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Development of a Glucuronoyl Esterase Assay

- biochemical characterization of three putative Glucuronoyl Esterases

Master Thesis in Biotechnology

AMANDA SÖRENSEN RISTINMAA

Department of Biology and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2017

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Supervisor: Jenny Arnling Bååth
Examiner: Lisbeth Olsson

Master Thesis 2017
Department of Biology and Biological Engineering
Division of Industrial Biotechnology
Chalmers University of Technology
SE-412 96 Göteborg
Telephone +46 31 772 1000

Printed by [Chalmers Reproservice]
Gothenburg, Sweden 2017

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Abstract

Glucuronoyl Esterases (GEs) have been proven to hydrolyze the ester bond between lignin and glucuronic acid units on the xylan chain. Separation of lignin and hemicellulose is vital for complete utilization of plant biomass for production of ethanol, chemicals and materials. Direct detection of enzymatic cleavage of the lignin carbohydrate ester bond is, however, difficult due the low concentration of bonds and the complex structure of the native material. Therefore, there is a need for a well-working GE assay with a variety of model substrates that imitate the natural substrate, for investigation of GE kinetics and substrate specificity.

In this study, the already established assay developed and validated by Sunner for commercially available substrates and the newly published Fraňová assay using a synthesized model substrate were used for characterization of three putative GEs. The Fraňová assay was for this work established and evaluated in terms of GE activity and pH working range. The assay was assessed in both a stopped and continuous mode. Due to a substrate containing p-nitrophenol, the assay can be used in a stopped mode from pH 5-9, however, not continuously at a pH below 6.

The kinetic parameters of the three GEs from the bacterium *Solibacter usitatus* differed significantly on various substrates. For all GEs, the highest specific activity was reached with the synthesized compound, methyl ester D-glucuronic acid 4-nitrophenol, using the Fraňová assay. The Fraňová assay is thus a suitable assay for activity measurements of GE activity. Moreover, kinetic parameters were determined for both methyl ester D-glucuronic acid (using the Sunner assay) and methyl ester D-glucuronic acid 4-nitrophenol (using the Fraňová assay) for one of the GE candidates (*SuC)*. The results showed a higher catalytic efficiency for the p-nitrophenol substrate compared to the substrate lacking p-nitrophenol.

In conclusion, the Fraňová assay was further developed into a continuous assay and validated. The assay is useful for GE research, since high specific GE activity was observed for the enzymes used in this study. Moreover, a range of model LC ester substrates can potentially, be synthesized.

Keywords: Lignin Carbohydrate Bonds, Glucuronoyl Esterase, CE15, Enzyme Assays, Model Substrates, Enzyme Assay Validation

Acknowledgements

I would like to thank my supervisor Jenny Arnling Bååth for guidance and discussions throughout this project. Great thanks to Scott Mazurkewich and Gunnar Westman for advice on numerous occasions. Last but not least, I would like to thank the Industrial Biotechnology group for good advice, fika and a great working environment in the lab.

So Long, and *Thanks for All* the Cake!

Amanda Sörensen Ristinmaa, Gothenburg, June 2017

Contents

Acronyms

AllylGlcA	Allyl-D-glucuronic acid ester
BnGlcA	Benzyl-D-glucuronic acid ester
CBM	Carbohydrate binding module
CV	Column Volumes
GE	Glucuronoyl esterase
GlcA-pNp	4-nitrophenyl-β-D-glucopyranosid urinate
GUS	β-gluconidase
HPLC	High Performance Liquid Chromatography
IEX	Ion Exchange Chromatography
IMAC	Immobilized Metal Affinity Chromatography
MeGalA	Methyl-D-galacuronic acid
MeGlcA	Methyl-D-glucuronic acid ester
MeGlcA-pNp	Methyl (4-nitrophenyl-β-D-glucopyranosid) urinate
NMR	Nuclear magnetic resonance
pNp	p-nitrophenol
pNp-Ac	p-nitrophenol Acetate
pNp-Ferulate	p-nitrophenol Ferulate
SDS-page	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TLC	Thin Layer Chromatography
UDH	Uronate dehydrogenase

1 Introduction

The world's primary energy source, fossil fuels have a great impact on the climate. For a sustainable future on earth, there is a demand for both increased resource availability and energy-efficiency. There is a need for renewable resources, which are not detrimental to the climate, and which are available in good supply. One such renewable resource is woody biomass.

Wood is mainly composed of cellulose, lignin, and hemicellulose. Cellulose forms a scaffold, upon which hemicellulose functions as a matrix, covered by lignin [1]. Complete separation of these three components would facilitate production of biomaterials, chemicals and biofuels from woody biomass. However, the separation is hindered by lignin and hemicellulose being interconnected through covalent lignin carbohydrate (LC) bonds. An enzyme, which has recently been proven to hydrolyze LC ester bonds and thus, would aid in this separation, is the glucuronoyl esterase (GE) [2].

To date, there are eight biochemically characterized GEs reported in the Carbohydrate Active Enzyme (CAZy) database, however, many more putative GEs exist, based upon sequence similarity [3]. Prior to applying GEs on native LCC material, it is necessary to determine enzyme activity and investigate substrate specificity. Therefore, both synthesized and commercially available model substrates, mimicking the native material have been used for screening and measuring activity. The commercially available substrate benzyl D-glucuronic acid ester (BnGlcA) has been used as a simple model substrate for GE assays, but for more complex LC-ester models, synthesis has to be performed [4]. Synthetic substrates such as methyl esters of 4-*O*-methyl-D-glucuronic acid (Me-GlcA) and methyl glycoside esters have been used, but are in many cases they are demanding to synthesize [5, 6]. Therefore, there is a necessity a variety of easily synthesized or commercially available model compounds, which can be used for simple screening of GE candidates and activity measurements.

Recently, Fraňová *et al.,* 2016 showed that an easily synthesized model compound MeGlcA-pNp could be used in a coupled assay for determining GE activity [7]. A few studies have investigated GEs using the commercially available models substrate BnGlcA, either qualitatively by TLC or quantitatively (spectrophotometry or HPLC) in a coupled GE assay developed by Sunner *et al.,* 2015 [4, 8]. Both assays are coupled, using the auxiliary enzymes Uronate dehydrogenase (UDH) or β-gluconidase (GUS), based upon formation of NADH or p-nitrophenol (pNp), respectively. These two assays fill the current void to easily measure and screen for GE activity, based upon spectrophotometric detection. By developing and validating the Fraňová *et al.,* 2016 assay and using it together with the Sunner assay, with several different model substrates, this project assesses a biochemical characterization of three GEs encoded by one bacterium.

1.1 Aim of thesis

The main purpose of this study was to establish and validate the Fraňová *et al.,* 2016 stopped, coupled assay for screening and determination of GE activity [7]. The assay was also developed in a continuous mode and evaluated upon usability. The enzyme activity of chosen GE candidates was determined using this new assay and compared on similar substrates with the already established assay developed by Sunner *et al.,* 2015. Both assays are coupled and measured spectrophotometrically [4, 7].

In order to test and compare the assays, three novel GEs of bacterial origin were expressed, purified and biochemically characterized with the Sunner assay on 4 different model substrates. Additionally, the putative GEs were tested for other esterase activities, on acetyl- and feruloyl-ester substrates, to evaluate their differences in activity and substrate specificity.

1.2 Delimitations

This thesis work has focused on development and validation of an assay to determine GE activity and did not cover enzyme discovery. Furthermore, because of time and availability limitations, only one model substrate was synthesized and validated for the Fraňová *et al.,* 2016 assay [7]. Even if expression and protein purification was a minor part of the project, no effort on optimization or development was worked on regarding this; e.g. already established protocols for immobilized metal ion affinity chromatography (IMAC) were used.

In addition, only three putative GEs, all of bacterial origin, were produced and tested. The enzymes were characterized upon activity on simple model substrates, but activity on more complex substrates and native LCCs were not investigated. Furthermore, enzyme structure determination, with e.g. X-ray crystallography, was beyond the scope of this thesis.

2 Theory

This section aims at providing an overview of the main components of wood: cellulose, hemicellulose and lignin and how they are interconnected through lignin-carbohydrate (LC) bonds. Furthermore, it will provide an introduction to the enzymes GEs, enzymatic assays and model substrates.

2.1 Wood structure

Cellulose is composed of chains of β-(1,4)-glucose molecules, and constitutes about 40-50 % of the total biomass content. Hemicelluloses are composed of hetero- and homo-polysaccharides. The type, amount and monosaccharide composition of hemicellulose vary depending on wood species, age of wood and the type of extraction method used. Lignin is built up from three different monomers, called monolignols. These are p-coumaryl-, coniferyl-, and synapyl-alcohol. They are all phenylpropane units, which differ by their methoxyl substitutions on the C-3 and C-5 positions on the aromatic ring [9].

The structure and chemical composition of the wood polymers also differ in the cell wall layers. The primary wall has cellulose, hemicelluloses, pectin and protein, completely embedded in lignin. The secondary wall has three layers: outer- (S_1), inner- (S_2) and a middle -layer (S_3). These layers are built up of fibrils, between which lignin and hemicelluloses are located. The middle lamella (ML) binds the cells together, see Figure 1 [1].

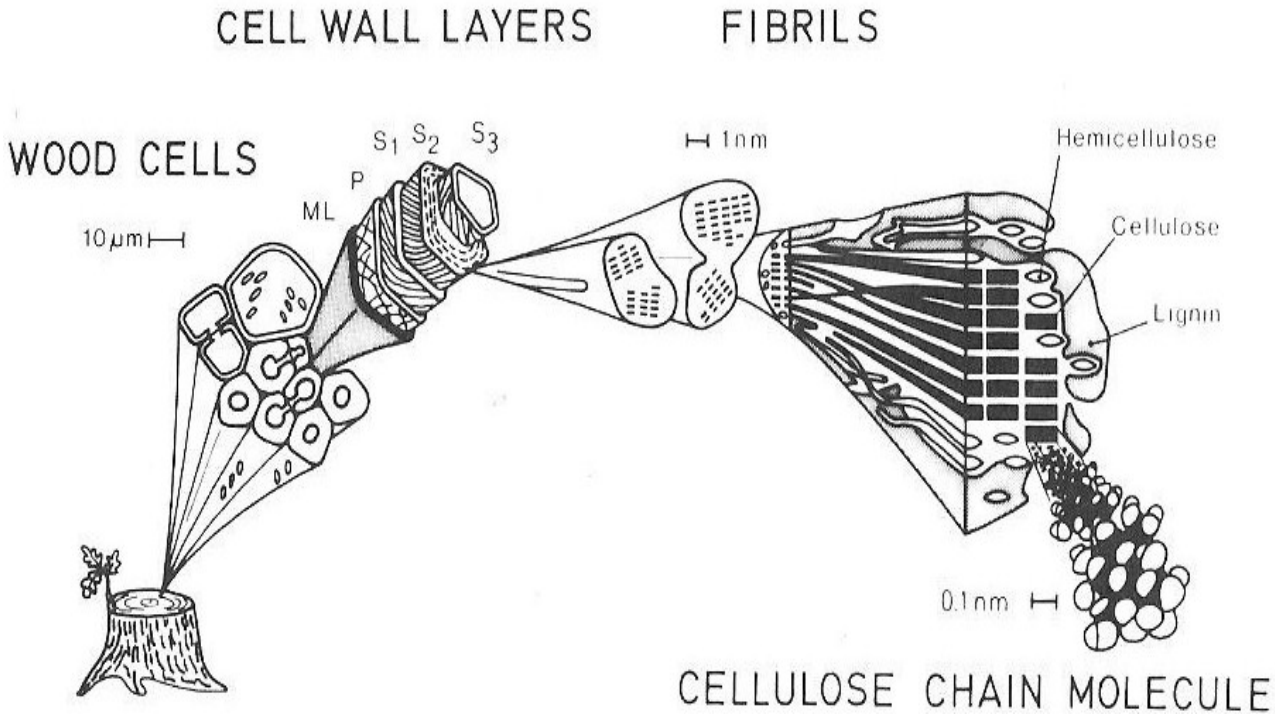


Figure 1. Structure of wood, ranging from wood cells on the micrometer scale, the three cell wall layers, fibrils to the thee major components: hemicellulose, cellulose and lignin on the nanometer scale [10].

2.2 Lignin-carbohydrate complexes (LCCs)

Hemicellulose and lignin are interconnected through LCCs [11]. Several covalent lignin-carbohydrate bonds have been proposed, such as the ether, ester, phenyl glycoside and the acetal bond [11]. However, the most studied is the ester bond. The ester bond ties the carboxyl on the 4-*O*-methyl-α-D-glucuronic acid side groups of glucuronoxylan together with either the α– or γ-carbon of the lignin moiety (Figure 2). It has been shown on model substrates that the γ–ester linkage might form from the α-ester linkages due to uronosyl migration [12]. Purification of the LC-ester bond from native material is considered difficult due to the low frequency and that the extraction method should not disrupt or change the covalent bonds in the native structure [13]. Many fractionation techniques include serial dissolution of ball-milled wood, which cannot separate a singular molecular species [11]. In many of these fractionation techniques, the yields are low. Structural analysis of the material by nuclear magnetic resonance (NMR) either by P^{31}-NMR or 2D-NMR is the only direct evidence of the connectivity between the carbohydrate fraction and lignin [2, 14]. The NMR results are difficult to analyze, and are indications rather then direct proof of the bonds.

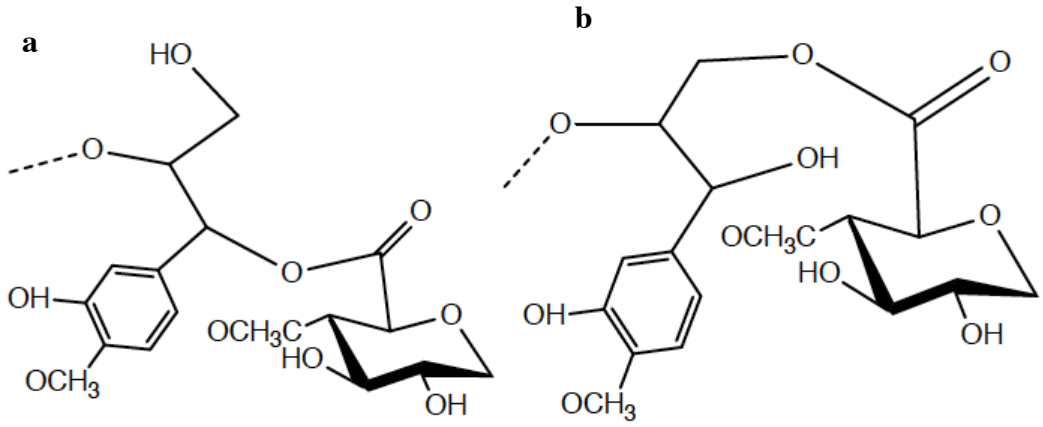


Figure 2. Schematic representation of the two different types of ester LCC bonds connecting the glucuronic acid group with the lignin: (a) α-ester or (b) γ-ester.

2.3 Glucuronoyl Esterases (GEs)

GEs are enzymes, which have been proven to hydrolyze the LC ester bond between lignin and hemicellulose [2]. They were first discovered in the wood-rotting fungus *Schizophyllum commune* in 2006 [5]. To date, eight GEs have been biochemically characterized in the Carbohydrate-Active Enzymes (CAZy) database, most of them of fungal origin. A central component for high catalytic efficiency of the GEs has seemed to be the 4-*O*-methyl substituent on the D-glucuronate. Ďurandová *et al.,* (2009) have showed that GEs from three different fungi exhibited a significantly higher specific activity on 4-*O*-methyl-D-glucuronic acid compared to D-glucuronic acid [15]. GEs from *Cerrena unicolor* and *Schizophyllum commune* have exhibited a higher affinity for the aromatic more bulky LC ester model substrates compared to less bulky LC ester model substrates [16]. The first support of GE activity on a polymeric substrate, methylated beech wood glucuronoxylan has been reported by Biely *et al.,* (2015) and observed by ^{1}H-NMR [17]. Some GEs have been reported to be bi- or multimodular-enzymes connected to CBMs (carbohydrate binding modules) and other catalytic modules [6, 15].

Recently, the first evidence of GE activity on native glucuoronic acid esters extracted from both birch and spruce has been published in 2016 by Arnling Bååth *et al.,* [2]. In addition, GE in synergy with either of the two commercially available enzyme mixtures on heat pretreated corn fiber have resulted in an increase in released sugars [16], showing the importance of GEs in degradation of the plant biomass.

Due to the difficulty in isolation of LCCs, because of the low frequency of LC bonds and complexity of woody biomass, detection of GE activity is simplified by the use of model substrates, typically used in enzyme assays.

2.4 Enzyme assays

Enzyme assays can be utilized for two different purposes: screening of enzyme activity or measuring level of enzymatic activity. As a consequence the requirements for preforming them also contrast. For qualitative detection, a positive and a negative control are required. However, when preforming a quantitative assay, further knowledge is required, which could include preparation of a standard curve and appropriate blank samples [18].

This section aims at providing an overview of enzymatic assays. Different types of enzyme assays and information about assay verification, buffer, pH, and temperature effects on enzyme activity are also presented in this section.

2.4.1 Conditions affecting enzyme activity

Enzyme activity depends upon substrate concentration, temperature, pH, type and strength of ions. Due to the fact that each enzyme has a particular optimum, these conditions change amongst enzymes. In most cases the specific qualities of the enzyme determine the optimal assay conditions [19].

The activity of an enzyme is defined as substrate converted or product formed per unit of time. IU or the International Unit is defined as μmol product/min, and is the unit used in this thesis. The purity of an enzyme is therefore, expressed by the specific enzyme activity, i.e. the enzyme units divided by the protein concentration of the enzyme preparation.

Effect of substrate concentration on enzyme activity

Varying the substrate concentration at a fixed enzyme concentration and observing the change in reaction rate is important for biochemical characterization of an enzyme. The obtained hyperbolic relationship is predicted by the Michelis-Menten equation 1.

$$\boldsymbol{v} = \frac{\boldsymbol{V_{max}}\cdot[\boldsymbol{S}]}{\boldsymbol{K_m}+[\boldsymbol{S}]} \quad [1]$$

Here, *v* is the measured velocity, V_{max} the maximum velocity of the reaction, [S] the substrate concentration and K_m the affinity of the enzyme to the substrate [20]. The velocity (observed in Figure 3) increases until it reaches a substrate-independent maximum at high substrate concentrations (V_{max}), creating a hyperbolic curve. This shape of the curve is hypothesized to arise because all binding sites are occupied or saturated with substrate at high substrate concentrations. However, due to the hyperbolic curve, complete saturation cannot be achieved.
The Michaelis-Menten constant K_m measures the affinity of the enzyme to a substrate.

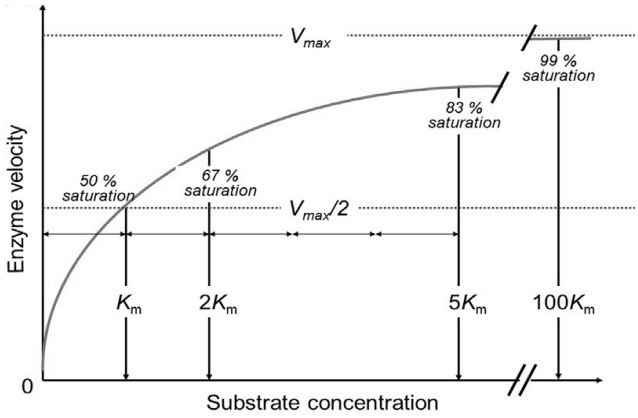


Figure 3. The change in velocity due to varying substrate concentration according to the Michaeli-Menten equation (Eq. 1). The Km value represents the substrate concentration at 1/2 [Vmax] [20].

K_m changes with both enzyme and substrate and therefore there is no general rule for which substrate concentration to use in an assay. This must be determined for each substrate and enzyme investigated. However, too high substrate concentrations might not be feasible because of high cost of substrate, substrate inhibition or the range of detection of the instrument. For the above reasons, it is suggested that the highest substrate concentration, to use should be $10K_m$, but deviations are frequently found in the literature [18,22].

From V_{max} and the enzyme concentration $[E]_t$ the turnover number k_{cat} can be determined. This is the total substrate molecules, which are converted to product per active site of the enzyme.

$$\mathbf{k_{cat}} = \frac{\mathbf{V_{max}}}{\mathbf{[E]_t}} \qquad [2]$$

Assuming only one active site per enzyme and that the rate-limiting step is the conversion of enzyme-substrate complex to enzyme (Eq 3), k_{cat} is the catalytic efficiency of the active site.

$$\frac{k_{cat}}{Km} \qquad [3]$$

k_{cat}/K_m is the specificity constant, and indicates an enzyme's overall ability to convert substrate to product, taking into account both the catalytic efficiency and the affinity to the substrate.

Effect of pH on enzyme activity

The pH dependency of enzyme activity is dependent upon the pKa of the particular enzyme. It will typically follow a bell-shaped curve, as observed in Figure 7, increasing from acidic conditions to the maximum velocity and later decreasing in the alkaline region. This is due to two main reasons: protonation of functional groups in the enzyme and cofactors involved in the reaction or changes in the native 3D structure of the protein [22].

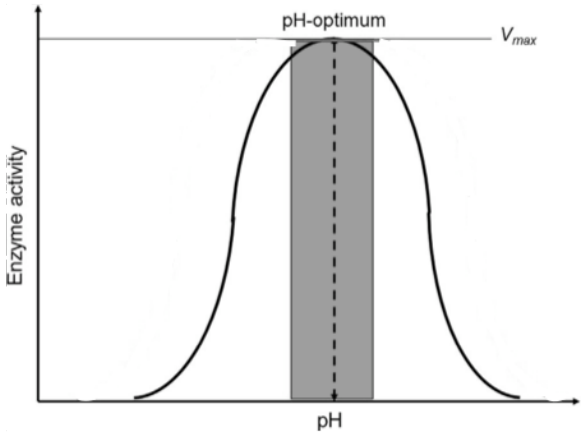


Figure 4. Enzyme activity as a function pH (black). pH sensitivity could either be due to protonation of functional groups on the active site or change in tertiary structure. Adapted from Bisswanger 2014 [20].

The pH at which the maximum velocity is reached is the pH-optimum, and is generally chosen as assaying pH for the enzyme of interest [19]. The optimal pH is generally also recommended for long time storage of an enzyme. However, care must be taken when adding aliquots of storage buffer to an assay, so that it does not affect the pH of the assay, if it deviates from that of the assay mixture.

Effect of buffers on enzyme activity

Buffers are used to adjust and stabilize pH during an enzyme assay and are composed of a weak acid and a strong base [18]. A buffer's ability to buffer the pH increases by its salt concentration; however, enzymes are generally inhibited at high salt concentrations (>0.5 M). Naturally, enzymatic activity also varies with varying salt concentration. This could be due to buffer components having stabilizing or destabilizing effects on the protein structure [18].

The buffering capacity of a buffer is usually narrow; therefore, if a broader range is required, a combination of many buffering systems is applicable, such as the TRIS/Act/MES buffering-system [21]. Such a system can be used when investigating protein pH optima. Furthermore, the effectiveness of the buffer also depends upon temperature. Therefore, the pH of a reaction also changes with temperature.

Effect of temperature on enzyme activity

The effect of temperature on any chemical reaction is normally an increased velocity of the reaction. However, too high temperatures cause enzyme denaturation due to the tertiary structure being thermally sensitive. As the tertiary structure differs between enzymes, each enzyme must be investigated individually to establish an appropriate assay temperature [22].

2.4.2 Stopped and continuous assays

Enzymatic activity can be measured in two modes; stopped or continuous. In a stopped assay, the reaction is stopped after a set time and product formation or substrate used is measured. Methods for stopping the reaction, which denature the enzyme, include heat, ice or alkaline treatment [22]. In a stopped assay, only one incubation time of the reaction is measured.

Therefore, it is important that stopped assays are checked by varying times of incubation; to ensure that the rate is linear and dependent on enzyme concentration through the measurement period.

The second approach of measuring enzymatic activity is the continuous assay. Here the time course of the reaction is followed, and the result is observed immediately over a chosen period of time [22].

All enzymes do not generate a detectable product. This can be overcome by using a coupled enzyme assay in both a stopped and continuous mode.

2.4.3 Coupled assays

In a coupled assay, the product is reacted on further (e.g. adding other enzymes), until a final product is formed, followed by a selected detection method [19]. The enzyme of interest should determine the measured rate; therefore, the coupling or auxiliary enzyme should not become rate limiting. The conversion rate exhibited by the coupling enzyme is dependent on the substrate concentration, which in turn is produced by the enzyme whose concentration is measured. At the beginning of the reaction, only small concentrations of intermediate substrate [B] will be present, and the coupling enzyme will be functioning at a fraction of its maximum conversion rate. This effect could result in a lag phase in the formation of product; therefore, enzyme activity must be studied over time.

$$[A] \rightarrow_{primary\ enzyme} [B] \rightarrow_{auxilrary\ enzyme} [C] \quad (1)$$

The efficiency of the coupling enzyme depends partially on its K_m. A lower K_m will contribute to a more efficient enzyme at lower substrate concentrations, however, this can also be overcome by using a large excess of the coupling enzyme. For an assay of this type it is necessary to determine the performance of the coupling assay, so that the coupling enzyme is not the rate-limiting step. This can be done by adding different concentrations of coupling enzyme and determine if the velocity of the reaction changes.

Continuous and stopped assays can both be coupled with an auxiliary enzyme, so that the rate of production of a chromophore can be detected. Examples of coupled assays for GE activity are the assays developed by Sunner *et al*., 2015 and by Fraňová *et al*. 2016 [4, 7].

The continuous, coupled Sunner assay can be used with the commercially available substrate benzyl glucuronate (BnGlcA). Hydrolysis of the benzyl ester group with a GE is followed by the action of the auxiliary enzyme uronate dehydrogenase (UDH), which enables the formation of NADH detected at 340 nm (Figure 5a).

The stopped, coupled assay developed by Fraňová uses the synthesized compound methyl (4-nitrophenyl-β-D-glucopyranosid) uronate (MeGlcA-pNp) for hydrolysis of a methyl ester group using a GE. Thereafter, the action of the auxilirary enzyme β-gluconidase (GUS) enables detection of pNp formation at 405 nm after the addition of the alkaline stop solution borax (Figure 5b).

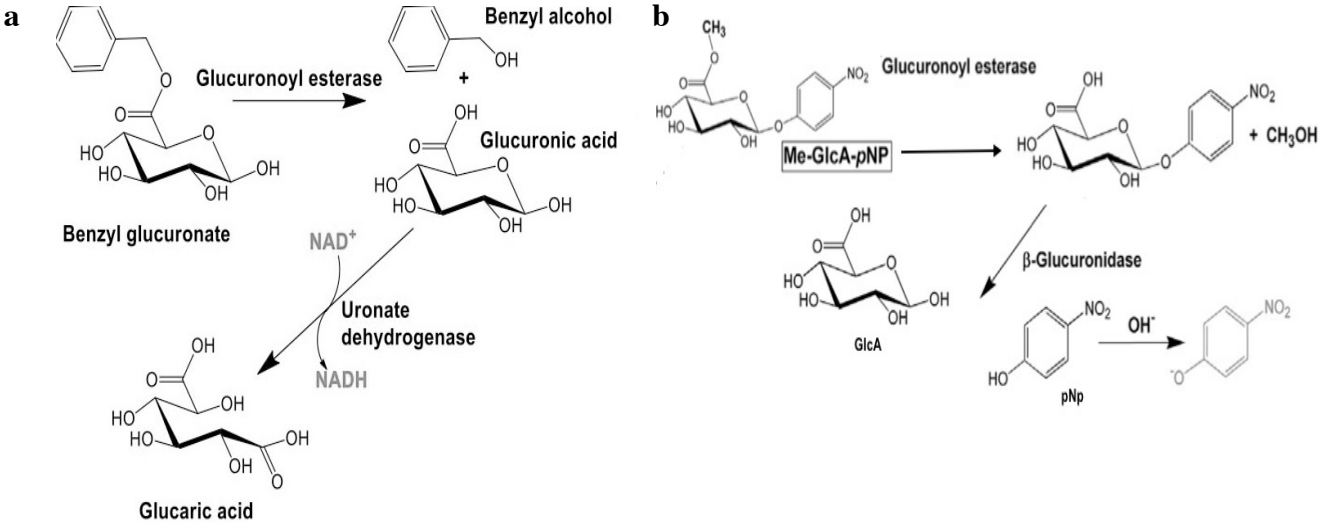


Figure 5. (a) Sunner assay overview, beginning with hydrolysis of the benzyl group by the GE and UDH oxidation of the glucuronic acid followed by colorimetric detection of the NADH formation at 340 nm (b) Fraňová assay overview, first, hydrolysis of the methyl group of the synthesized substrate MeGlcA-pNp by GE and thereafter, β- glucuronidase cleavage of the GlcA, followed by colorimetric detection of pNp at 405 nm [4, 7].

2.4.4 Enzyme assay verification

In development of enzyme assays, it is important to validate the assay based upon its performance. However, it is important to emphasize that the type of validation is highly dependent upon the type of assay to be performed. A stopped assay needs to be stopped at different time pointes to ensure the linearity of the assay and for a coupled assay, the concentration of coupling enzyme needs to be varied to ensure that the same conversion rate is achieved [22].

One aspect that is valid for both modes of assays, is to ensure that detection of the product formation or the substrate depletion differs in the observed feature, such as extinction coefficient. In addition, the decrease or increase of enzyme concentration should exhibit a linear response to enzyme velocity if the other components are kept at a constant concentration. This can be observed in Figure 6.

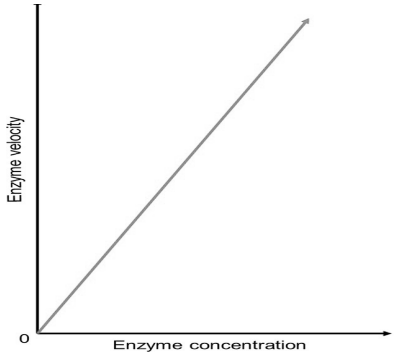


Figure 6. Schematic representation of linear response to reaction velocity with varying enzyme concentrations. A common verification procedure for enzymatic assays [18].

Another variable, which needs to be taken into account when verifying an enzyme assay, is the pH. The pH working range of an assay is important to gauge, as this influences the usefulness of the assay. Briefly, since enzymes are sensitive to changes in pH it is vital that the buffer used in the reaction can keep the pH constant. The pH can also influence the absorbance read of the assay. Therefore, when preforming any enzyme assay blank samples generating background rate, which correct for non-enzymatic conversion of the substrate are needed.

2.5 Model substrates for enzyme assays

Model substrates are simplified imitations of a natural substrate (as an example, see Figure 7). A natural material often has a complex molecular structure. Therefore, model substrates, with important structural features, are used for assaying purposes and they are often more suitable for quantification and detection of enzyme activity.

Activity of enzymes can be determined through chromatographic methods (HPLC or TLC) or in spectroscopic enzyme assays. For the use in spectroscopic enzyme assays, an important feature of the model substrate is that enzyme activity and the chemical reaction leads to a change in absorbance. Examples of compounds, which can give rise to this sort of measurable change, are NADH and pNp. Generally, the absorbance of NADH is measured at 340 nm and pNp at 405 nm [19]. Furthermore, the pNp extinction coefficient is pH dependent (Eq. 4).

$$\varepsilon_{pH} = 18\,000 * [10^{-7.17}/(10^{-pH} + 10^{-7.15})] \quad [4]$$

Thus, the same concentration of pNp will give rise to a different absorbance depending on the pH of the reaction. Both pNp and NADH are products formed in the GE assays studied in this thesis, for spectroscopic detection [4,7].

For GE research, model substrates of esters of glucuronic acid and 4-*O*-Me glucuronic acid mimicking the lignin moiety to different degrees have been used. Overall, the compounds have an ester moiety, representing the lignin part e.g. with a methyl or a lignin-like alcohol as can be observed in Figure 7b. Moreover, at the anomeric group present on the C1-carbon of the glucuronic acid, a molecule such as pNp (p-nitrophenol) can be attached for detection purposes.

The GE model substrates used in this study mimic the α-ester bound lignin moiety (Figure 2). In addition, the anomeric group present on the C-1 carbon of the GlcA is either hydroxyl or p-nitrophenol, these substrates measure GE activity. To observe or exclude any additional enzyme activities, the model substrates methyl-D-galacuronic acid (MeGalA), p-nitrophenol-Ferulate and p-nitrophenol-Acetate (pNp-Ac) have also been included. These substrates screen for various ester activities, which are interesting to investigate because certain GE could be promiscuous and hydrolyze multiple types of ester-bonds.

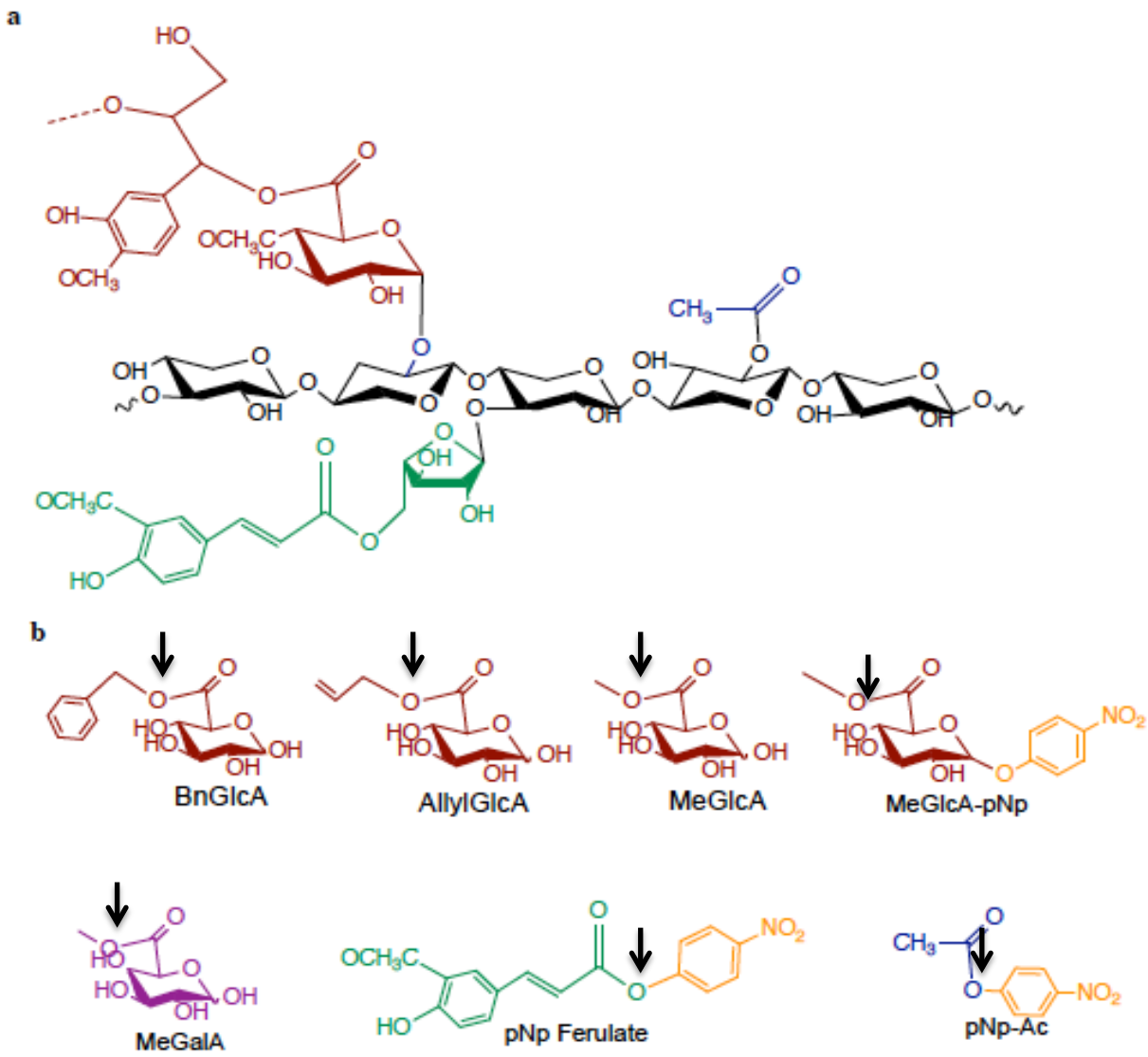


Figure 7. (a) schematic representation of the structure in woody biomass, featuring a xylane backbone (black) with an acetyl- (blue), ferulate –(green) and glucuronic acid (red) bound with an α-ester bond to the lignin moiety (b) The seven model substrates used in this thesis: benzyl-D-glucuronic acid (BnGlcA), allyl-D-glucuronic acid (allylGlcA), methyl-D-glucuronic acid (MeGlcA) (all red), Methyl (4-nitrophenyl-β-D-glucopyranosid) urinate (MeGlcA-pNp) (red-yellow), methyl-D-galacuronic acid (MeGalA) (purple), p-nitrophenol-Ferulate (pNp-Ferulate) (green-yellow) and p-nitrophenol-Acetate (pNp-Ac) (blue-yellow). The site of enzymatic hydrolysis is marked with an arrow.

3 Materials and methods

This section aims at providing an overview of the methods and materials used in this thesis. An overview of the workflow is summarized in Figure 8. Starting with expression and purification of enzymes, basic characterization such as measuring protein content, purity and storage stability, followed by biochemical characterization with the Sunner assay as well as checking for additional esterase activities. Thereafter, the Fraňová assay was established, developed and validated and finally, used for measurement of enzyme activity and kinetic parameters[7].

Figure 8. Experimental workflow starting with protein production and purification, measuring protein content and purity, biochemical characterization of the putative GEs and finally, establishment and validation of the Fraňová assay [7].

3.1 Protein production and purification

The three different GEs (*Su*A, *Su*B and *Su*C) from the bacterium *Solibacter usitatus* were produced in *E. coli* BL21 cells, purified using IMAC and checked for purity using SDS-page.

3.1.1 Expression and Purification of GEs

Recombinant *E. coli* BL21 cells with inserted genes encoding for the putative GEs *Su*A, *Su*B and *Su*C were pre-cultured in three different culture tubes with 10 mL LB media for 24 hours, 37 °C, 200 rpm with the addition of 50 µg/mL kanamycin. In order to express *Su*B in a soluble form, coexpression with chaperons was necessary. For this purpose, 35 µg/mL chloramphenicol was added to this culture. Thereafter, the cells were propagated in 1 L shake flasks of and grown for 3 hours in the same conditions as the pre-cultures. After two hours, chaperons were induced for the *Su*B expression by the supplement of 10 ng/mL tetracycline, next, after 3 hours 0.2 mM IPTG was added to all 3 shake flasks to induce protein expression. The cultures were incubated at 17°C at 200 rpm overnight, and the cells were harvested by centrifugation at 9500 rpm for 15 min. The cell pellets were re-suspended in 50 mL 50 mM NaP_i, 250 mM NaCl buffer containing 10 µg/mL lysozyme and 5 µg/mL DNAse, and disrupted with a Branson 250 sonicator (Branson Ultrasonic SA, Carouge, Switzerland). The settings were 1 min with a 2 sec pulse on, 2 sec pulse off using the amplitudes: 25, 30 and 35 %. Thereafter, the cells were centrifuged at 9500 rpm for 25 min and the supernatant filtered through a 0.45 µl filter, and loaded onto a HisTrap Excel™ column (GE Healthcare, Freiburg, Germany).

3.1.2 Immobilized Metal Affinity Chromatography (IMAC)

Immobilized Metal Affinity Chromatography (IMAC) was performed with a 5 mL HisTrap Excel columns (GE Healthcare, Freiburg, Germany). The loading buffer used was 50 mM TRIS, 250 mM NaCl, pH 8. The column was washed with 5 Column Volumes (CV) of 5 % elution buffer (50 mM TRIS, 250 mM NaCl, 250 mM Imidazole, pH 8) at a rate of 1 mL/min, followed by elution with 100 % buffer at 3 mL/min. Fractions of flow-through, wash and elution were evaluated using SDS-PAGE (Bio-Rad, California, USA). The elution fraction, containing the pure GEs, was buffer exchanged to 25 mM TRIS, pH 8 using Amicon Ultra spin columns (10 kDa cutoff; Millipore, Cork, Ireland).

3.2 Protein concentration and purity

Protein purity was evaluated through a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, California, USA) followed by imaging on a Chemidoc Touch Stain-free Imager. Protein concentration was measured using a NanoDrop 2000 spectrophotometer (Thermofisher, Wilminton, USA), by using NanoDrop 2000 software and predicted molecular weight and extinction coefficient of the GEs.

3.3 Biochemical GE characterization

For biochemical characterization of the three GEs, the Sunner assay was predominantly used developed in a continuous mode [4]. This assay was used for measuring the effect of pH on enzyme stability, activity and kinetic measurements on the four different D-glucuronic acid substrates and one D-galacturonic acid substrate. For measurements of Acetyl and Feruloyl esterase activities assays developed by Biely *et al*., 1988 and Matishuba *et al.,* 2001 were used [23, 24].

3.3.1 GE activity measurements

The substrates BnGlcA, AllylGlcA, MeGlcA, MeGalA (Carbosynth, Compton, UK) were dissolved in 100 % DMSO to a 100 mM concentration and stored at -20°C. For activity measurements the stopped, coupled assay established by Sunner was developed in a continuous mode [4], performed in 200 µL reactions, containing 50 mM potassium phosphate buffer pH 7.5, 2 mM substrate, 2 µL UDH (Megazyme, Ireland) and 16 µL NAD^+. For reaction overview see Figure 9. Reactions were run in 96-well flat-bottomed microplates (Sarstedt 82.1581, Nümbrecht, Germany) in absorbance mode for 7 min at 25 °C. One unit of GE activity is defined as the amount of enzyme producing 1 µmol of NADH in 1 min. NADH oxidation was monitored at 340 nm and the extinction coefficient of NADH was taken to be 6220 $M^{-1}cm^{-1}$.

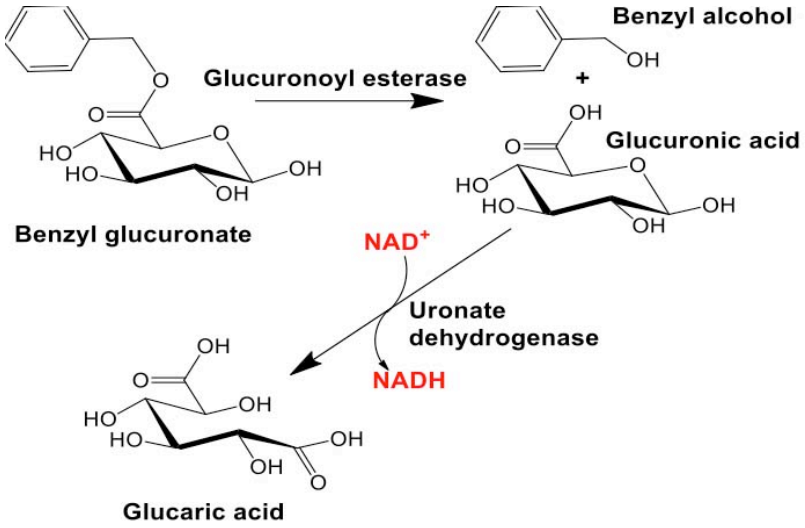


Figure 9. Sunner assay, beginning with hydrolysis of the benzyl group by the GE and UDH hydrolysis of the glucuronic acid followed by spectrophotometric detection of the NADH at 340 nm [4]

3.3.2 Effect of pH on enzyme stability

To determine the optimal storage conditions, 10 µL of *Su*A and *Su*C were stored in 100 µL aliquots at 4 °C for 4 weeks in five different buffers: 50 mM sodium acetate, pH 4.5, MES pH 5.5 Sodium phosphate pH 6.5, HEPES pH 7.5 or TRIS pH 8.5. To each buffer 10 µL of either three additives were added: glycerol, trace metals, or water. Activity was measured on BnGlcA after 1 day and 4 weeks using the developed assay described in 3.3.1. Activity was also measured after freeze-thawing of the enzymes to -20 °C.

3.3.3 Effect of pH on enzyme activity

Activity was measured using the developed assay described in section 3.3.1, however, the buffer was changed to a three-component constant ionic strength buffer containing 50 mM Tris, 25 mM acetic acid, and 25 mM MES over the pH range 4.5-9.5.

3.3.4 Kinetic measurements using the continuous assay

For kinetic measurements, the developed assay described in section 3.3.1 was used. The substrate concentration of BnGlcA, AllylGlcA, MeGlcA and MeGalA was varied between 0-20 mM. Kinetic parameters (K_m, k_{cat}) were obtained through Michaelis-Menten nonlinear regression using GraphPad Prism version 7 for Mac (GraphPad Software, La Jolla California USA).

3.3.5 Kinetics measurements using the stopped assay

The assay was performed according to Sunner *et al.,* 2015 [4]. 200 µL reactions containing 50 mM potassium phosphate buffer pH 6 were incubated for 10 min, 35 °C with the substrate MeGlcA. The substrate concentration was varied between 0-10 mM. 50 µL of the reaction mix was transferred to a microplate in technical replicates and the concentration of released GlcA was determined in a 250 µL detection assay (K-URONIC kit) at 25°C for 10 min in absorbance mode.. Kinetic parameters were obtained through nonlinear regression using GraphPad Prism version 7 for Mac (GraphPad software, La Jolla California, USA).

3.3.6 Acetyl esterase activity

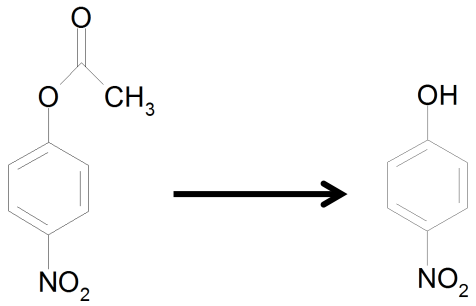


Figure 10. Schematic picture of the acetyl esterase reaction. Acetyl esterases convert Acetyl-pNp into acetyl and pNp which is detected spectrophotometrically at 405 nm [25].

The pNp-Acetate assay was performed according to Biely *et al.,* 1985 [23]. Briefly, pNp-Acetate (Sigma) was dissolved in 100% MeOH to a 40 mM substrate stock and stored at -4°C. Assays were performed in 200 µL reactions containing 50 mM sodium phosphate buffer, pH 7.5 and 1.5 mM Acetyl-pNp substrate at 25 °C. Released 4-nitrophenol was monitored at 405 nm in absorbance mode and extinction coefficient of 4-nitrophenol was taken to be 1190 $M^{-1}cm^{-1}$. One unit of acetyl esterase activity is defined as the amount of enzyme producing 1 µmol of 4-nitrophenol in 1 min.

3.3.7 Feruloyl esterase activity

A Feuloyl esterase assay was performed according to Matishuba *et al.,* 2002 [24]. pNp-Feruloate (Sigma) was dissolved in 100% DMSO to a 10 mM substrate stock and stored at -4°C. Assays were performed in 200 µL reaction volumes, containing 50 mM sodium phosphate buffer pH 7.5 or pH 6 and 0.06 mM pNp-Feruloate at 25 °C. Released 4-nitrophenol was monitored at 405 nm in absorbance mode and extinction coefficient of 4-nitrophenol was taken to be 1190 $M^{-1}cm^{-1}$. One unit of feruloyl esterase activity is defined as the amount of enzyme producing 1 µmol of 4-nitrophenol in 1 min.

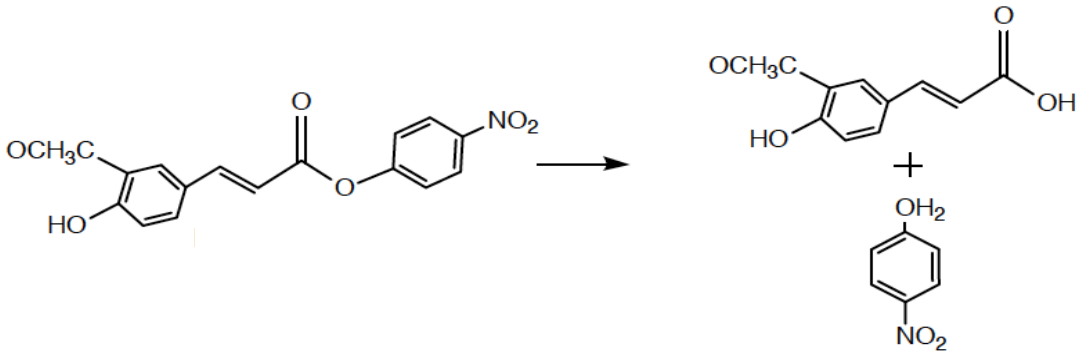


Figure 11. Schematic picture of the feruloyl esterase reaction. Hydrolysis of pNp Ferulate is followed by detection of pNp formation at 405 nm [26].

3.4 The Fraňová GE assay

The assay developed by Fraňová *et al*., 2016 for measurements of GE activity was used as a stopped assay and developed into a coupled assay [7]. The two modes were used for two different purposes, the continuous assay was used for screening of activity and the stopped assay was used for kinetics.

The coupled colorimetric assay described in Figure 12 is dependent upon the auxiliary enzyme, β-gluconidase and the chromophoric model substrate methyl (4-nitrophenyl β -D-glucopyranosid)uronate (MeGlcA-pNp) see Figure 12 [7].

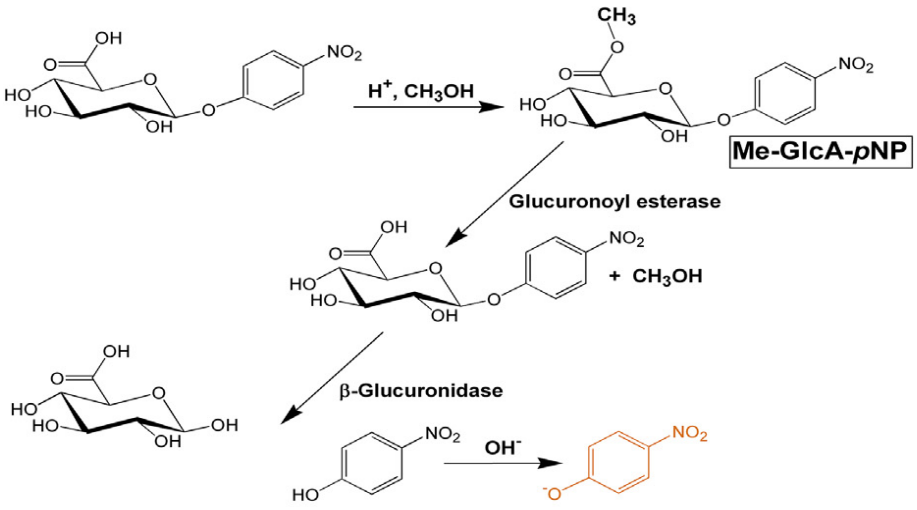


Figure 12. Overview of the Fraňová GE assay, beginning with synthesis of the chromophoric model substrate, MeGlcA-pNp followed by hydrolysis of the methyl group by a GE and β-gluconidase cleavage of the p-nitrophenol and finally, colorimetric detection of pNp formation [7].

3.4.1 Substrate synthesis

The substrate (MeGlcA-pNp) was synthesized according to the first step in Figure 12, using methyl (4-nitrophenyl-β-D-glucopyranosid)uronate (GlcA-pNp) from Megazyme. 200 mg of GlcA-pNp was dissolved in 15 ml of absolute methanol and 2 g of a strong cation exchanger Dowex 50WX8 (Sigma, H+ form) was added. The suspension was stirred at 400 rpm at room temperature in the dark. After 24 h, the suspension was filtered and the filtrate evaporated, and analyzed by TLC (described in section 3.4.3) and NMR in an Agilent 400-MR NMR Spectrometer with a 5 mm PFGPZT One NMR Probe (Agilent, California, USA).

3.4.2 Chromatography of the synthesized substrate

Post synthesis chromatography was performed to remove any impurities such as incomplete reactions. A glass column was filled with a Merck silica gel 60 matrix, wetted with 1-butanol:ethanol:water (10:5:2, v/v) at a ratio of 1:1 (v/v). Substrate for the synthesis was added and 1-butanol:ethanol:water (10:5:2, v/v) used as mobile phase. Aliquots of 1 mL were collected and analyzed by TLC.

3.4.3 Thin layer chromatography (TLC)

An aliquot of the synthesized substrate was blotted onto Merck silica gel 60 F254-precoated plates in 1-butanol:ethanol:water (10:5:2, v/v) and analyzed. UV light was used as to visualize the product.

3.4.4 Kinetic measurements in the stopped assay mode

Quantitative detection of GE activity on MeGlcA-pNp was conducted according to Fraňová *et al.,* 2016 [7]. Briefly, GE activity was determined at 35 °C in 50 mM NaP*i*, pH 6.0, using 0-8 mM MeGlcA-pNp in a total volume of 200 µl. The reaction was started by addition of the GE and terminated by addition of 1200 µl of a saturated borax ($Na_2B_4O_7$) solution after 10 min. Released 4-nitrophenol was determined in a spectrophotometrically at 405 nm in endpoint mode. One unit of GE activity is defined as the amount of enzyme producing 1 µmol of 4-nitrophenol in 1 min. Kinetic measurements were conducted at pH 6 due to high substrate hydrolysis at higher pH.

3.4.5 The Fraňová assay developed into a continuous mode

The stopped assay by Fraňová *et al.,* 2016 was developed into a continuous mode [7]. Briefly, GE activity was determined at 25 °C in 50 mM 50 mM NaP*i*, pH 6.0, using 0.5 mM Me-GlcA-pNP as the substrate in a total volume of 200 µl. Released 4-nitrophenol was monitored in absorbance mode at 405 nm in a spectrophotometer for 12 min. One unit of GE activity is defined as the amount of enzyme producing 1 µmol of 4-nitrophenol in 1 min. The continuous mode was used for activity screening of GE candidates, but not for kinetic measurements. The continuous assay was conducted at pH 6 due to high background absorbance at higher pH.

4 Results

This section aims at providing an overview of the results obtained. The results encompass three major parts: production and biochemical characterization of GEs, establishment of the newly published stopped Fraňová assay, including validation and development of the assay into a continuous mode and lastly, comparison of the GEs on model substrates using both the Sunner assay and the Fraňová assay [4,7].

4.1 Expression and purification of three putative GEs

Three annotated CE15 family members, and thereby, putative GEs encoded by the bacterium *Solibacter usitatus* were selected for this study, to investigate the diversity of GEs from one organism with two different assays. *Su*A and *Su*C consist of putative GE domains, while *Su*B has a SGNH domain coupled to the GE domain (predicted by Phyre[2]). Proteins with this type of domain have been found in a variety of hydrolases, and enzymes containing this domain exhibit esterase or lipase activity [25].

These three GEs were expressed in *E. coli* BLT21 and purified with IMAC. Purity of the fractions was assessed by SDS-page (Supplementary data, Figure S1 & Figure S2). Overall, the observed and predicted MW corresponded well with theoretical data for all proteins (Table 1). In the SDS-page gel of *Su*B a band was observed at 50 kDa for both the cell extract (CE) and the flow through (FT) (Supplementary data, Figure S2). The molecular weight of that band corresponds well to the GE domain of the *Su*B (50 kDa) without the predicted SGNH domain, suggesting a partial cleavage between the GE and SGNH domain in these fractions. However, this will have to be further investigated. Protein concentration of the pure fractions was determined using the predicted MW and extinction coefficient in a NanoDrop 2000 spectrophotometer.

Table 1. Theoretical MW (kDa), observed MW (kDa) and extinction coefficient in the purified samples. The theoretical MW and the extinction coefficient was predicted using Prot Param.

GE construct	Predicted MW [kDa][1]	Observed MW [kDa]	Extinction coefficient[1] [$mM^{-1}cm^{-1}$]
*Su*A	44	45	69
*Su*B	70	77	89
*Su*C	43	40	64

4.2 Biochemical characterization of GEs

In this section, biochemical characterization of the three GEs is presented, including storage stability at 4 °C and -20 °C, pH optimum, buffer optimum and kinetics on four different model substrates. In the experiments, the already established Sunner assay, described in 3.3.1 was used to determine enzyme activity. Furthermore, acetyl- and ferouyl-esterase activities (Table 2) were assayed for all GE candidates, described in section 3.3.6 and 3.3.7. Due to the low quantity of *Su*B storage test was not performed on this enzyme.

Table 2. Overview of the five types of biochemical characterization performed on the three different GEs.

GE construct	pH stability	pH profile	Buffer optimum	Kinetics	Additional esterase activities
*Su*A	✓	✓	✓	✓	✓
*Su*B		✓	✓	✓	✓
*Su*C	✓	✓	✓	✓	✓

[a] The biochemical characterization was conducted with the Sunner assay 2015 [7], with the exception for additional esterase activities. Stability tests was not preformed on *Su*B due to low quantity.

4.2.1 Effect of pH on enzyme stability

pH storage stability was determined by incubating *Su*A and *Su*C in either: 50 mM sodium acetate pH 4.5, MES pH 5.5, sodium phosphate (NaP*i*) pH 6.5, HEPES pH 7.5 or TRIS pH 8.5 at 4 °C for 4 weeks and at -20 °C. The additives used were 10 % glycerol, trace metals (TM) or water. *Su*A was most stable at 4 °C in pH 5.5 MES with both glycerol and water, but also exhibited a high stability at alkaline pH with all additives (Figure 13a). The same trend can be observed for the -20 °C test (Figure 13b). *Su*C showed higher specific activity in storage with glycerol in both acidic and alkaline pH (Figure 14a). In contrast, there is no bigger difference between the samples with different additive for the -20 °C test (Figure 14b). Both GEs keep high levels after freezing, which is useful for storage of enzymes.

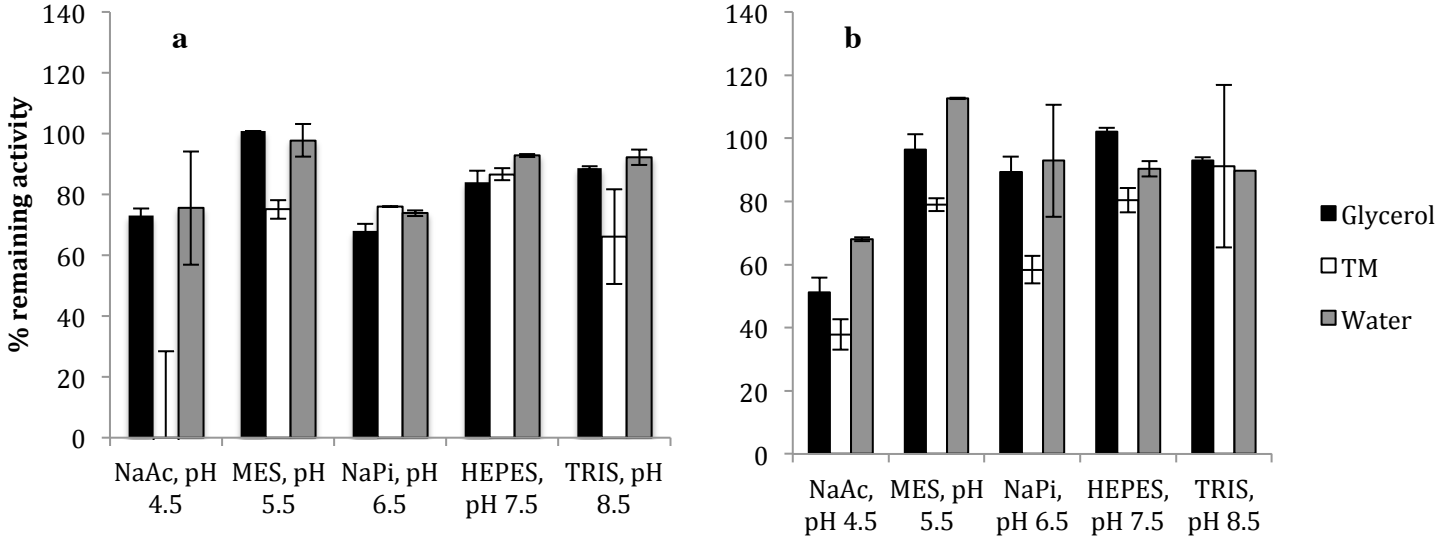


Figure 13. pH stability of *SuA*. pH stability was determined by incubating the enzyme in 50 mM sodium acetate, pH 4.5, MES pH 5.5, sodium phosphate pH 6.5, HEPES pH 7.5 and Tris pH 8.5 at 4 °C for 4 weeks (a) and at -20 °C (b) with either glycerol (black), trace metals (TM) (white) or water (gray). The specific activity was assessed on 2 mM BnGlcA in 50 mM sodium phosphate buffer pH 7.5, 25 °C. Enzyme activity was normalized to enzymatic activity measured at day 1. Error bars represent the standard error from two independent measurements.

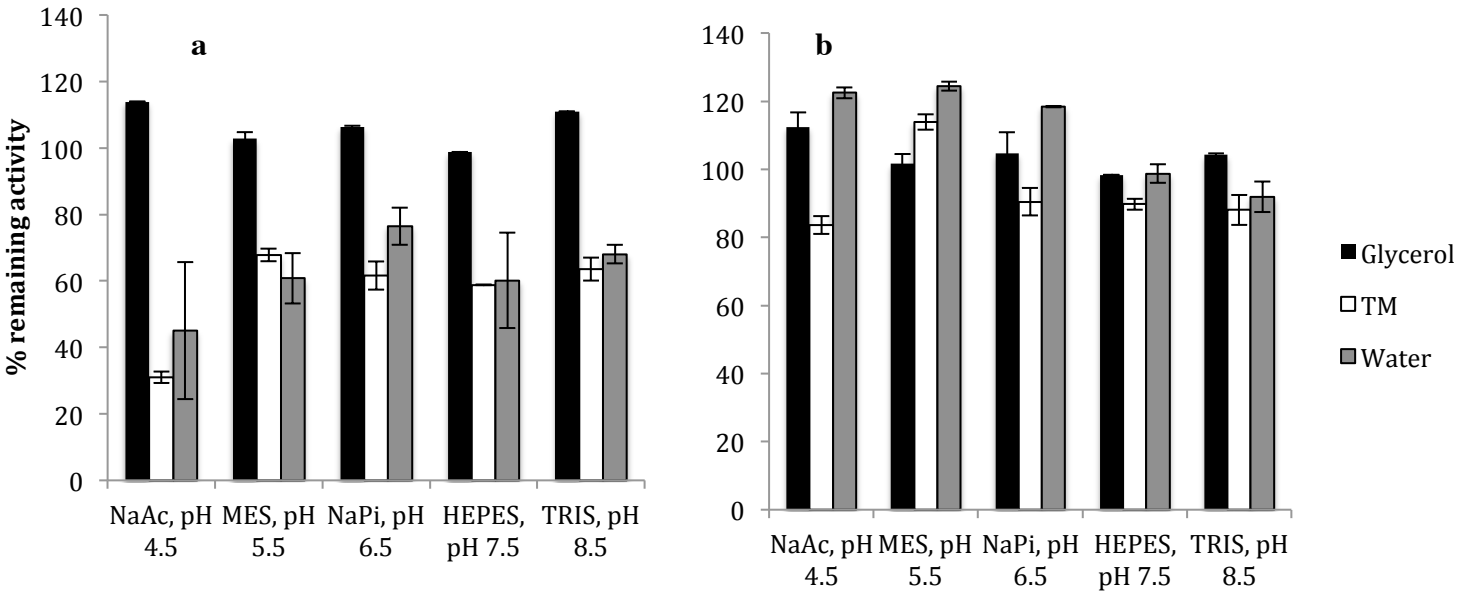


Figure 14. pH stability of *Su*C. pH stability was determined by incubating the enzyme in 50 mM sodium acetate, pH 4.5, MES pH 5.5, sodium phosphate pH 6.5, HEPES pH 7.5 and Tris pH 8.5 at 4 °C for 4 weeks (a) and at -20 °C (b) with either glycerol (black), trace metals (TM) (white) or water (gray). The specific activity was assessed on 2 mM BnGlcA in 50 mM sodium phosphate buffer pH 7.5, 25 °C. Enzyme activity was normalized to enzymatic activity measured at day 1. Error bars represent the standard error from two independent measurements.

4.2.2 Effect of pH on enzyme activity

pH profiles for *Su*A, *Su*B and *Su*C were created using a three-component constant ionic strength buffer containing 50 mM Tris, 25 mM acetic acid, and 25 mM MES. Activity above pH 9.5 was not determined because of substrate instability and high background hydrolysis. All three GEs exhibited a pH optimum in the alkaline region. *Su*B and *Su*C exhibited a higher specific activity at pH 7.5 (Figure 15b, c). In contrast, *Su*A exhibited a higher specific activity at pH 8.5 (Figure 15a).

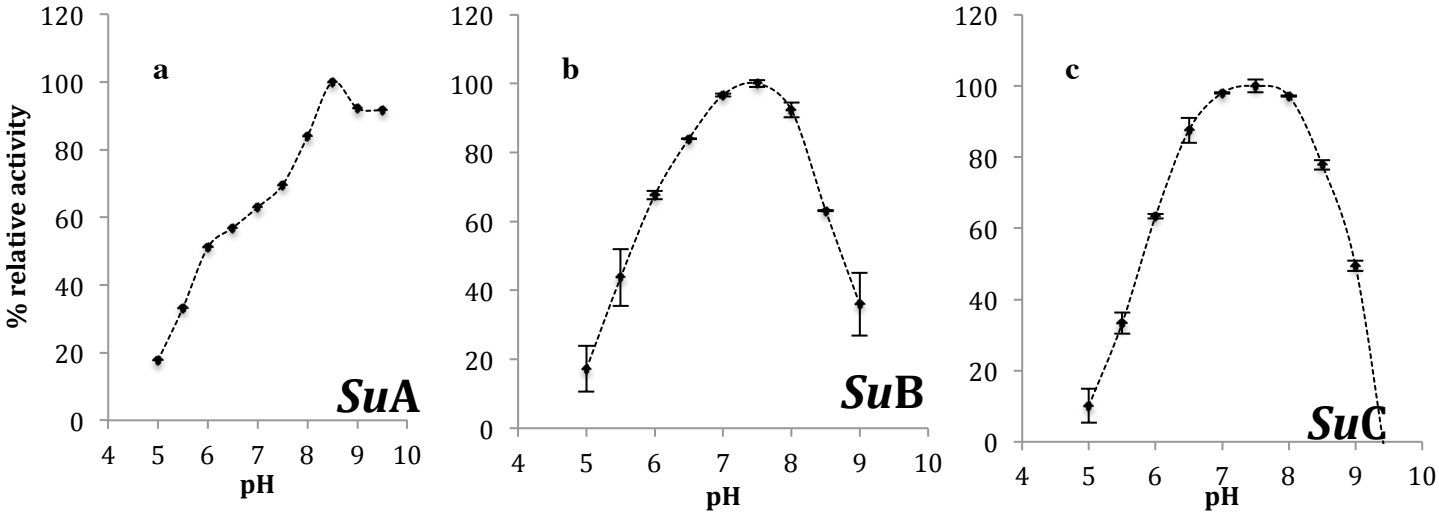


Figure 15. Optimal pH of the 3 different GEs (a-c). pH stability was determined using a three-component constant ionic strength buffer containing 50 mM Tris, 25 mM acetic acid, and 25 mM MES. Enzyme activity was normalized to the highest observed activity. Error bars represent the standard error from two independent measurements.

4.2.3 Effect of buffers on enzyme activity

To determine the most suitable buffer for activity measurements five different buffers were investigated: 50 mM TRIS-HCl, MOPS, HEPES, sodium phosphate (NaP*i*) and 50 mM TRIS, 25 mM MES and 25 mM acetic acid (TMA) in the pH range 7-8.5 (Based upon the results from the optimal pH for enzyme activity). *Su*A was investigated in the pH range of 7-8.5, and *Su*B and *Su*C in the range of 7-8. All GEs exhibited the highest activity in 50 mM sodium phosphate buffer (Figure 16), and therefore, this buffer was chosen for assigning kinetic parameters for all GEs.

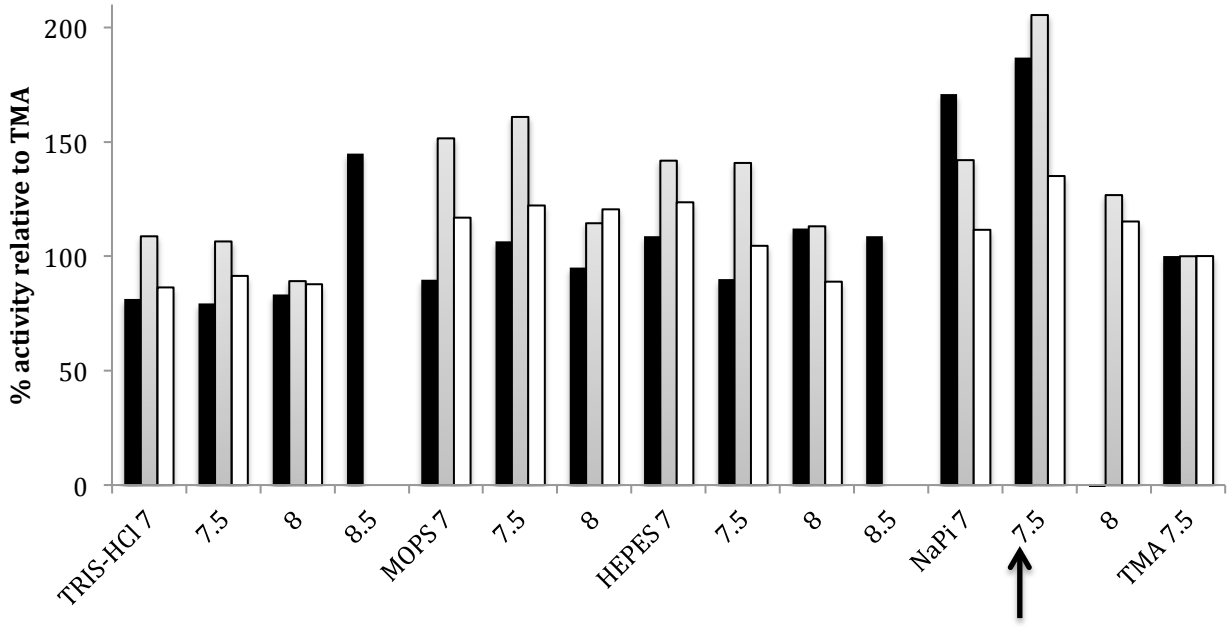


Figure 16. Determination of the most suitable buffer for activity measurements. Relative activity of *Su*A (black) *Su*B (gray) and *SuC* (white) in five different buffers 50 mM TRIS-HCL, MOPS, HEPES, sodium phosphate (NaPi) and 50 mM Tris, 25 mM MES and 25 mM acetic acid (TMA) in the range of 7- 8.5. Enzyme activity was normalized to pH 7.5 TMA buffer activity. The arrow indicates the buffer used for determining kinetic parameters.

4.2.4 Determination of kinetic parameters on four model substrates

The three GEs were assayed on four different substrates: BnGlcA, AllylGlcA, MeGlcA and MeGalA, to determine their kinetic parameters. Both *Su*A and *Su*C showed a considerably higher kinetic efficiency [k_{cat}/K_m] for BnGlcA than *Su*B (Table 3). Due to low kinetic efficiency for *Su*B on BnGlcA, the other substrates were not investigated.
Generally, *SuA* exhibited a higher kinetic efficiency and affinity for the BnGlcA substrate compared to the other D-glucuronic acid substrates. In contrast, *SuC* displayed an equal high catalytic efficiency and affinity for all GlcA substrates.

Table 3. Kinetic parameters for the three GEs on Bn-, Ally-, Me-GlcA and Me-GalA. Assay conditions are described in materials and methods section 3.3.4. Standard errors in parenthesis were estimated using the software GraphPad Prism.

Substrate	Enzyme ID	K_m [mM]	k_{cat} [sec^{-1}]	k_{cat}/K_m [mM^{-1} sec^{-1}]
BnGlcA	*Su*A	0.4 (0.1)	14.3	34.5
	*Su*B	14 (2.2)	22.6	1.6
	*Su*C	1.9 (0.4)	76.2	40.2
AllyGlcA	*Su*A	1.9 (0.9)	17	8.9
	*Su*C	2.1 (0.4)	82.5	39.8
MeGlcA	*Su*A	2.2 (0.4)	12	5.6
	*Su*C	2.2 (0.2)	68.1	31
MeGalA	*SuA*	1.8 (0.3)	4	2.4
	*Su*C	10.4 (1.5)	17.9	1.7

[a] *Su*A has a higher kinetic efficiency for BnGlcA compared to the other GlcA substrates, *SuB* has a low kinetic efficiency and *SuC* exhibits an equal kinetic efficiency for all GlcA substrates

4.2.5 Additional esterase activities

The three GEs were tested on Ac-pNp and pNp-ferulate in sodium phosphate buffer pH 7.5 to investigate additional esterase activities. No activity was observed for pNp-ferulate. Specific acetyl esterase activity for *Su*A and *Su*C pNp-Ac was in all cases very low, 113 (±8) and 76 (±3) mU/mg. For *SuB* no activity was observed.

4.3 Establishment, development and validation of the Fraňová assay

In this section, data from verification of the newly established stopped assay and the developed continuous assay are presented. Both assays are described in section 3.4.4 and 3.4.5, respectively. The GE *Su*C was used for assay verification in both continuous and stopped mode. The reason for choosing *SuC* was that this GE displayed the highest k_{cat}/K_m for the substrate MeGlcA in the continuous Sunner assay, which is structurally similar to the substrate used in the Fraňová assay [4,7].

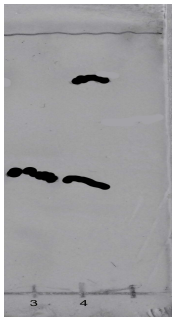
4.3.1 Substrate synthesis and verification of substrate purity

Synthesis of methyl esters of D-glucuronic acid was performed in methanol with an acid catalyst, using a cation exchanger with the aim of producing a methylated ester model substrate for the GE assay. The reaction product was further purified by silica gel chromatography (Supplementary data, Figure S 6b), because of impurities observed in the TLC (Supplementary data, Figure S 6a). The addition of an ester group was confirmed with ^{1}H-NMR at 3.75 ppm (Table 4 and Supplementary data, Figure S 7) and purity was verified by TLC (Figure 17a). However, the lower band to the right in Figure 17 indicates that the methylated substrate is impure, as it has moved the same distance as the unmethylated substrate in the left lane. The quantity of methylated substrate was 52 %. This was determined by using the coupling enzyme GUS, that hydrolyzes GlcA-pNp, to measure the amount of unmethylated substrate in the methylated- and unmethylated-substrate mixture (Supplementary data, Table S 2).

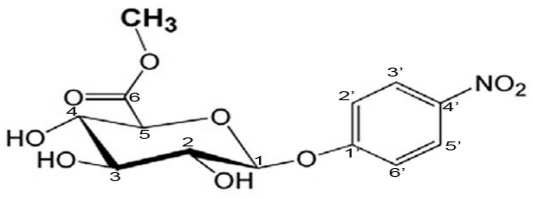
Table 4. ^{1}H-NMR chemical shifts (in ppm) of MeGlcA-pNp after chromatography, the addition of a ester group can be observed at 3.75 ppm.

	H-1	H-2	H-3	H-4	H-5	O-Me	H-2', H-6'	H-3', H-5'
MeGlcA-pNp	5.16	3.63	3.57	3.39	4.14	3.75	7.20	8.17

[a] These were assigned based upon Fraňová *et al*., 2016. . The corresponding carbon assignments can be observed in figure 17b [7]



a



b

Figure 17. (a) TLC analysis of GlcA-pNp (left) and MeGlcA-pNp (right) an impure substrate can be observed due to the lower band on the right (b) The synthesized substrate MeGlcA-pNp with assigned carbons based upon Fraňová *et al*,. [7].

4.3.2 Stopped assay verification

In this section, the verification of the newly established stopped Fraňová assay (described in section 3.4.4) is presented [8]. To verify that the assay is working it is vital to distinguish product from substrate, the substrate conversion rate is dependent on the enzyme of interest concentration, and a stable substrate. For the stopped assay, additional tests are needed (in comparison to the continuous assay) to ensure that the rate is linear through the measurement period as well as verification of a well-working stop solution.

pH range of stopped assay

In an assay, it is important to be able to distinguish product from the substrate. Therefore, the absorbance of product (pNp), substrate (MeGlcA-pNp) and intermediate (GlcA-pNp) was investigated at 405 nm in a three-component ionic strength buffer containing 50 mM Tris, 25 mM acetic acid, and 25 mM MES, with the aim of determining the limit of detection of product in a coupled assay. Taking into account that the product should exceed the background by a factor of two in absorbance, the lowest concentration of pNp, which could be detected was 0.1 mM at pH 5-6.

Linearity and enzyme concentration determination

To establish that the GE concentration determined the substrate conversion rate, the GE was added in different concentrations. The linear response in Figure 18a confirms that the conversion rate is dependent on the GE concenetration. In addition, to control that the coupling enzyme, GUS, did not determined the substrate conversion rate, different concentrations of GUS was added. The non-linear response to a increase in GUS concenetration in Figure 18b confirms that the GUS concentration is not limiting the convertion rate. The arrows in the graphs specify the concentration of *Su*C and GUS used in this assay.

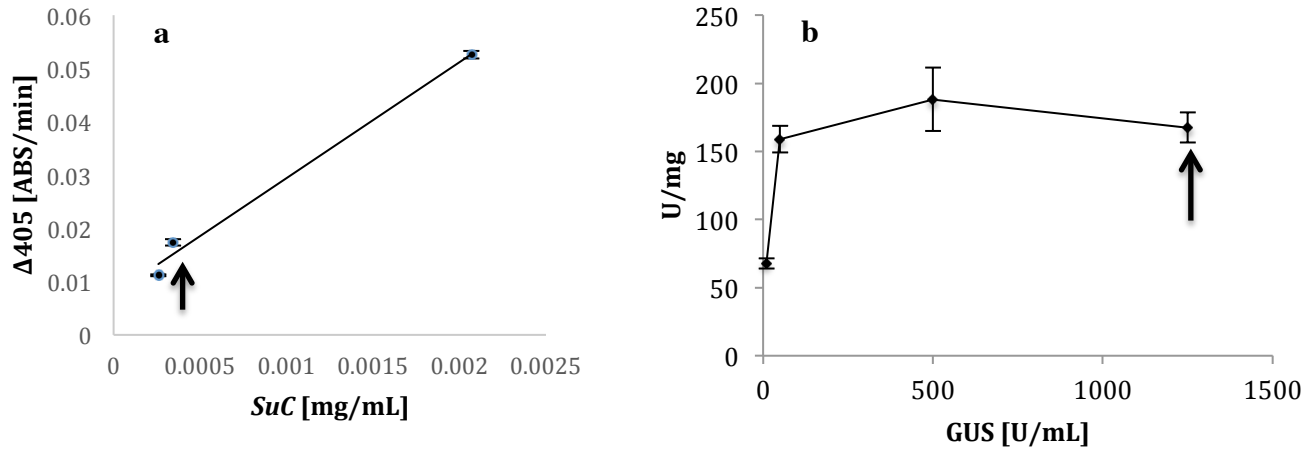


Figure 18. (a) Linear response in absorbance/min as a function of enzyme concentration (*SuC)* (b) evaluation of assay performance based upon varying GUS concentration. Arrows indicate the enzyme concentration used in the assay. Error bars represent the standard error from two independent measurements.

Control of stop solution

To control if borax, used as stop solution, terminates the reaction instantaneously, a reaction with MeGlcA-pNp, coupling enzyme (GUS) and *Su*C was stopped at time zero (Figure 19). The amount of pNp produced in a 200 µl reaction is in the same range as the sample with only MeGlcA-pNp and buffer. Thus, the stop solution terminates the reaction for both the GE and the coupling enzyme immediately. Samples with MeGlcA-pNp and GUS and a sample with MeGlcA-pNp, GUS and *SuC* were used as positive controls.

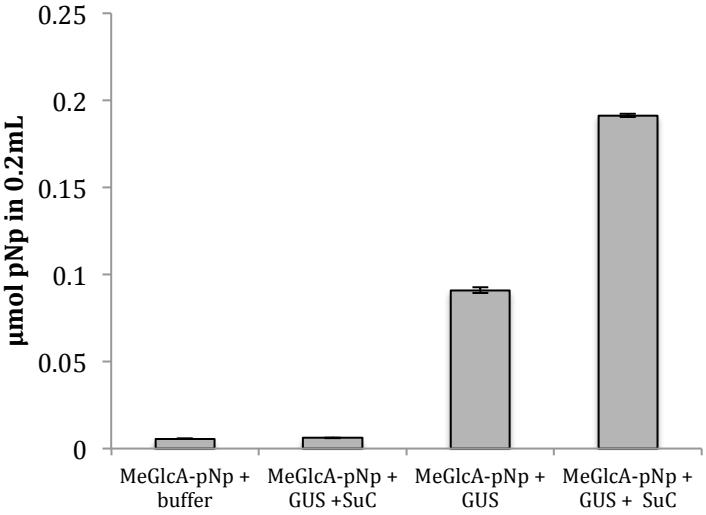


Figure 19. Control experiments for *Su*C in the stopped assay, ensuring that borax stopped the reaction. The µmol pNp produced in the MeGlcA-pNp together with coupling and GE is in the same range as MeGlcA-pNp in buffer. Error bars represent the standard error from two independent measurements

Control for linearity over time

For a stopped assay, it is vital that the reaction runs at a constant rate over the period of the assay. Therefore, the reaction was stopped at incubation times: 3, 5 and 10 min to ensure a linear rate (Figure 20). As can be observed in Figure 20, the formation of the product is linear over the period of the incubation time.

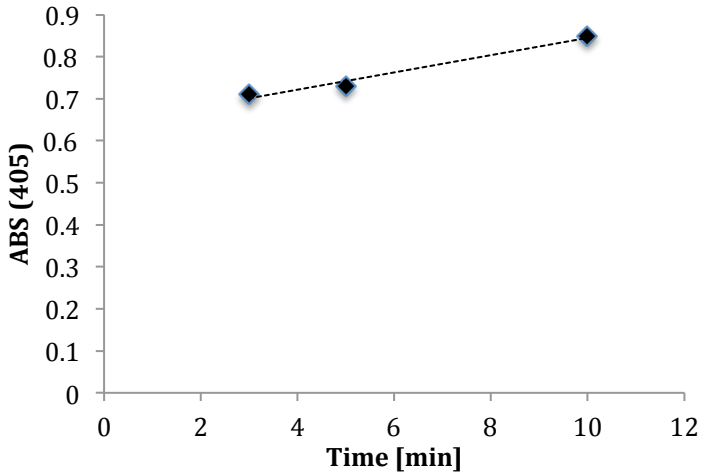


Figure 20. Linear response in absorbance as a function of reaction time, the concentration of *Su*C is kept constant. pNp formation was detected using 0.5 mM MeGlcA-pNp in NaP*i*, pH 6.

Effect of pH on substrate hydrolysis

For evaluation of substrate stability for different pH values, 0.5 mM MeGlcA-pNp was incubated at 35 °C for 10 min with only the coupling enzyme (GUS). It can be observed that at alkaline pH, the substrate hydrolyses more than at acidic pH (Figure 21). At pH 9, the background hydrolysis is almost twice as high compared to at pH 5.

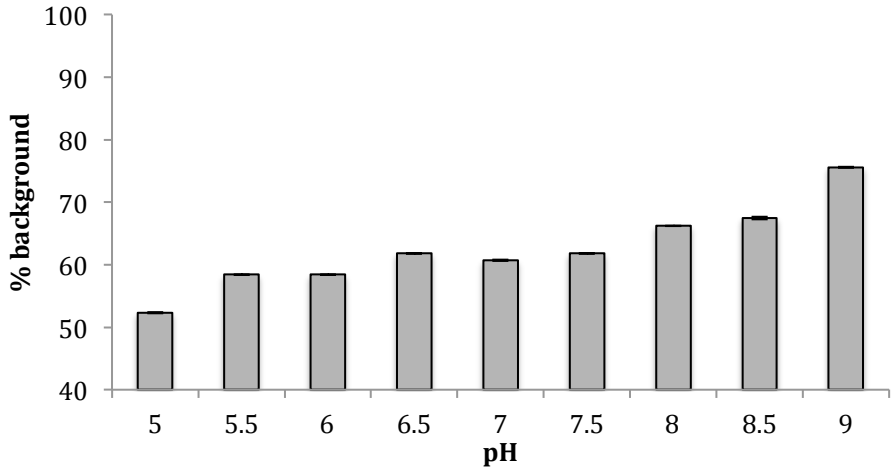


Figure 21. Spontaneous substrate hydrolysis at pH values 5-9.5 using a three-component constant ionic strength buffer containing 50 mM Tris, 25 mM acetic acid, and 25 mM MES with the addition of 1200 µl saturated borax solution. At higher pH the substrate hydrolysis increases. Error bars represent the standard error from two independent measurements

4.3.3 Continuous assay verification

In this section, the verification of continuous Fraňová assay developed in this thesis as described in section 3.4.5. To verify that the assay is working it is vital to distinguish product from substrate, and that the substrate conversion rate is dependent on the enzyme of interest concentration. Lastly, the limitations of the continuous assay are discussed.

pH range of continuous assay

As described in the stopped assay verification the product should exceed the background by a factor of two in absorbance. An absorbance that is higher for substrate and intermediate compared to product means that you cannot distinguish the product from background. At pH 5 you cannot distinguish 0.12 mM pNp from the substrate MeGlcA-pNp and intermediate, GlcA-pNp. However, a higher concentration of pNp can be distinguished from this background. Thus, the lowest concentration of pNp, which can be detected in the continuous assay is 0.5 mM at pH 5, 0.3 mM at pH 5.5 and 0.12 mM at pH 6.

Linearity and enzyme concentration determination

Similarly to the stopped assay verification it is also vital for the continuous assay to establish that the GE concentration determined the substrate conversion rate. The linear response in Figure 22a confirms that the substate conversion rate is dependent on the GE concenetration. In addition, to control that the coupling enzyme, GUS, did not determined the substrate conversion rate, different concentrations of GUS was added (Figure 22b).

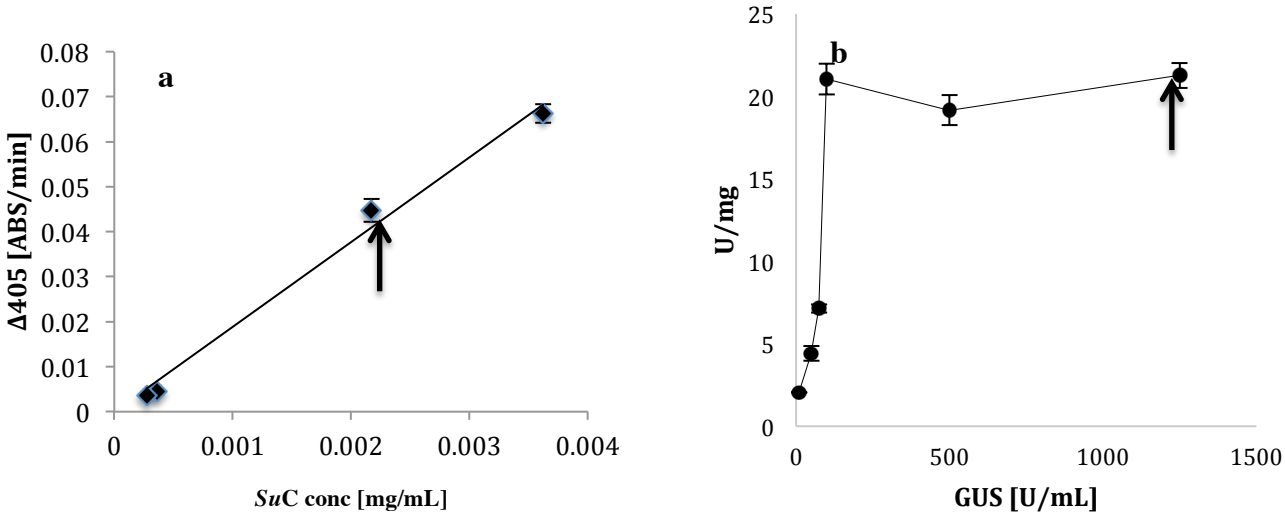


Figure 22. (a) Linearity of the GE assay evaluated by the effect of *SuC* concentration on reaction velocity. (b) Evaluation of assay performance based upon varying GUS concentration, to make sure that the coupling enzyme is not rate limiting. Arrows indicate the enzyme concentration used in the assay. Error bars represent the standard error from 2 independent measurements

Assay limitation – impure substrate

To determine an acceptable MeGlcA-pNp concentration, leading to an absorbance below 1.5 (the linear range limit for the spectrometer), the impure substrate MeGlcA-pNp was incubated with only the coupling enzyme (GUS). At higher substrate concentrations (1 mM and above), the background noise of the impurities increases, making the absorbance read higher than 1.5 (Figure 23a). Therefore, the highest acceptable substrate concentration is around 0.5 mM.

For investigation of the change in absorbance of pNp depending on the pH of the reaction, 0.1 mM MeGlcA-pNp was incubated with GUS in the pH range 5-7.5. At a higher pH, the absorbance is higher (Figure 23b) which means that at an alkaline pH and a high substrate concentration the absorbance will no longer be within in the linear range of the spectrophotometer.

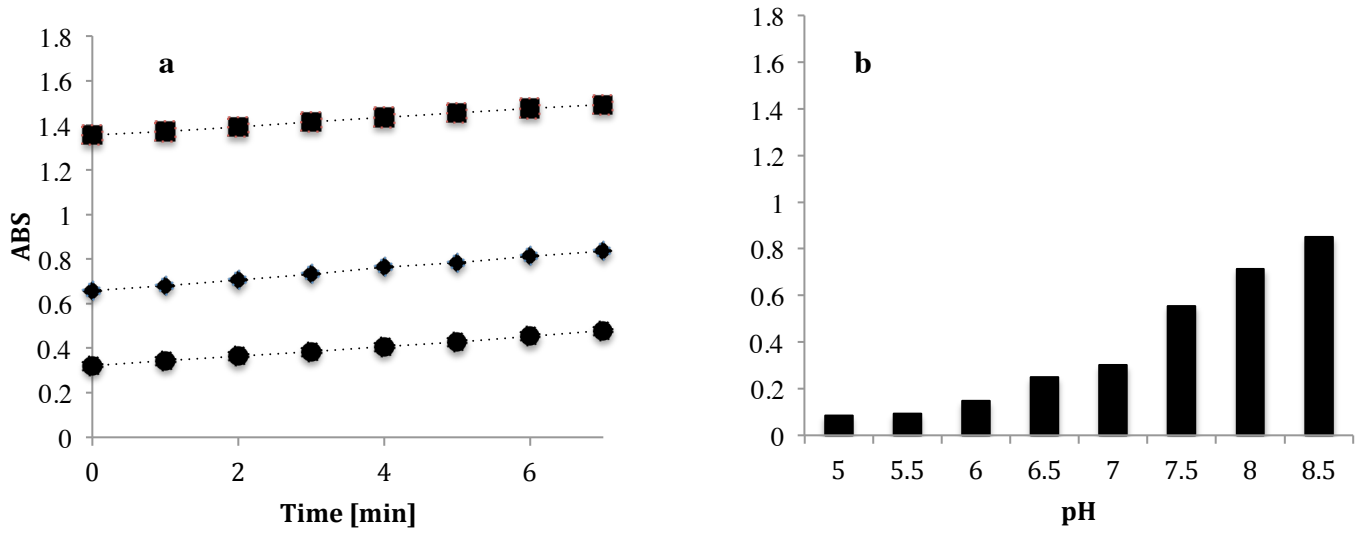


Figure 23. (a) Effect of the concentration of impure substrate on absorbance. An absorbance bellow 1.5 absorbance units is within the linear range of the spectrophotometer This was investigated using 3 different substrate concentrations: (◆) 1 mM (■) 0.5 mM and (•) 0.25 mM MeGlcA-pNp in NaP*i*, pH 6 (b) Effect of pH on absorbance unit of 0.1 mM MeGlcA-pNp after hydrolysis with GUS in the pH range of 5-8.5, showing an increasing absorbance with higher pH.

4.4 GE activity and specificity of different model substrates with two different assays

To compare the activity on the commercial GlcA substrates with the synthesized compound MeGlcA-pNp, the three GEs were assayed on all substrates. Enzyme reactions were performed with 0.5 mM substrate at 25 °C in continuous mode for 12 min in NaP*i*, pH 6 for both assays, to obtain similar conditions. For all commercially available compounds, BnGlcA gave rise to the highest activity, followed by AllylGlcA, MeGlcA and MeGalA (as seen in section 4.2.4). The synthesized compound MeGlcA-pNp exhibited a higher or equivalent activity to BnGlcA for all GEs (Figure 24). Linear response for variation of enzyme concentration was checked for all GEs on MeGlcA-pNp (Figure 22a, and Supplementary data, Figure S 9)

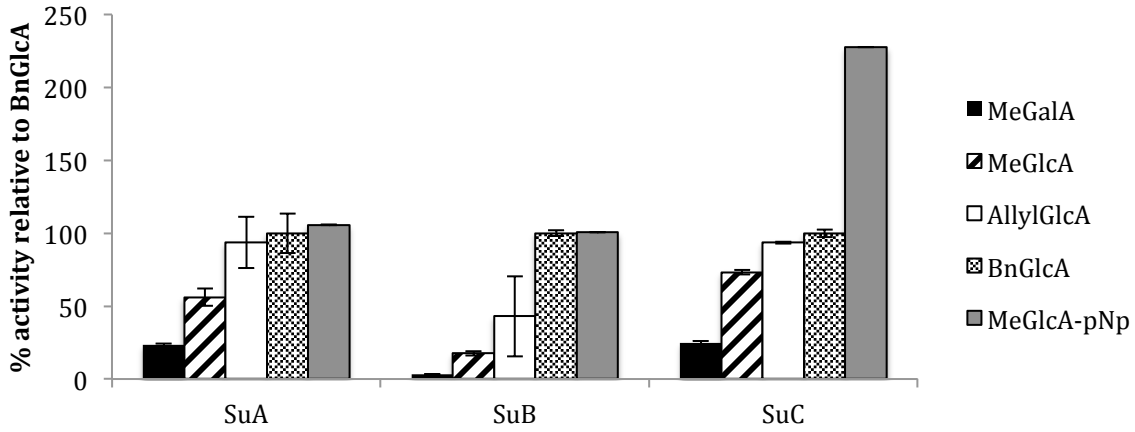


Figure 24. Activity of *Su*A, *Su*B and *Su*C on 5 different model substrates in sodium phosphate buffer pH 6, screened at 0.5 mM substrate concentration. Activity is normalized to relative BnGlcA activity. The lowest and highest activity for all GEs can be observed for MeGalA and MeGlcA-pNp, respectively. Error bars represent the standard error from two independent measurements

4.5 Kinetics on two methylated glucuronic acid substrates with two different assays

Kinetics on the commercially available substrate MeGlcA using the Sunner assay and the synthesized compound MeGlcA-pNp using the Fraňová assay were compared using the GE *Su*C because of its high specific activity in the coupled assay upon the synthesized substrate [4,7]. This can be observed in Figure 24. Enzyme reactions were performed at 35 °C for 10 min, thereafter stopped with borax or transferred and developed with the commercially available Megazyme UDH kit. The linear response throughout the reaction was checked for MeGlcA-pNp (Supplementary data, Figure S 10). For both compounds, the K_m is in the same range, however, for MeGlcA-pNp the V_{max} is six times higher leading to a higher k_{cat}/K_m for MeGlcA-pNp (Supplementary data, Figure S 11).

Table 5. Kinetic parameters for *SuC* on MeGlcA-pNp and MeGlcA determined using 50 mM sodium phosphate, pH 6..

Substrate	K_m [mM]	V_{max} [U/mg]	k_{cat} [sec^{-1}]	k_{cat}/K_m [mM^{-1} sec^{-1}]
MeGlcA-pNp	1.2 (0.2)	170 (14.5)	122.8	102.3
MeGlcA	1.5 (0.18)	39.4 (1.5)	28.6	19

[a] A higher kcat/Km can be observed for pNp substrate compared to the non-pNp substrate. Standard errors in parenthesis were estimated using the software GraphPad Prism.

5 Discussion

In this section, significant results obtained in the result section are discussed and compared with resent findings from the field of GE research. Also, suggestions for future research are discussed.

5.1 Characterization and diversity of three putative GEs

Most studies of GE activity have been conducted on GEs of fungal origin with the exception of the characterized GE of marine bacterial origin by De Santi *et al.,* 2016 [8]. In this study, three different GEs from the bacterium *Solibacter usitatus* were characterized this regard to pH optima, kinetics and other esterase activities, which adds novel information to bacterial CE15 family members.

The pH optima of 7.5 for *Su*B and *Su*C are in the same range as reported for other GEs [8, 26, 27]. Due to high substrate hydrolysis at alkaline pH, the majority of studies have been reported GE activity at pH 6 or lower [28, 29]. However, because of the pH optima of 7.5 for both *Su*B and *Su*C, this pH value was tested and selected for determination of kinetic parameters in this work. Nonetheless, for *Su*A, pH 8 should in future be used for kinetic measurements. The high pH optima of all these GEs highlight the fact that enzyme assays should be able to operate within the alkaline region, despite significant substrate hydrolysis at this pH with the substrates used so far for assaying GE activity. The pH dependence of GEs highlights the usefulness of the Sunner assay, as it does operate at an alkaline pH.

The three GEs displayed different catalytic efficiencies (k_{cat}/K_m) for all model substrates. *Su*A displayed a strong preference for BnGlcA with a k_{cat}/K_m 3 times higher than for AllylGlcA, MeGlcA and MeGalA, indicating the preference for more bulky substrates, an effect similarly observed by d'Errico *et al*., with two fungal GEs [16]. On the contrary, *Su*C displayed a k_{cat}/K_m in the same range for all glucuronic acid substrates, but considerable lower for the galacturonic acid substrate. These results indicate that the enzyme has high affinity to C-4 in the glucuronic acid as opposed to the galacturonic acid C-4 group. An effect also reported by Ďuranová in 2009 investigated four fungal GEs [15]. Finally, *Su*B exhibited the lowest k_{cat}/Km for the BnGlcA substrate, 30-40 times lower than *Su*A and *Su*C, respectively. This low k_{cat}/K_m has also been observed in *Nc*GE and Cip2 [27, 30] but on a different substrate. The reason could be that the synthetic substrates do not represent the natural bonds in the target compounds for this enzyme, and thus, that the target compound remains to be elucidated.

Lastly, *Su*A and *Su*C exhibited low activity upon pNp-Ac. Recently, De Santi *et al.,* 2016 demonstrated that a bacterial GE of marine origin had little activity upon glucuronic ester compounds, however, the enzyme exhibited activity upon pNp-Ac [8]. In contrast to De Santi *et al.,* 2016 the three GEs in this study exhibited lower activity upon pNp-Ac. In future, testing of a range of acetate compounds such as pNp-butyrate and pNp-octanoate would also be of interest.

The variety in pH optima and catalytic efficiency of the three GEs show the diversity amongst these enzymes and could imply activation at different time points and regulatory control. The three different GEs in *Solibacter usitatus* might work in synergy with GEs or

other carbohydrate acting enzymes, however, this remains to be elucidated in future experiments. Future work could therefore, include the study of enzyme synergy (either the three GEs combined or in combination with other carbohydrate active enzymes) to observe boosting effect of the GEs on carbohydrate active enzyme action, on either a natural or a complex synthesized substrate. This boosting effect could be useful in industry to achieve higher hydrolysis yields.

5.2 Advantages and limitations with the Fraňová assay

Model substrates, which have been used in GE research, are to some extent commercially available but are often difficult to synthesize. Therefore, in this study, the recently published assay with the easily synthesized methylated-D-glucuronic acid compound was studied for its potential in GE research. However, the MeGlcA-pNp compound obtained in this study was impure due to incomplete methylation, despite efforts for purification through chromatography. A possible reason could be the presence of water in the reaction, thus lowering the yield of the esterified compound. In the future, impure substrates could be avoided through continuous TLC monitoring of the reaction and the use of freshly distilled methanol. If an impure substrate is obtained, HPLC could aid in purification. As mentioned in the background, an assay can both be run continuously and stopped, therefore, this assay was both evaluated in a stopped and continuous manner.

The stopped already established Fraňová assay is convenient to use for screening of GE activity, once an appropriate setup has been validated [7]. An advantage of the stopped assay compared to the continuous is the use of the stop solution borax, which can be used to dilute the high background (obtained due to an impure substrate). Thus, the high background reached at higher substrate concentrations can be decreased to an appropriate absorbance. In addition, the stop solution, which raises the pH, allows the assay to be used in a wide pH range, provided that the substrate does not completely hydrolyze during the reaction. But due to the health risks involved in handling borax, it would for future use recommend substituting it to for another alkaline solution. Even if the assay is well-working, stopping an enzymatic reaction is a laborious procedure and if incubation time or substrate concentrations are altered in the assay setup, validation of the assay must be redone (checking for linearity over time).

Continuous assays are convenient and easy to use, because you do not have to handle the stop solution. Errors in the experimental procedure are easily spotted and remedied, because the reaction is monitored in real time. However, if an impure substrate is obtained, such as in this study, the continuous mode is problematic due to high background of impurities and the limit of detection is reached at moderate substrate concentrations. In practice, this means that the substrate cannot be varied to high substrate concentrations for determination of kinetic parameters. In addition, the detected molecule pNp in itself becomes a limiting factor in this assay, due to the pH dependence of its extinction coefficient. At a lower pH 5-5.5, the absorbance of the pNp molecule cannot be distinguished from the intermediate GlcA-pNp. Despite this, an impure substrate can be used for activity measurements in a continuous fashion.

The Fraňová assay can be used for measuring GE activity in both a continuous and a stopped manner, despite an impure substrate [7]. If a pure substrate had been obtained from the start of this project, kinetics would have been applicable also in a continuous fashion. However, due to the pH dependence of pNp, a continuous assay cannot be used at a pH below 6 at 405

nm. A broader pH range can be used in the stopped assay mode, due to the addition of the alkaline stop solution before measuring the absorbance.

5.3 GE activity and specificity on different model substrates with two different assays

Due to the pH dependent extinction coefficient of pNp, the substrate MeGlcA-pNp could not with ease be screened at 0.5 mM in a continuous fashion at pH 7.5, despite this being the pH at which the GEs exhibited the highest activity. This is because of the high background due to an impure substrate and the pH dependent extinction coefficient of pNp (the absorbance read increases with the pH even if the substrate concentration is constant). Therefore, the activity of the various GEs on commercial and the synthesized compound was compared in NaP*i* buffer, pH 6 for both assays.

It has been shown in a study by Hüttner *et al.,* 2016 that three fungal GEs all hydrolyzed glucuronic acid esters of BnGlcA, AllylGlcA and MeGlcA at pH 6 [31]. Analogously to these results all of the three GEs in the present study showed activity upon these compounds, both at pH 7.5 and 6. In particular, *Wc*GE1 show similar effects to that of *SuA* and *SuC*, which both had a high activity upon both BnGlcA and AllylGlcA.

The substrate MeGlcA-pNp was used by Ďuraranová in 2009 to study four fungal GEs on both MeGlcA and MeGlcA-pNp [15]. Generally, it has been found that the GEs were more active upon the MeGlcA-pNp substrate than MeGlcA. In the present study, this trend was also observed for all GEs, which had a greater activity on MeGlcA-pNp then MeGlcA and equivalent or larger activity than upon BnGlcA. Thus, the introduction of a hydrophobic group to the substrate increased the rate of hydrolysis.

5.4 Kinetics on two Methylated Glucuronic Acid Substrates with two different assays

Because of high substrate background for the synthesized MeGlcA-pNp substrate, kinetics was, for this purpose performed in a stopped mode for both the synthesized substrate and the commercially available MeGlcA with the GE *Su*C. There is a considerable difference in the k_{cat}/K_m between the two methylated glucuronic acids for *Su*C. The K_m is in the same range, however, the k_{cat} is larger for MeGlcA-pNp, indicating the similar substrate affinity but a more efficient substrate conversion.

The addition of the pNp hydrophobic group to the D-glucuronic acid ester might have contributed to the higher substrate conversion of the pNp substrate with the Fraňová assay compared to the non-pNp substrate with the Sunner assay. In the Sunner assay, the model substrate methyl-D-glucuronate is a mixture of both α- and β-anomers, which are de-esterified by the GE to glucuronic acid. The coupling enzyme UDH, only hydrolyzes the β-anomer of the glucuronic acid. Therefore, upon comparison with the Fraňová assay (that uses a substrate with only the β-anomer present) it might contribute to a bias result [4,7].

6 Conclusion

The three bacterial GEs investigated in this study, differed in pH optima and substrate specificity for commercially available substrates through the use of the Sunner assay. However, within the field of GE research the lack of a wide range of commercially available model substrates has driven the search for easily synthesized model substrates. One such substrate is the synthesized MeGlcA-pNp compound used in the novel GE assay developed by Fraňová [7]. This assay has been verified and validated in both a stopped and in a continuous mode. Due to the detection of the pH dependent p-nitrophenol, the assay can be used in a stopped mode from pH 5-9 (with addition of alkaline stop solution), however, not continuously at a pH below 6.

The GEs studied, all exhibited a high specific activity upon the synthesized compound compared to the commercially available model substrates used, which makes the novel assay useful for GE research. The Fraňová assay is further useful due to a range of model compounds that could be synthesized by substitution of different alcohols during synthesis [7]. With the use of a wide range of substrates, more information about GE substrate specificity may be collected and thereby contributing to discovery of novel GEs.

Before the use of enzymes in an industrial process with structurally complex biomass, it is necessary to understand their mechanism of action. Therefore, synthesizing new model substrates with different key features is of importance. One such feature that could provide vital information about substrate specificity would be model substrates with both γ- and the α-ester LC-bonds and α- and β-anomers.

The field of GE research is relatively new and therefore, there is a need of a range of model substrates to gain insight into GE activity and diversity. Further on, structure elucidation of GEs through the use of X-ray crystallography and NMR would also contribute to the understanding of enzyme-substrate interactions.

7 Bibliography

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8 Supplementary data

This section provides supplementary results obtained in this work.

8.1 Expression and purification of putative GEs

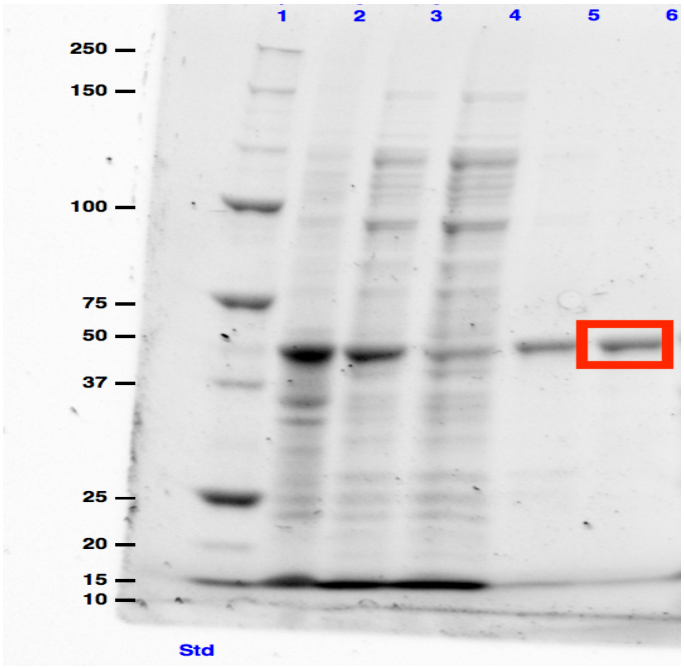

Figure S1. SDS-page of *SuA* fractions collected by IMAC and investigated for purification. The gel was loaded with samples of *Su*A from insoluble fraction (lane 2) crude extract (lane 3), flow through from IMAC (lane 4), wash from IMAC (lane 5) and elution from IMAC (lane 6). The molecular mass (kDa) of the standards (lane 1) is indicated beside the gel.

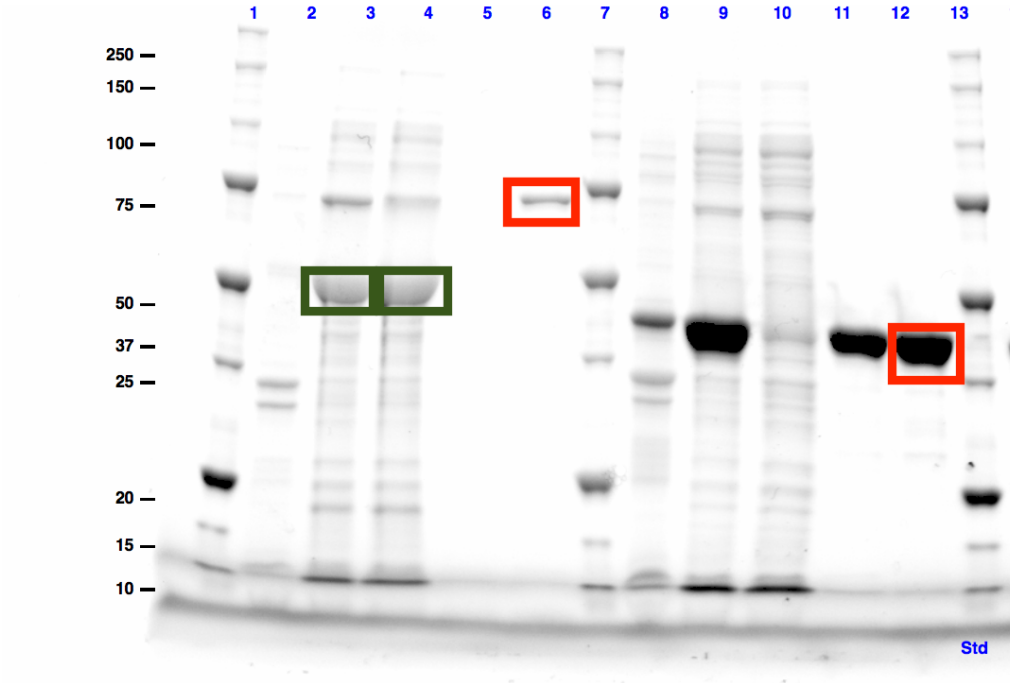

Figure S2. SDS-page of *SuB* and *SuC* fractions collected by IMAC and investigated for purification. The gel was loaded with samples of *SuB* in lane 2-6 from insoluble (lane 2), crude extract (lane 3), flow through from IMAC (lane 4), wash from IMAC (lane 5) and elution from IMAC (lane 6). The molecular mass (kDa) of the standards (lane 1, 7 and 13) is indicated beside the gel. The gel was loaded with samples of *Su*C in lane 8-12 in a similar fashion.

8.2 Biochemcial characterization

8.2.1 Determination of kinetic parameters

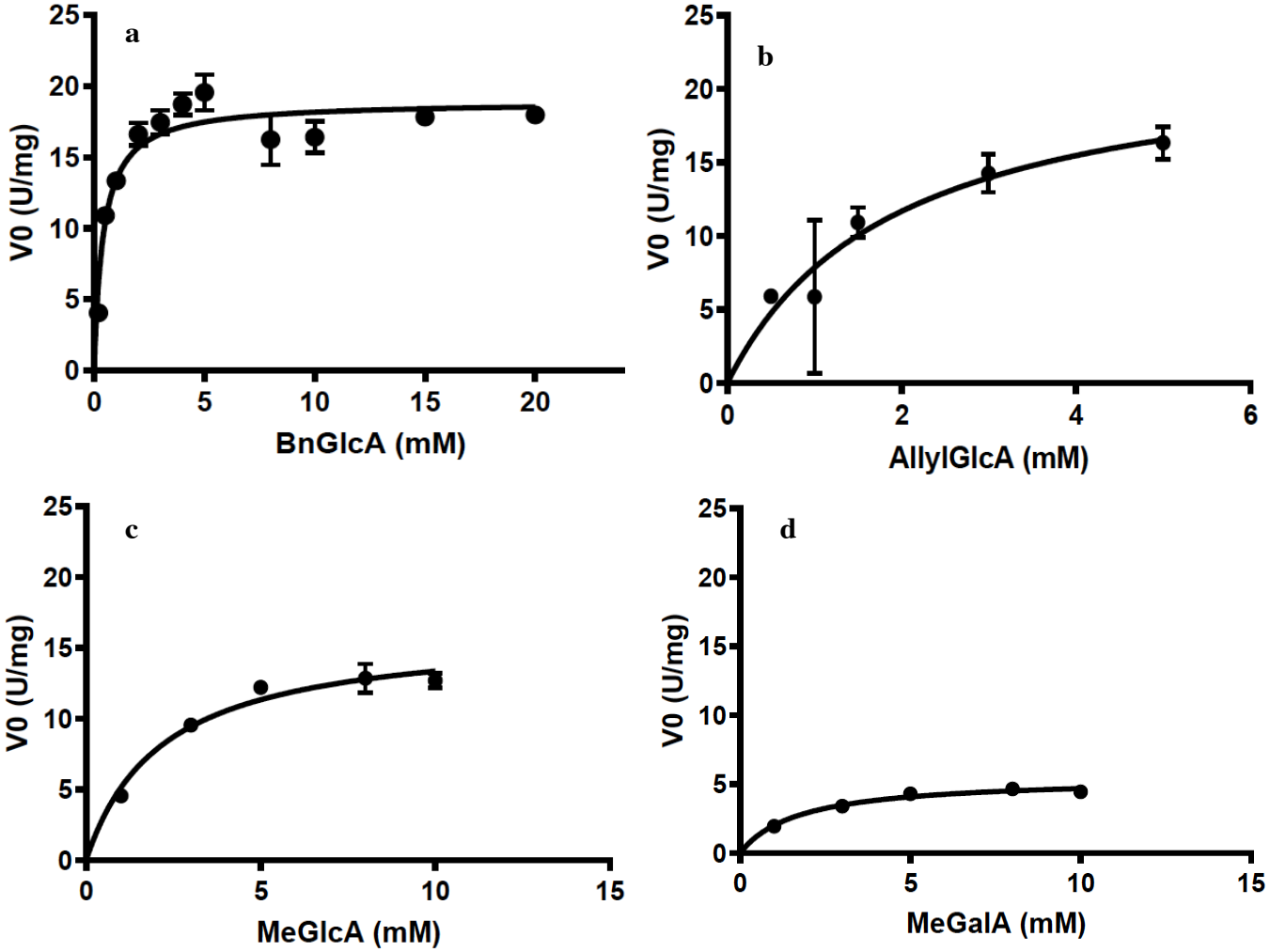

Figure S3. Enzyme activity of *SuA* on 4 different substrates BnGlc (a) AllylGlcA (b) MeGlcA (c) and MeGalA (d) in sodium phosphate buffer, pH 7.5. Nonlinear regression was obtained by fitting to the Michaelis-Menten equation, for determination of kinetic parameters, Standard deviations of duplicates are represented as error bars.

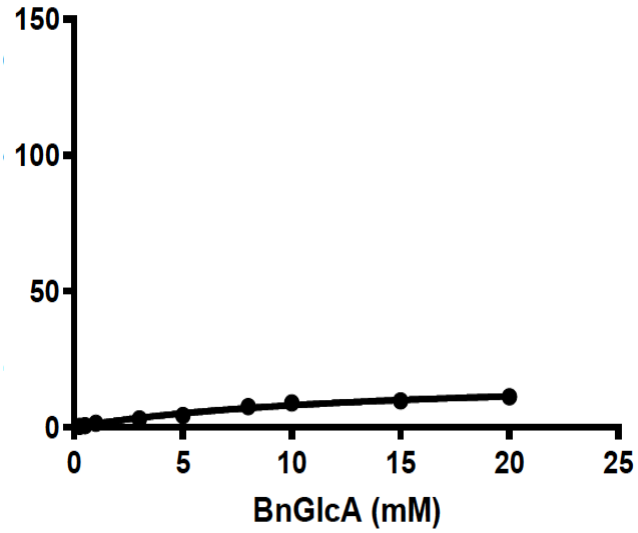

Figure S4. Enzyme activity of *SuB* on BnGlc in sodium phosphate buffer, pH 7.5. Nonlinear regression was obtained by fitting to the Michaelis-Menten equation, for determination of kinetic parameters, Standard deviations of duplicates are represented as error bars.

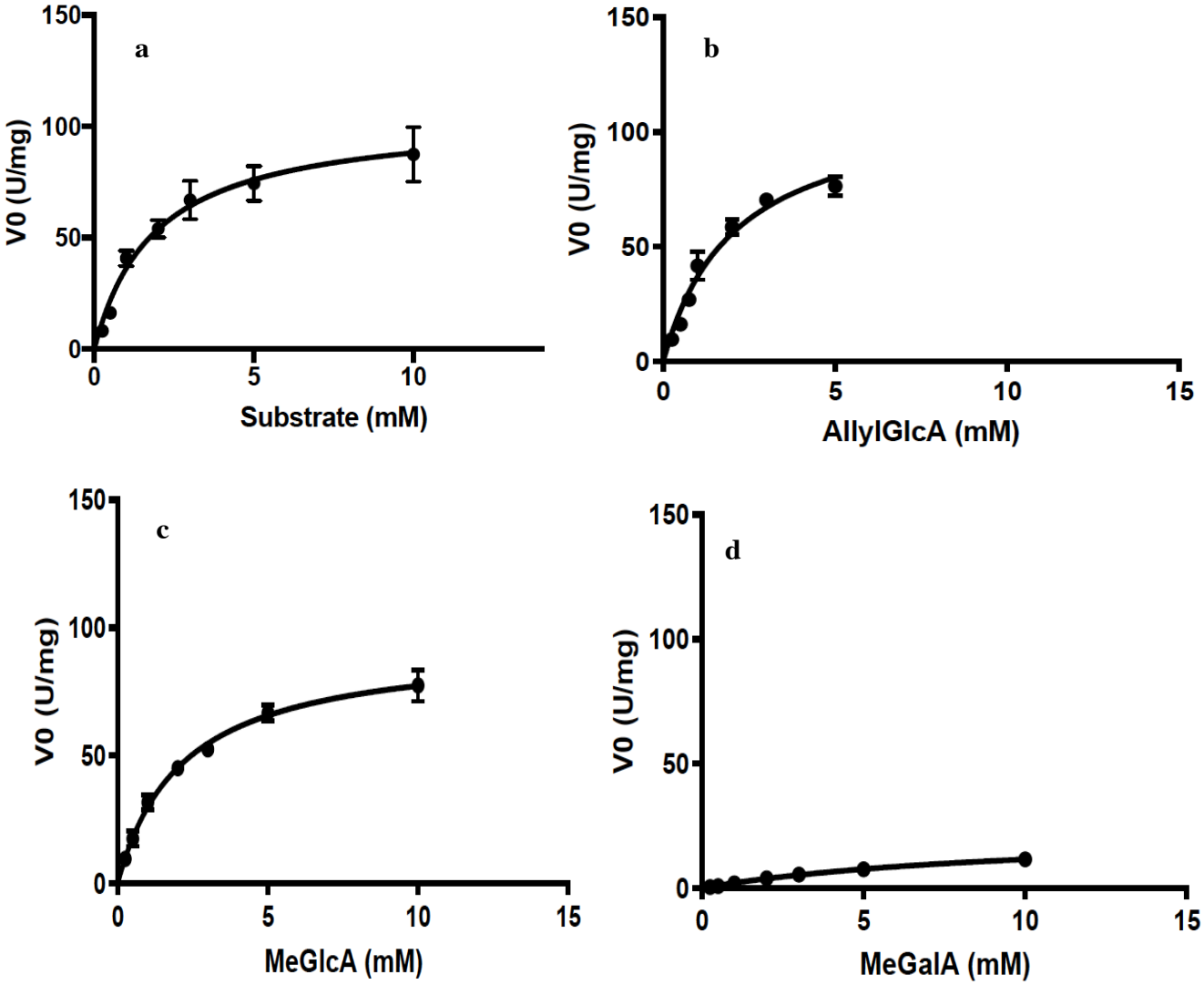

Figure S5. Enzyme activity of *SuC* on 4 different substrates BnGlc (a) AllylGlcA (b) MeGlcA (c) and MeGalA (d) in sodium phosphate buffer, pH 7.5. Nonlinear regression was obtained by fitting to the Michaelis-Menten equation, for determination of kinetic parameters, Standard deviations of duplicates are represented as error bars.

8.3 Fraňová assay validation

8.3.1 Substrate synthesis

Table S 1. 1H-NMR chemical shifts (in ppm) of MeGlcA-pNp after synthesis, assined based upon Fraňová [7].

	H-1	H-2	H-3	H-4	H-5	O-Me	H-2', H-6'	H-3',H-5'
MeGlcA-pNp	5.18	3.63	3.54	3.637	4.18	3.78	7.23	8.23

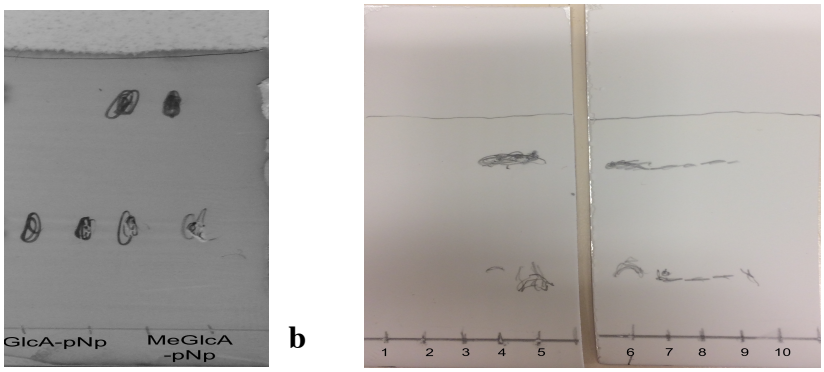

Figure S 6. TLC of: (a) The synthesized MeGlcA-pNp substrate before chromatographic purification (b) fractions collected after chromatigraphic purification. Fractions 4-9 were pooled together and used for the assay.

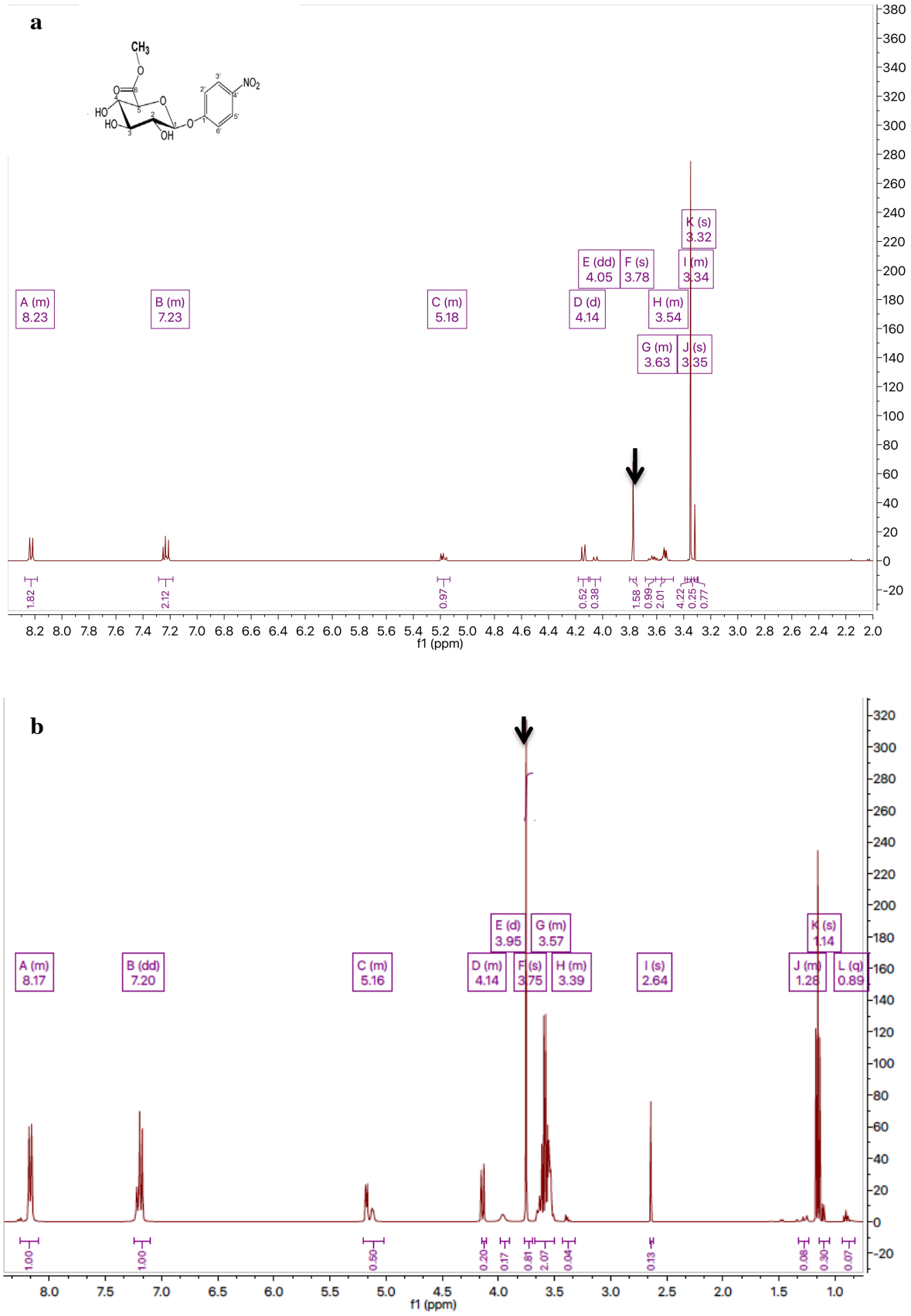

Figure S 7. H[1]-NMR spectra of: (a) The synthesized MeGlcA-pNp substrate before chromatographic purification (b) MeGlcA-pNp after chromatographic separation. The arrows mark the successful addition of the O-Me group to the D-glucuronic acid.

8.3.2 Substrate concentration

Table S 2. Substrate concentration and purity determination of the impure newly synthesized MeGlcA-pNp substrate.

Expected MeGlcA-pNp (mM)	GlcA-pNp (mM)	MeGlcA-pNp (mM)	% methylated substrate
0.25	0.13	0.13	52
0.5	0.22	0.22	50
1	0.44	0.56	54
3	1.3	1.5	54
5	2.5	2.5	50

8.3.3 Continuous assay validation

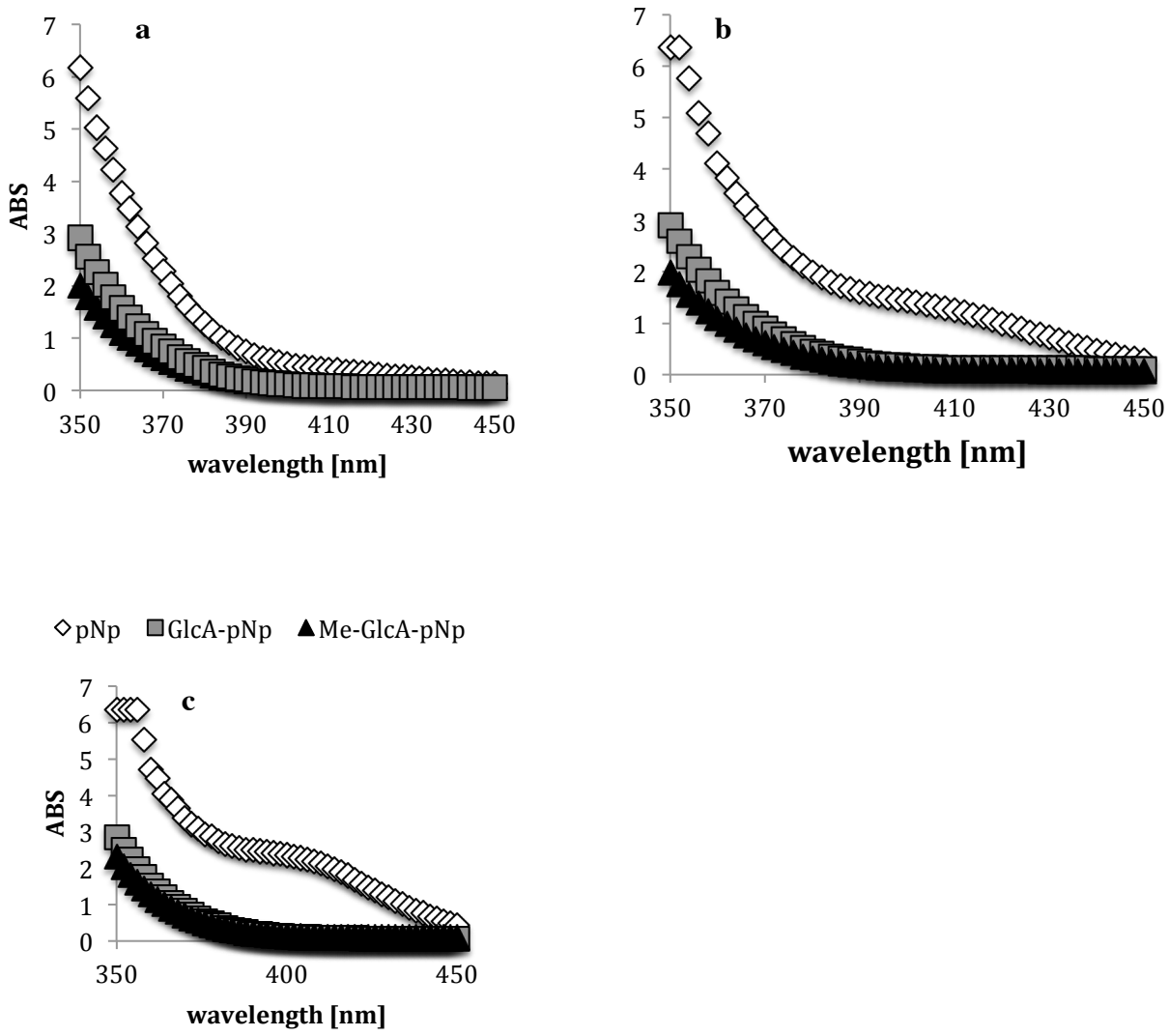

Figure S 8. Spectral scans of pNp (◆) GlcA-pNp (■) and MeGlcA-pNp (Δ) pH 5 (a) pH 5.5 (b) and pH 6 (c) from 350-450 nm.

Range of linearity GE concentration

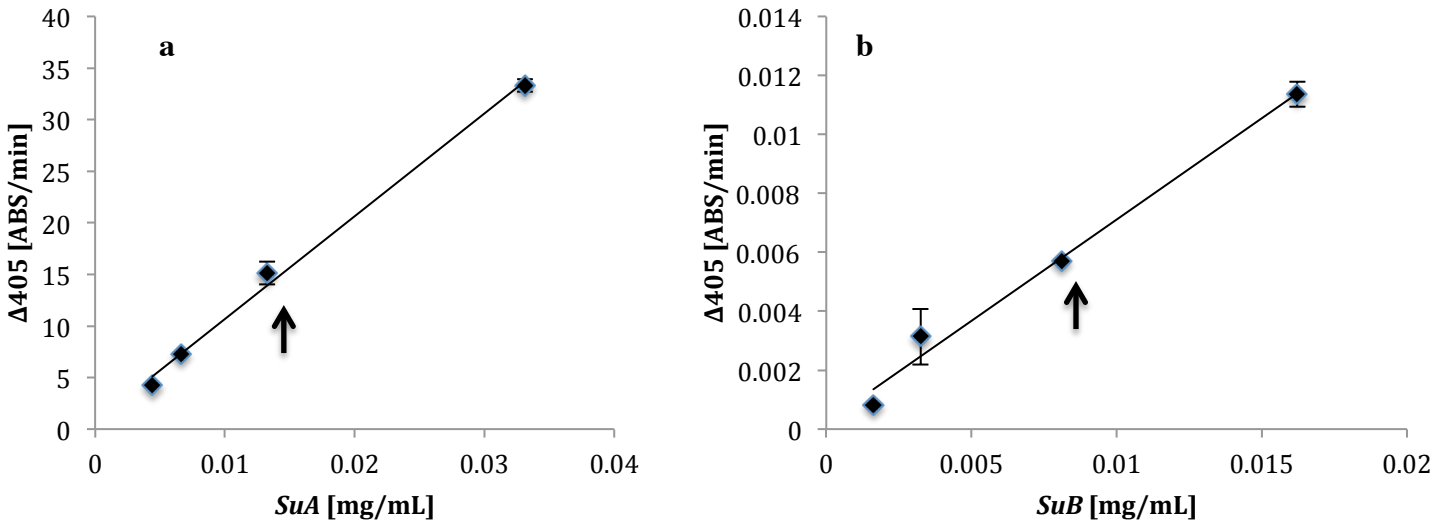

Figure S 9. Absorbance response as a function of GE concentration for: (a) *SuA* (b) *SuB*. Error bars represent the standard error from two independent measurements. Arrows indicate the GE concentration used in the assay.

8.3.4 Stopped assay validation

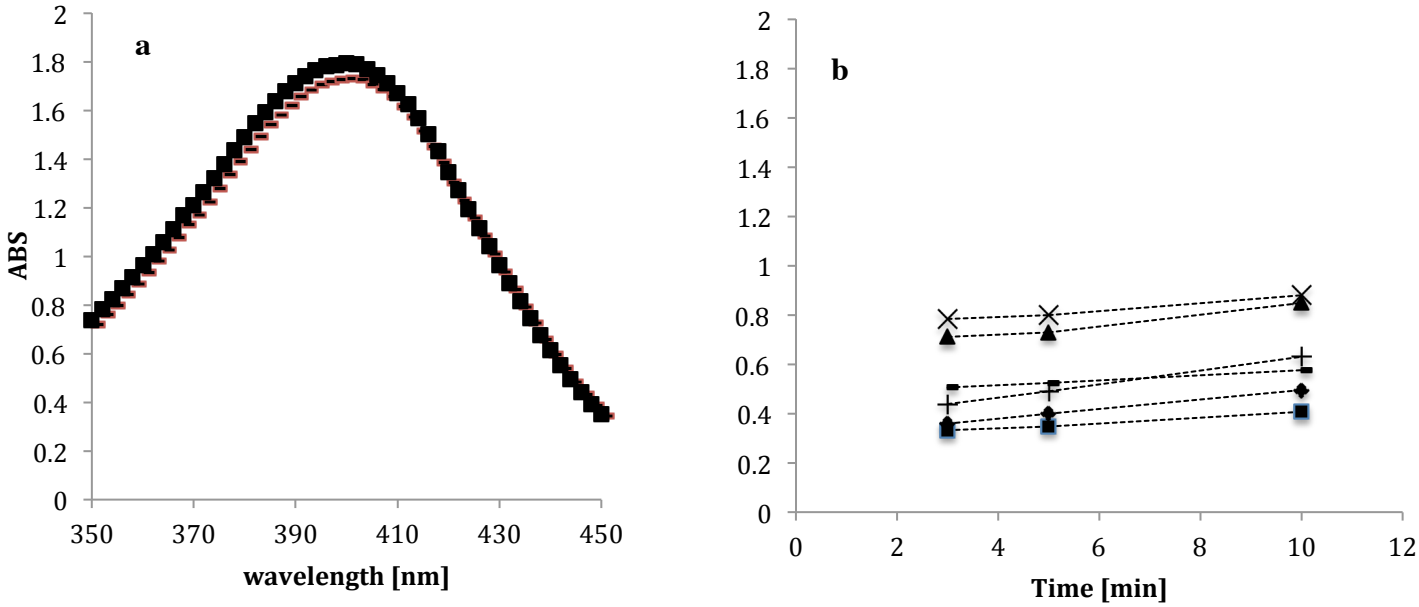

Figure S 10. (a) spectral scan of pNp (1 mM) in the pH range 5-8 with the addition of 1200 ul borax from 350-450 nm. (b) kinetic measurements stopped at 3,5 and 10 min with (■) 0.5 mM (◆) 0.75 mM (Δ) 1 mM (–) 3 mM (×) 5 mM (+) 8 mM MeGlcA-pNp substrate.

8.4 Kinetic comparison between MeGlcA and MeGlcA-pNp

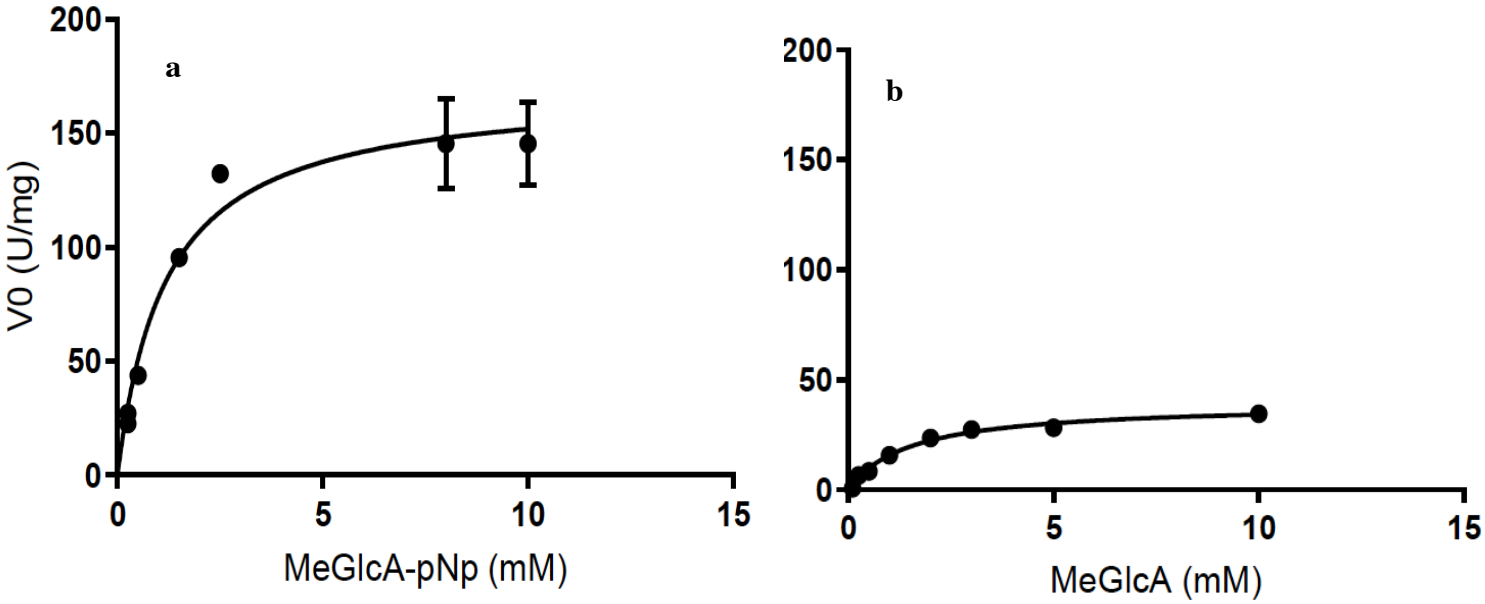

Figure S 11. Enzyme activity of *SuC* on (a) MeGlcA-pNp (b) MeGlcA in NaP*i*, pH 7.5 Nonlinear regression was obtained by fitting to the Michaelis-Menten equation, for determination of kinetic parameters, Standard deviations of duplicates are represented as error bars.