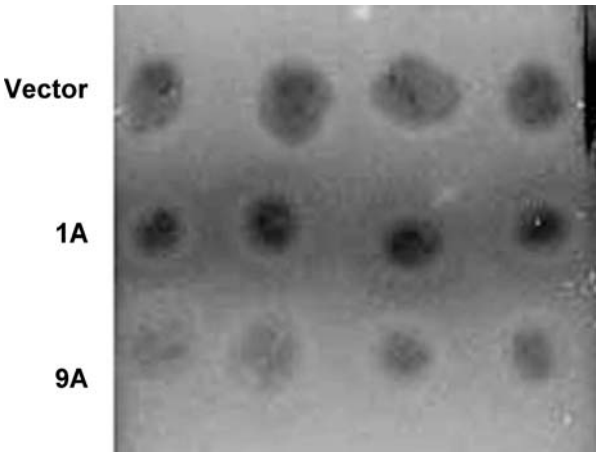

Fig. 1 α-Glucuronidase solid phase activity assay. *E. coli* SOLR transformants were spotted and grown on membrane and placed onto detection medium. *Vector*, cells carrying pBS vector control; *1A*, cells carrying α-glucuronidase gene in same direction as P*lac* promoter; *9A*, cells carrying α-glucuronidase gene in opposite direction as P*lac* promoter

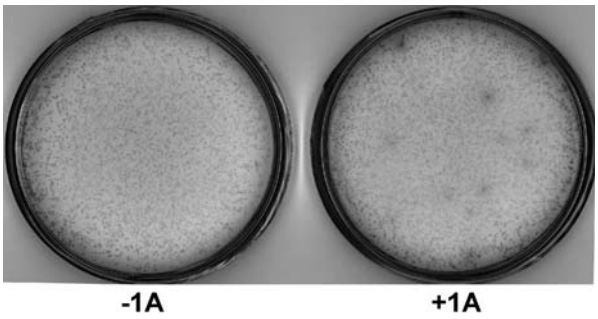

Fig. 2 High density α-glucuronidase solid phase assay. Each membrane on a 10-cm petri dish was spread with approximately 22,000 *E. coli* SOLR transformants carrying pBS vector control. Approximately ten SOLR transformants carrying α-glucuronidase gene were (*+1A*) or were not (*−1A*) mixed with the pBS vector control before spreading on the membrane

monitored by absorption measurements every 10 min. The window of time in which the rate of the reaction is constant can be easily determined (Fig. 3). The precise ratio of the various aldouronic acid chemical entities is unknown. However, if each chemical species were purified and used as substrate at defined concentrations, then this assay could be used to conduct real time kinetic measurements using natural substrates. Although only the aldobiouronic acid substrate would be expected to release xylose, α-glucuronidase hydrolysis of the larger species could also be monitored by supplementation with β-xylosidase enzyme. For instance, α-glucuronidase hydrolyzes aldotriouronic acid to release glucuronic acid and xylobiose. The xylobiose can then be hydrolyzed by β-xylosidase to xylose that can be visualized by the detection reagents. Indeed, when liquid activity assay using the mixed aldouronic acid native substrate is supplemented with β-xylosidase, a significant increase in signal is observed (data not shown).

Discussion

Despite the central role that α-glucuronidase enzymes have in biomass degradation, relatively few genes have been described. The α-glucuronidase genes that are currently known were isolated by several methods. Genes were discovered serendipitously because they were located on the same genomic DNA fragment as another hemicelluloytic gene that was the original target [29, 30]. Other α-glucuronidase genes have been tentatively identified based on homology to data from microbial genome sequencing databases.

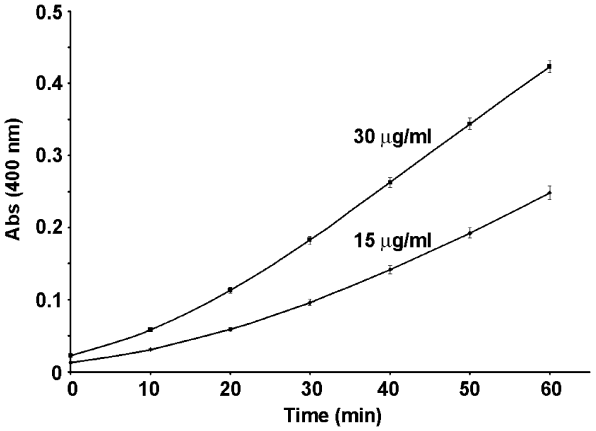

Fig. 3 α-Glucuronidase liquid phase activity assay. Activity of various amounts of α-glucuronidase enzyme (15 and 30 μg/ml) was monitored by periodic measurement of optical density at 400 nm

Another approach has been to use degenerate oligonucleotides to probe or amplify a DNA library for the specific gene. The information used to design the oligonucleotides can be derived from analyzing conserved regions by comparing multiple α-glucuronidase enzymes [20]. Alternatively, the endogenous α-glucuronidase enzyme can be purified and peptide sequence determined to provide more specific information to design the degenerate oligonucleotides [31]. If purified endogenous enzyme is available, it is also possible to generate antibodies against the protein, and then screen a DNA library for the expressed recombinant polypeptide [32]. None of the aforementioned strategies directly target α-glucuronidase activity; thus, genes that might encode α-glucuronidase activity but do not share the significant homologies would be missed. The α-glucuronidase gene from *Thermotoga maritima* is the one example of an activity-based cloning of which we are aware [33]. Pools of *E. coli* bacteria transformed with *T. maritima* genomic DNA fragments were screened for α-glucuronidase activity in a liquid format. Although relatively few clones were analyzed (<7,000), the α-glucuronidase gene was isolated.

There are several assays that measure α-glucuronidase activity. The most commonly used are those based on the copper reagent protocol of Milner and Avigad [34]. This assay measures the release of glucuronic acid from native substrate using a copper reagent that reacts strongly with hexuronic acids but very poorly to xylose. High performance liquid chromatography has also been used to assay enzymatic release of glucuronic acid [19, 35, 36]. A drawback to these protocols is that they are endpoint assays. Another more recent assay involves the development of an artificial chromogenic substrate [37]. Unfortunately, this substrate is not commercially available and is not native. More importantly, all these assays are based in liquid phase. A major limitation of a liquid phase activity assay is the relatively low throughput. In contrast, solid phase assays which exist for other hemicellulolytic genes, such as xylanase, allow for screening mixed genome libraries at densities up to 50,000 clones per 15-cm petri dish. The existence of such activity assays is a major factor in the great number of known genes for enzymes such as xylanase.

In summary, we have demonstrated a new assay for α-glucuronidase activity that can be adapted to a high-throughput, solid phase screen. Using this protocol, one can subject a bacteriophage genomic DNA library to mass excision to obtain a large, mixed population of SOLR cells, each containing a different fragment of DNA. These cells can be plated to high densities and screened directly for α-glucuronidase activity. The development of this assay should allow for rapid discovery of new α-glucuronidase genes from mixed genomic DNA libraries.

References

1. Bhat, M. K. (2000). *Biotechnology Advances*, *18*, 355–383. doi:10.1016/S0734-9750(00)00041-0.
2. Ohmiya, K., Sakka, K., Karita, S., & Kimura, T. (1997). *Biotechnology & Genetic Engineering Reviews*, *14*, 365–414.
3. Somerville, C., Bauer, S., Brininstool, G., Facette, M., Hamann, T., & Milne, J. (2004). *Science*, *306*, 2206–2211. doi:10.1126/science.1102765.
4. Ward, O. P., & Moo-Young, M. (1989). *Critical Reviews in Biotechnology*, *8*, 237–274. doi:10.3109/07388558909148194.
5. Saha, B. C., & Bothast, R. J. (1999). In S. H., Imam, R. V., Greene, B. R., & Zaidi (Eds.), *Biopolymers* pp. 167–194. Washington, DC: American Chemical Society.
6. Biely, P., MacKenzie, C. R., Puls, J., & Schneider, H. (1986). *Bio/Technology*, *4*, 731–733. doi:10.1038/nbt0886-731.
7. Bajpai, P. (1997). *Advances in Applied Microbiology*, *43*, 141–194. doi:10.1016/S0065-2164(08)70225-9.
8. Poutanen, K., Tenkanen, M., Korte, H., & Puls, J. (1991). In G. F., Leatham, M. E., & Himmel (Eds.), *Enzymes in biomass conversion* pp. 426–436. Washington, DC: American Chemical Society.

9. Timell, T. E. (1964). *Advances in Carbohydrate Chemistry*, *19*, 247–302.
10. Timell, T. E. (1965). *Advances in Carbohydrate Chemistry*, *20*, 409–483.
11. Wilkie, K. C. B. (1979). *Advances in Carbohydrate Chemistry and Biochemistry*, *36*, 215–264. doi:10.1016/S0065-2318(08)60237-1.
12. Wong, D. W. (2006). *Applied Biochemistry and Biotechnology*, *133*, 87–112. doi:10.1385/ABAB:133:2:87.
13. Roy, N., & Timell, T. E. (1968). *Carbohydrate Research*, *6*, 482–487. doi:10.1016/S0008-6215(00)81244-6.
14. Das, N. N., Das, S. C., Dutt, A. S., & Roy, A. (1981). *Carbohydrate Research*, *94*, 73–82. doi:10.1016/S0008-6215(00)85597-4.
15. Das, N. N., Das, S. C., & Mukherjee, A. K. (1984). *Carbohydrate Research*, *127*, 345–348. doi:10.1016/0008-6215(84)85369-0.
16. Takahashi, N., & Koshijima, T. (1988). *Wood Science and Technology*, *22*, 231–241. doi:10.1007/BF00386018.
17. de Wet, B. J. M., & Prior, B. A. (2004). In B. C., Saha, K., & Hayashi (Eds.), *Lignocellulose biodegradation* pp. 241–254. Washington, DC: American Chemical Society.
18. Biely, P., de Vries, R. P., Vrsanska, M., & Visser, J. (2000). *Biochimica et Biophysica Acta*, *1474*, 360–364.
19. Nagy, T., Emami, K., Fontes, C. M., Ferreira, L. M., Humphry, D. R., & Gilbert, H. J. (2002). *Journal of Bacteriology*, *184*, 4925–4929. doi:10.1128/JB.184.17.4925-4929.2002.
20. de Wet, B. J. M., van Zyl, W. H., & Prior, B. A. (2006). *Enzyme and Microbial Technology*, *38*, 649–656. doi:10.1016/j.enzmictec.2005.07.018.
21. Zaide, G., Shallom, D., Shulami, S., Zolotnitsky, G., Golan, G., & Baasov, T. (2001). *European Journal of Biochemistry*, *268*, 3006–3016. doi:10.1046/j.1432-1327.2001.02193.x.
22. Tenkanen, M., & Siika-aho, M. (2000). *Journal of Biotechnology*, *78*, 149–161. doi:10.1016/S0168-1656(99)00240-0.
23. Khandke, K. M., Vithayathil, P. J., & Murthy, S. K. (1989). *Archives of Biochemistry and Biophysics*, *274*, 511–517. doi:10.1016/0003-9861(89)90464-5.
24. Johnson, K. G., Silva, M. C., MacKenzie, C. R., Schneider, H., & Fontana, J. D. (1989). *Applied Biochemistry and Biotechnology*, *20–21*, 245–258. doi:10.1007/BF02936486.
25. Mierzwa, M., Tokarzewska-Zadora, J., Deptula, T., Rogalski, J., & Szczodrak, J. (2005). *Preparative Biochemistry & Biotechnology*, *35*, 243–256. doi:10.1081/PB-200065648.
26. Siika-aho, M., Tenkanen, M., Buchert, J., Puls, J., & Viikari, L. (1994). *Enzyme and Microbial Technology*, *16*, 813–819. doi:10.1016/0141-0229(94)90041-8.
27. de Vries, R. P., Kester, H. C., Poulsen, C. H., Benen, J. A., & Visser, J. (2000). *Carbohydrate Research*, *327*, 401–410. doi:10.1016/S0008-6215(00)00066-5.
28. Wagschal, K., Franqui-Espiet, D., Lee, C. C., Robertson, G. H., & Wong, D. W. (2005). *Applied and Environmental Microbiology*, *71*, 5318–5323. doi:10.1128/AEM.71.9.5318-5323.2005.
29. Shulami, S., Gat, O., Sonenshein, A. L., & Shoham, Y. (1999). *Journal of Bacteriology*, *181*, 3695–3704.
30. Suzuki, T., Kitagawa, E., Sakakibara, F., Ibata, K., Usui, K., & Kawai, K. (2001). *Bioscience, Biotechnology, and Biochemistry*, *65*, 487–494. doi:10.1271/bbb.65.487.
31. de Vries, R. P., Poulsen, C. H., Madrid, S., & Visser, J. (1998). *Journal of Bacteriology*, *180*, 243–249.
32. Margolles-Clark, E., Saloheimo, M., Siika-aho, M., & Penttila, M. (1996). *Gene*, *172*, 171–172. doi:10.1016/0378-1119(96)00167-9.
33. Ruile, P., Winterhalter, C., & Liebl, W. (1997). *Molecular Microbiology*, *23*, 267–279. doi:10.1046/j.1365-2958.1997.2011568.x.
34. Milner, Y., & Avigad, G. (1967). *Carbohydrate Research*, *4*, 359–361. doi:10.1016/S0008-6215(00)80191-3.
35. Castanares, A., Hay, A. J., Gordon, A. H., McCrae, S. I., & Wood, T. M. (1995). *Journal of Biotechnology*, *43*, 183–194. doi:10.1016/0168-1656(95)00128-X.
36. Wood, T. M., & Wilson, C. A. (1995). *Applied Microbiology and Biotechnology*, *43*, 893–900. doi:10.1007/BF02431925.
37. Biely, P., Hirsch, J., la Grange, D. C., van Zyl, W. H., & Prior, B. A. (2000). *Analytical Biochemistry*, *286*, 289–294. doi:10.1006/abio.2000.4810.