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A long-wavelength fluorescent substrate for continuous fluorometric determination of α-mannosidase activity: Resorufin α-D-mannopyranoside

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ABSTRACT

A simple and reliable continuous assay for measurement of α-mannosidase activity is described and demonstrated for analysis with two recombinant human enzymes using the new substrate resorufin α-D-mannopyranoside (Res-Man). The product of enzyme reaction, resorufin, exhibits fluorescence emission at 585 nm with excitation at 571 nm and has a pK_a of 5.8, allowing continuous measurement of fluorescence turnover at or near physiological pH values for human lysosomal and *Drosophila* Golgi α-mannosidases. The assay performed using recombinant *Drosophila* Golgi α-mannosidase (dGMII) has been shown to give the kinetic parameters K_m of 200 μM and V_{max} of 11 nmol/min per nmol dGMII. Methods for performing the assay using several concentrations of the known α-mannosidase inhibitor swainsonine are also presented, demonstrating a potential for use of the assay as a simple method for high-throughput screening of inhibitors potentially useful in cancer treatment.

Introduction

The N-glycosylation pathway is a target for chemical intervention in a number of pathological conditions, including cancer. Cells that have undergone oncogenic transformation often display abnormal cell surface oligosaccharides [1–4]. These changes in glycosylation are important determinants of tumor progression. Inhibition of the mannose trimming enzyme Golgi α-mannosidase II (GMII,[2] mannosyl-oligosaccharide 1,3- and 1,6-α-mannosidase II, EC 3.2.1.114) is a potential route for blocking the changes in cell surface oligosaccharides [5,6]. GMII is the final glycoside hydrolase in the N-glycosylation pathway and is responsible for the formation of the "core" tri-mannose structure. GMII catalyzes the hydrolysis of both an α-1,6- and α-1,3-linked mannose from GlcNAc-Man5-GlcNAc$_2$-Asn to form GlcNAc-Man$_3$-GlcNAc$_2$-Asn [7,8]. Subsequently, a series of glycosyl transferases add a variety of carbohydrates (including *N*-acetyl glucosamine, galactose, and sialic acid) to this tri-mannose core to form the completed complex carbohydrate structure.

The α1,3- and α1,6-mannosidases, including GMII and lysosomal α-mannosidase (LM, EC 3.2.1.24), are members of the family 38 glycoside hydrolases. These enzymes cleave between two mannose residues with a net retention of configuration and operate via a mechanism involving formation of a covalent glycosyl enzyme intermediate. LMs are involved in degradation of complex sugars derived from glycoproteins within the low-pH environment of the lysosome. Impairment of LM activity, through genetic mutation or environmental toxicity, leads to the buildup of partially processed oligosaccharides in large vacuolar structures within cells and has severe neurological consequences [9,10]. Initial clinical tests indicated that the GMII inhibitor swainsonine, which inhibits in the nanomolar range, had promise as a cancer treatment agent [8]. However, side effects, possibly caused by the inhibition of

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[2] *Abbreviations used:* GMII, Golgi α-mannosidase II; LM, lysosomal α-mannosidase; dGMII, catalytic domain of *Drosophila melanogaster* GMII; MU-Man, 4-methylumbelliferyl α-D-mannopyranoside; PNP-Man, *p*-nitrophenyl α-D-mannopyranoside; X-Man, 5-bromo-4-chloro-3-indolyl α-D-mannopyranoside; Res-Man, resorufin α-D-mannopyranoside; HLM, human lysosomal α-mannosidase; NMR, nuclear magnetic resonance; TLC, thin layer chromatography; CH$_2$Cl$_2$, dichloromethane; MeOH, methanol; R_f, retention factor; HCl, hydrochloric acid; CDCl$_3$, deuterochloroform; SiO$_2$, silica; EtOAc, ethylacetate; Ni–NTA, nickel–nitrilotriacetic acid; DMSO, dimethyl sulfoxide; 4-MU, 4-methylumbelliferone; HPLC, high-pressure liquid chromatography; Res-CB, resorufin β-D-cellobioside; HTS, high-throughput screening.

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LM by swainsonine, severely limit its usefulness, necessitating the search for more specific inhibitors.

Thus, GMII is a target for a number of synthetic efforts aimed at providing more selective and effective inhibitors. The catalytic domain of the *Drosophila melanogaster* enzyme (dGMII) has been studied extensively due to the ease of obtaining high-resolution structural data [11]. Structural details of the substrate cleavage events have been elucidated [12–14]. GMII employs a two-stage mechanism involving two carboxylic acids positioned within the active site that act in concert—one as a catalytic nucleophile and the other as a general acid/base catalyst. Protonation of the exocyclic glycosyl oxygen of a substrate molecule leads to bond breaking and simultaneous attack of the catalytic nucleophile to form a glycosyl enzyme intermediate [12]. Hydrolysis of the covalent intermediate by a nucleophilic water molecule gives an α-mannose product. Substrate rearrangement in the active site repositions the second mannose for a second round of cleavage [13,14].

The pH optimum of dGMII is low at 5.7 [15]. An existing fluorogenic substrate, 4-methylumbelliferyl α-D-mannopyranoside (MU-Man) [16] cannot be used for continuous fluorometric measurement because of the high pK_a value of the released product, 4-methylumbelliferone, which has a pK_a of 7.8 [17]. This substrate does not allow continuous measurement of activity because it is not substantially fluorescent at these low-pH values if 360 nm excitation is used. α-Mannosidase has also been assayed for previously using a chromogenic substrate, *p*-nitrophenyl α-D-mannopyranoside (PNP-Man) [15,16]. However, chromogenic substrates suffer from low sensitivity, and the absorbance readings of released PNP also cannot be recorded continuously at low pH because PNP has a pK_a of 7.1 [18]. The addition of a high-pH stop buffer, such as 0.5 M sodium bicarbonate [15] or glycine–NaOH buffer [16], is required prior to measurement when using either substrate. 5-Bromo-4-chloro-3-indolyl α-D-mannopyranoside (X-Man) is a chromogenic substrate that releases a precipitating blue dye on cleavage, and although activity can be observed at low pH [19], the solid nature of the product makes activity measurements difficult to quantitate. To overcome these limitations, a long-wavelength fluorescent substrate, resorufin α-D-mannopyranoside (Res-Man), was prepared and used to obtain continuous fluorometric measurement of α-mannosidase activity. This substrate releases the red fluorescent fluorophore resorufin (excitation = 571 nm, emission = 585 nm) with a pK_a of 5.8 [20]. The use of resorufin as the released product for low-pH enzymes has previously been found to be effective in measuring cellulase activity continuously [21]. Here we present several examples of continuous α-mannosidase activity measurement from recombinant samples of purified α-mannosidase, α-mannosidase II from dGMII [15], and human lysosomal α-mannosidase (HLM) at pH 6.0. The results indicate that the new Res-Man substrate can be used to provide continuous fluorometric measurement, kinetic analysis, and inhibition screening of α-mannosidase activity at physiological pH.

Materials and methods

Chemicals and instruments

Resorufin sodium salt was obtained from Sigma Chemical (St. Louis, MO, USA). Hydrochloric acid, acetic anhydride, and dry pyridine were obtained from Mallinckrodt Chemicals (Phillipsburg, PA, USA). Hydrobromic acid (33 wt% solution in glacial acetic acid), silver carbonate, Amberlite IRC-50 ion exchange resin, sodium methoxide (25 wt% solution in methanol), and anhydrous dichloromethane were obtained from Aldrich Chemical (Milwaukee, WI, USA). Sym-collidine was obtained from Acros Organics (Morris Plains, NJ, USA). All chemicals were used without further purification. ^{1}H nuclear magnetic resonance (NMR) spectra were obtained using a Varian Inova 300-MHz nuclear magnetic resonance instrument. Microtiter plates used were obtained from Becton Dickinson (BD Falcon, product no. 351172, clear, flat-bottomed, 96-well plates). Fluorescence readings were obtained using a PerkinElmer HTS 7000 Plus BioAssay Reader and HTSoft analysis software.

Synthesis

Resorufin-free acid

Resorufin sodium salt (3.00 g, 12.75 mmol) was dissolved in ice-water (150 ml), and concentrated hydrochloric acid (6 ml) was added with stirring to pH 2.0. After stirring at 0 °C (3 h), the red–brown precipitate was filtered and washed with water until the filtrate was neutral. The resulting resorufin-free acid was dried in air and in vacuo overnight to yield a dark red solid (2.64 g, 97%), homogeneous by thin layer chromatography (TLC) analysis (9:1 CH_2Cl_2/MeOH, $R_f = 0.43$).

Resorufin α-D-mannopyranoside, tetra-O-acetate

Under an anhydrous N_2 (g) atmosphere, a mixture of acetobromomannoside (prepared by per acetylation of D-mannose using excess acetic anhydride and pyridine [22] followed by treatment with HBr in glacial acetic acid) (2.37 g, 5.77 mmol), anhydrous CH_2Cl_2 (20 ml), anhydrous acetonitrile (20 ml), resorufin (370 mg, 1.74 mmol), dry silver carbonate (855 mg, 3.10 mmol), and sym-collidine (275 μl) were allowed to stir under anhydrous conditions at room temperature overnight, protected from light. After this time, the reaction mixture was filtered through a Celite pad and the gray solids were washed with additional CH_2Cl_2 (30 ml). The clear orange filtrate was evaporated to near dryness and redissolved in CH_2Cl_2 (50 ml) washed with water (2 × 50 ml), saturated aqueous sodium bicarbonate solution (1 × 50 ml), water (1 × 50 ml), 1 N HCl/H_2O (1 × 50 ml), 0.2 N sodium thiosulfate solution (1 × 50 ml) and water (1 × 50 ml). The resulting organic layer was dried over anhydrous sodium sulfate, filtered, evaporated, and dried in vacuo overnight to yield an orange powder (3.65 g). Purification by column chromatography (SiO_2, 70–230 mesh, 25–75 mm, irrigant = 9:1 CH_2Cl_2/EtOAc) produced a bright orange solid that was recrystallized from ethylacetate to give amorphous reddish crystals (0.89 g, 95%) ($R_f = 0.16$, irrigant = 9:1 CH_2Cl_2/EtOAc). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.72 (d, 1H), 7.43 (d, 1H), 6.95 (dd, 1H), 6.83 (dd, 2H), 6.33 (d, 1H), 6.09 (d, 1H), 5.35 (m, 1H), 5.25 (d, 1H), 4.30 (dd, 1H), 4.10 (m, 2H), 3.50 (m, 1H), 2.18 (s, 3H), 2.10 (s, 3H), 2.06 (s, 3H), 2.01 (s, 3H).

Resorufin α-D-mannoside

Resorufin mannoside, tetraacetate (417 mg, 0.50 mmol), was suspended in anhydrous methanol (15 ml), and sodium methoxide (25%, w/v) solution in methanol (300 μl) was added (to pH 10.0). This reaction was stirred overnight at room temperature, and the resulting bright orange product was filtered, washed with minimum anhydrous methanol, and dried in vacuo overnight to produce an orange solid that was recrystallized from acetonitrile. Yield = 268 mg, 99% TLC ($R_f = 0.83$, irrigant = 7:3 EtOAc/MeOH). ^{1}H NMR (DMSO-d6, 300 MHz): δ = 7.80 (d, *J* = 7.8 Hz, 1H), 7.52 (d, *J* = 8.5 Hz, 1H), 7.17 (d, *J* = 0.8 Hz, 1H), 7.10 (dd, *J* = 7.8, 0.8 Hz, 1H), 6.79 (dd, *J* = 7.8, 0.84 Hz, 1H), 6.39 (d, *J* = 1.3 Hz, 1H), 5.61 (d, *J* = 6.5 Hz, 1H), 5.21 (dd, *J* = 13.7, 5.9 Hz, 2H), 5.02 (dd, *J* = 9.1, 5.9 Hz, 2H), 4.83 (d, *J* = 0.1 Hz, 1H), 4.65 (m, *J* = 7.8 Hz, 2H), 4.28 (d, *J* = 0.7 Hz, 1H), 3.63 (m, 4H), 3.45 (m, 3H), 3.10 (m, 6H).

Protein purification

The purification and characterization of full-length HLM will be described in more detail in a subsequent publication (M. Venkatesan, D.A. Kuntz, and D.R. Rose, manuscript submitted). Briefly, HLM containing a C-terminal His6 tag was expressed in a secreted form in *Drosophila* S2 cells using the *Drosophila* Expression System (DES, Invitrogen, Carlsbad, CA, USA). Then 2.5 mM $CoCl_2$ was added to the medium containing secreted HLM. This was batch bound to Chelating Sepharose Fast Flow (GE Biosciences, Montreal, Quebec, Canada). Bound protein was eluted with decreasing pH buffers added stepwise. The pooled dialyzed fractions were then resolved by cation exchange on SP HiTrap (GE Biosciences). HLM was eluted over a gradient of 0.2–0.6 M NaCl. The enzyme was concentrated to 1 mg/ml before being snap frozen in liquid nitrogen for storage.

dGMII purification was essentially as described previously [11] with an added anion exchange step to further purify the enzyme. Briefly, the soluble catalytic domain of *Drosophila* GMII (residues 76–1108) containing an N-terminal His6 tag was expressed in a secreted form in S2 cells. The medium was batch bound to Blue F3GA agarose (Sigma Chemical). dGMII was eluted with 0.35 M NaCl and directly batch bound to nickel–nitrilotriacetic acid (Ni–NTA) agarose (Qiagen, Montreal, Quebec, Canada) from which it was eluted with 30 mM imidazole. After dialysis, the protein was further purified by salt elution from a MonoQ anion exchange column (GE Biosciences), followed by subsequent dialysis, concentration, and freezing in liquid N_2.

Enzyme assays

Continuous assay of α-mannosidase enzymes

For all assays, the enyzmes dGMII and HLM were diluted to 67 μg/ml in 100 mM sodium acetate buffer (pH 6.0) (reaction buffer). Each enzyme preparation was added in triplicate (10 μl) to wells in a clear, flat-bottomed, 96-well polystyrene microtiter plate (BD Falcon). Then 40 μl of reaction buffer was added to each sample. A blank sample containing no enzyme (50 μl of reaction buffer) was also prepared in triplicate wells. A 0.5-mM substrate reagent solution was prepared by diluting a 5-mM dimethyl sulfoxide (DMSO) stock (1:10) in reaction buffer. The substrate reagent solution (50 μl) was added to each well to a final concentration of 0.25 mM. Final concentrations were 6.7 μg/ml for both enzymes (55–60 nM). Fluorescence was measured using a PerkinElmer HTS 7000 Plus BioAssay Reader in top read mode (excitation filter = 550 nm, emission filter = 595 nm). Fluorescence was recorded at room temperature for 40 cycles with a cycle time of 1 min. All readings were performed in triplicate and averaged.

Continuous kinetic assay

Four Res-Man substrate reagent solutions (1.0, 0.60, 0.30, and 0.20 mM) were prepared by diluting DMSO stocks 1:5 in reaction buffer. The substrate reagent solutions were added in triplicate (50 μl) to wells in a clear, flat-bottomed, 96-well polystyrene microtiter plate. After that, 40 μl of reaction buffer was added to each well, and then 10 μl of diluted dGMII was added to the above wells. The final substrate concentrations were 0.50, 0.30, 0.20, and 0.10 mM. The final enzyme concentration was 56 nM. Fluorescence was measured as above. Fluorescence was recorded at 30-s intervals for 210 s, and the mean fluorescence value of a blank (50 μl of substrate reagent added to 50 μl of reaction buffer in triplicate wells) was subtracted from the value of each sample well to normalize data at each time point. A standard curve was generated by plotting fluorescence of four concentrations of resorufin standards (50, 20, 10, and 5 μM) prepared by diluting DMSO stocks in sodium acetate buffer and adding to microtiter plate wells in triplicate (100 μl). All readings from triplicate wells were averaged. The curve generated from the standards was used to convert raw fluorescence data into μmol/ml/min resorufin produced. Initial velocities were determined from the linear portion of this curve using GraFit (Erithacus Software) and Microsoft Excel graphing software. Kinetic parameters K_m and V_{max} were calculated using GraFit employing a nonlinear regression analysis.

Continuous assay using known mannosidase inhibitor

Diluted dGMII (10 μl) was added to 27 wells in a clear, flat-bottomed, 96-well polystyrene microtiter plate. Swainsonine (H_2O stock) (Sigma Chemical, product no. 068K8721) was added to wells at eight different concentrations in triplicate, and well volume was adjusted to 50 μl with reaction buffer. A set of triplicate wells received no swainsonine, and well volume was adjusted to 50 μl with reaction buffer. Final concentrations of swainsonine were 10.0 μM, 1.00 μM, 500 nM, 250 nM, 100 nM, 50.0 nM, 25.0 nM, 10.0 nM, and 0.00 nM. A 0.5-mM substrate reagent solution was prepared by diluting a 5-mM DMSO stock 1:10 in reaction buffer. Then 50 μl of the substrate reagent solution was added to each well to give a final concentration of 0.25 mM. The final concentration of enzyme in all wells was 56 nM. Fluorescence was measured using a PerkinElmer HTS 7000 Plus BioAssay Reader in top read mode (excitation filter = 550 nm, emission filter = 595 nm). Fluorescence was recorded at room temperature for 30 cycles with a cycle time of 1 min. All readings were performed in triplicate and averaged. The mean fluorescence value of a blank (50 μl of substrate reagent added to 50 μl of reaction buffer in triplicate wells) was subtracted from the value of each sample well to normalize data at each time point. IC_{50} was determined as the concentration of inhibitor that results in an initial velocity 50% that of the sample containing no inhibitor. IC_{50} was used along with previously calculated K_m to determine K_i.

Results and discussion

Several fluorescent and chromogenic mannopyranosides are commercially available for use in detection of α-mannosidase activity, including MU-Man, PNP-Man, and X-Man. However, kinetic assays using these substrates are inconvenient. PNP-Man and X-Man hydrolysis release a chromogenic product, which is less sensitive than using fluorescence spectroscopy and can be difficult to quantitate if enzyme activity is low. In addition, quantitation of PNP requires the addition of a high-pH buffer (i.e., a “stop” buffer) before absorbance can be recorded, elevating the pH of the reaction mixture above the pK_a of PNP (i.e., 7.1) [18]. To obtain kinetic data, aliquots must be removed and measured at defined intervals. The product of X-Man hydrolysis is a dark blue precipitate that is difficult to quantitate. Assays using MU-Man must be obtained photometrically (resulting in reduced sensitivity) or, to perform more sensitive fluorescence readings, aliquots must be removed at several time points, at which time a stop buffer is added [16], elevating the pH of the reaction mixture above 7.8, the pK_a of the product, 4-methylumbelliferone (4-MU) [17]. 4-MU gives minimal fluorescence at pH 5.7, the reported optimum for dGMII [15]. Other methods of continuous quantitation using these substrates involve analysis of product turnover using cumbersome TLC or high-pressure liquid chromatography (HPLC) analysis of aliquots removed at various time points. These methods are time-consuming and limit the ability to perform sensitive kinetic analyses of the enzymatic reaction at early time points of the reaction.

Previous success has been achieved in measuring activity of cellulases, which also have optimal activities at acidic pH, by using a substrate that releases the fluorophore resorufin, resorufin β-D-cellobioside (Res-CB) [21]. Resorufin is a long-wavelength, red fluorescent fluorophore (excitation = 571 nm, emission = 585 nm) with a pK_a of 5.8 [16]. As demonstrated previously [21], its low pK_a relative to 4-MU allows resorufin to retain appreciable fluorescence at low pH values. Very good results were achieved previously using Res-CB for continuous fluorometric determination of cellulase activity at pH 6.0 [21]. Thus, it was reasoned that a resorufin-based substrate for α-mannosidase, Res-Man, could be effective in obtaining continuous fluorometric measurements and kinetic enzyme data for α-mannosidases at or near their physiological pH values using long-wavelength excitation and emission wavelengths that help to avoid (albeit to a limited to extent) background fluorescence from high-throughput screening (HTS) library compounds and endogenous cell lysate components.

Synthesis of Res-Man

Our synthetic method was similar to the methods used for other resorufin glycosides (Fig. 1) [23]. The starting material was the free phenol form of resorufin, prepared by acid treatment and crystallization of commercially available resorufin sodium salt (Sigma Chemical). The glycosylation reaction used the peracetylated 1-deoxy-1-bromo derivative of D-mannose that could be prepared by sequential acetylation and treatment of the anomeric mixture of α- and β-mannose pentaacetates with HBr in glacial acetic acid at 0 °C. Using these methods, high yields of the reactive bromo-sugar product were produced. The anomeric configuration of the acetobromo-D-mannose produced was found to be a mixture of α and β anomers, as evidenced by the ^{1}H NMR analysis.

Glycosylation was carried out using a modified Koenigs–Knorr methodology [24] employing silver carbonate as catalyst and sym-collidine as proton scavenger to give the protected intermediate resorufin α-D-mannoside, tetraacetate. Zemplen deprotection [25] with catalytic sodium methoxide in methanol afforded the desired Res-Man substrate as a single product that crystallized from the reaction mixture in virtually quantitative yield.

The Res-Man substrate produced in this way was found to be stable and could be stored for long periods of time (>6 months) at −18 °C, desiccated in a powdered form or in anhydrous DMSO solution (5 mM), without noticeable decomposition (TLC analysis).

Enzymatic assays of recombinant α-mannosidases

Our results demonstrate the ability of this new substrate to provide continuous fluorometric measurement of α-mannosidase activity from different sources. At pH 6.0, a fluorescence increase over time was observed in assays using recombinant dGMII (Fig. 2), which has an optimum pH of 5.7 [15], and recombinant HLM, which has an optimum pH of 4.5 (Fig. 2).

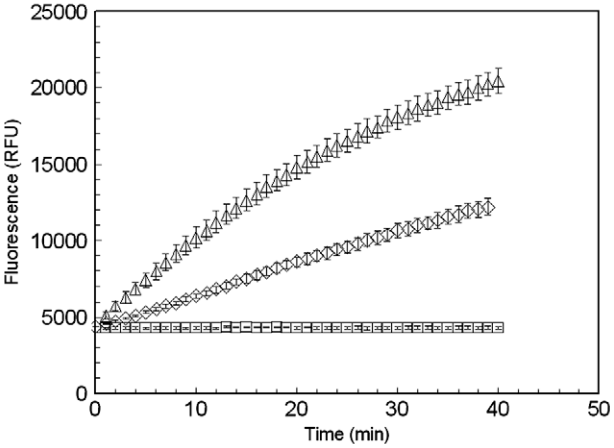

Fig. 2. Continuous fluorescent analysis of recombinant dGMII (Δ) and recombinant HLM (◇) using the substrate Res-Man. Res-Man (500 μM, diluted from 5 mM DMSO stock using 100 mM sodium acetate buffer, pH 6.0) (50 μl) was added to the enzyme samples (50 μl) to a final concentration of 250 μM. Enzyme samples were prepared in 100 mM sodium acetate buffer and diluted to a final concentration of 6.67 μg/ml in the reaction mixture. Fluorescence of a blank (no-enzyme) sample (□) was also analyzed over the same time period. Fluorescence emission was measured at 590 nm with excitation at 550 nm for 40 min at 1-min intervals. Data points are means, and error bars represent standard errors ($n = 3$). RFU, relative fluorescence units.

Inhibition assay

Inhibition of α-mannosidase has been indicated as important in controlling tumor progression [1–4,26]. A sensitive continuous assay of α-mannosidase activity could prove to be invaluable in high-throughput screening of inhibitors. Activity of dGMII in the presence of swainsonine, a natural inhibitor of type II α-mannosidases [8,15,26], at several different concentrations was assayed using a continuous assay format. The results suggest that the Res-Man substrate would be suitable for inhibitor screening given that decreasing activity was observed with increasing swainsonine concentrations (Fig. 3) and complete inhibition of activity was observed at concentrations greater than 1 μM (Fig. 3). Using the Res-Man substrate, IC_{50} was determined to be 100 nM and K_i was calculated to be 44.7 nM. IC_{50} of swainsonine with dGMII has been reported to be in the range of 12–20 nM [15] using PNP-Man as a substrate.

In previous studies, the substrate MU-Man has been used for determination of α-mannosidase activity using fluorescence end-

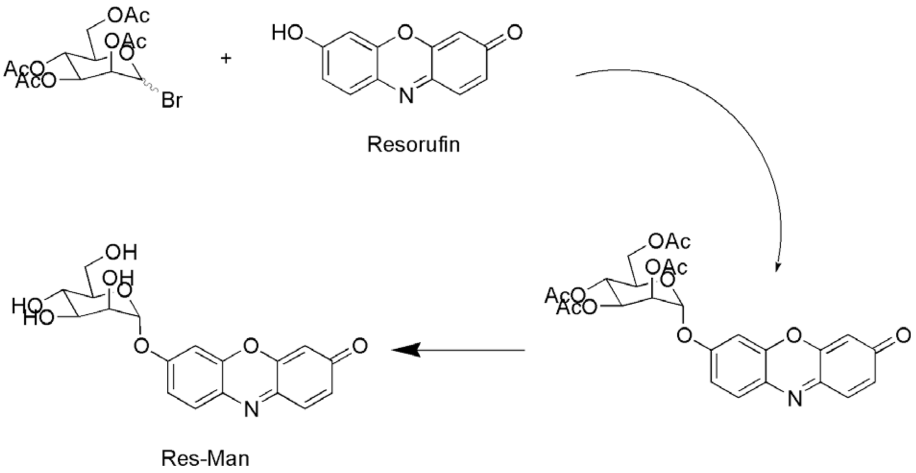

Fig. 1. Res-Man synthetic scheme.

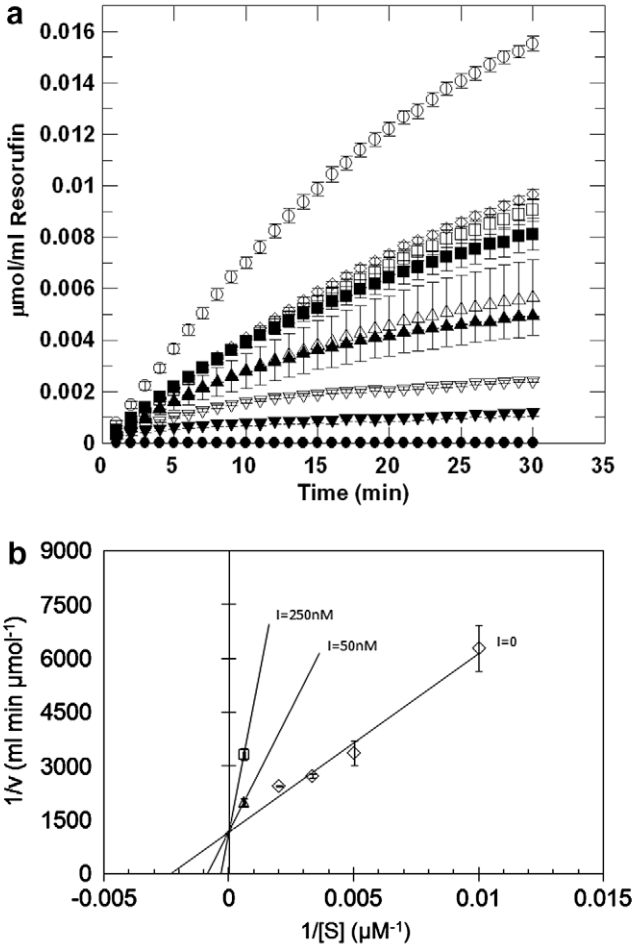

Fig. 3. (a) Inhibition of recombinant dGMII by swainsonine using the substrate Res-Man. IC_{50} was determined to be 100 nM, and K_i was determined to be 44.7 nM. dGMII was diluted to 6.67 µg/ml in 100 mM sodium acetate (pH 6.0). Swainsonine was added at concentrations of 10 µM (●), 1 µM (▼), 500 nM (▽), 250 nM (▲), 100 nM (△), 50 nM (■), 25 nM (□), and 10 nM (◇). An enzyme sample containing no inhibitor (○) was also prepared. Res-Man (500 µM, diluted from a 5-mM DMSO stock using sodium acetate buffer) (50 µl) was added to the enzyme samples (50 µl) to a final concentration of 250 µM. Fluorescence of all assays was recorded using excitation of 550 nm and emission of 595 nm. Data were converted to micromoles (µmol) of resorufin product produced using a standard curve created by measuring fluorescence of several known concentrations of resorufin in sodium acetate buffer (pH 6.0). Data points are means, and error bars represent standard errors ($n = 3$). (b) Lineweaver–Burk plots generated from kinetic assay using the substrate Res-Man. The kinetic parameters K_m and V_{max} were determined via computer-assisted nonlinear regression analysis using GraFit software. Values for K_m and V_{max} for the uninhibited assay were determined by GraFit to be 202.05 µM and 0.00059442 µmol/ml/min, respectively. The inset shows competitive inhibition by swainsonine at 50 nM (Δ) and 250 nM (□) concentrations.

point measurements at various time points with the addition of a high pH "stop buffer" to terminate the reaction and increase the fluorescence of the released fluorophore [16]. This method has been used to determine the activity of α-mannosidase at low pH values (6.0) [16], albeit not in a continuous format [16]. In addition, the products from these reactions (e.g., 4-MU, mannose) have also been measured by cumbersome TLC or HPLC analysis techniques.

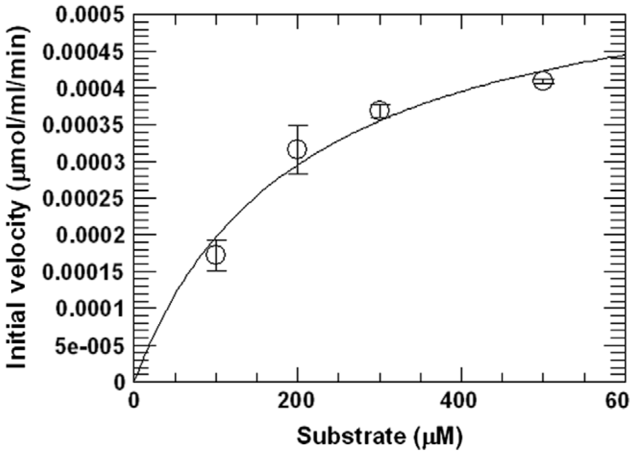

Fig. 4. Computer-assisted nonlinear regression kinetic analysis of Res-Man. The assay method was performed by adding recombinant dGMII (6.67 µg/ml final concentration) to four concentrations (0.50, 0.30, 0.20, and 0.10 mM) of Res-Man in 100 mM sodium acetate buffer (pH 6.0). Fluorescence was recorded (excitation = 550 nm, emission = 595 nm) at 30-s time intervals for 210 s. Data were converted to micromoles (µmol) of resorufin product produced using a standard curve created using known concentrations of resorufin in sodium acetate buffer (pH 6.0). Data points are means, and error bars represent standard errors ($n = 3$).

Analysis of the affinity of dGMII for MU-Man ($K_m = 5$ mM [H. Strachan and K. Moremen, personal communication]) and PNP-Man ($K_m = 50$–60 mM [unpublished]) indicates that the previously used artificial substrates are poor mimics of the natural substrate ($K_m = 85$ µM [H. Strachan and K. Moremen, personal communication]).[3]

A continuous kinetic assay using dGMII, performed using a pH 6.0 buffer, resulted in a K_m of 200 ± 73 µM and a V_{max} of 11 ± 1.5 nmol/min per nmol dGMII. using the Res-Man substrate (Fig. 4). Thus, this new substrate has an affinity at least 250-fold better for dGMII than the commonly used PNP-Man substrate, as well as being approximately 20-fold better than the 4-MU-Man substrate, and is similar in affinity to naturally occurring substrates. These data suggest that the continuous assay performed at pH 6.0 with the new Res-Man substrate can be used to obtain reliable kinetic data at or near physiological pH values. Fluorescence emission of the released resorufin product at pH 6.0 is somewhat reduced (~30%) relative to its fluorescence at a higher pH [21]. However, previous assays with Res-CB [21] suggest that the kinetic parameters obtained are essentially unaffected relative to an assay performed using a stop buffer. Furthermore, because resorufin is a relatively pH-insensitive fluorophore, fluorescence emission and excitation wavelengths remain fairly uniform over a large range of pH values from 3.0 to 9.0 [27] that allow the same filter sets and instrumental setup methods to be used for assays run with different samples having a variety of pH values. It is noted that fluorescence measurements of free resorufin standards should be measured over the range of concentrations and at the same pH as those used for test samples to obtain quantitative turnover rates at specific pH values.

Uses of the new Res-Man substrate for continuous assay of α-mannosidase enzymes derived from additional species, toward

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[3] Heather Strachan and Kelley Moremen (Complex Carbohydrate Research Center and Department of Biochemistry and Molecular Biology, University of Georgia), in a personal communication, reported a value for the K_m of 4-MU-Man, used as a substrate when measuring inhibitory compounds from Geert-Jan Boon's lab using the same dGMII enzyme preparation, of 4.9 ± 2.6 mM with MU-Man (pH 5.5) in a total concentration range of 0.1875–3 mM and reported a value for the K_m of HLM of 0.8 ± 0.4 mM with MU-Man (pH 4.0) in a total concentration range of 0.1875 to 3 mM. Attempts to measure the K_m for PNP-Man using dGMII indicated that it was potentially in the range of 50–60 mM. However, this is well above the solubility limit of PNP-Man in aqueous solutions, so an exact determination of the K_m could not be determined. Measured K_m values for several preferred natural substrates were found to be as follows: $K_m = 89.6$ µM with GlcNAc-Man5-GlcNac$_2$ for dGMII and $K_m = 85.8$ µM for Man5-GlcNac$_2$ for HLM.

various α-mannosidase isozymes, as well as analysis of activity and inhibition in live cell and live tissue formats are underway. Continued work with the Res-Man substrate holds the promise for analysis of α-mannosidase activities and identification of potential inhibitors or modulators of α-mannosidase activity that may prove to be useful for therapeutic intervention in a variety of important disease systems.

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