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An approach for fluorometric determination of glycosyltransferase activities

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A new strategy for the fluorometric determination of glycosyltransferase activities is reported. The method involves dansyl chloride derivatization of the reduced form (pNH_2phenyl) of a hydrophobic, aglycon moiety covalently linked to a number of acceptor substrates (pNO_2phenyl). Focusing on the Golgi enzyme core 2 *N*-acetylglucosaminyltransferase, we found that synthesis and fractionation of the dansylated substrate derivative were rapid, easy and inexpensive. Additionally, the corresponding enzyme assay proved reproducible and very sensitive, as 0.4 pmol of reaction product were readily detected. This fluorometric approach appears therefore to be a valid tool for investigating the monitoring differential expression of glycosyltransferases exhibiting low levels of enzyme activity.

Keywords: glycosyltransferases, assays, fluorescence, dansyl chloride, β1,6 *N*-acetylglucosaminyltransferase

Abbreviations: T, transferase, Gal, D-*N*-galactose; GlcNAc, D-*N*-acetylglucosamine; GalNAc, D-*N*-acetylgalactosamine; HPLC, high pressure liquid chromatography; UDP, uridine diphosphate; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; pNp, *para*-nitrophenyl; NMR, nuclear magnetic resonance; DMSO, dimethyl sulphoxide.

Introduction

It is well known that cell surface complex carbohydrates are subject to structural variations during development, differentiation and oncogenic transformation [1–2]. In this regard, it was observed that changes in cellular oligosaccharide profiles correlated with differential expression of specific glycosyltransferases [3], the enzymes which catalyse the individual sugar transfer steps during processes of protein and lipid glycosylation [4]. Glycosyltransferases are commonly assayed by radiochemical procedures, where labelled products are separated from precursors by chromatographic, electrophoretic or immunological methods [5–8]. Recently, however, growing interest in their regulation, mode of action and biological significance has prompted the development of novel, improved detection methods [9–12].

Fluorometric assays for glycosyltransferases have been based upon tagging substrate acceptors with fluorogenic compounds, such as 2-aminopyridine [13, 14] and tetramethylrhodamine [12]. In general, the usefulness of a fluorescent substrate depends on several factors: i) fluorescence intensity; ii) purity and stability; iii) effective substrate-product separation; and iv) enzyme affinity. The assay rationale must be broadly applicable and performed with relatively inexpensive materials and equipment. Useful application of such assays would be possible with their adaptation for automated systems.

In a previous work, we described a fluorescent assay for the Golgi enzyme core 2 *N*-acetylglucosaminyltransferase (core 2 GlcNAc-T) which was based upon derivatization of its reaction product (i.e. Galβ1,3[GlcNAcβ1,6]GalNAcα-pAminophenyl) with dansyl chloride followed by separation via reverse-phase HPLC [15]. The method was found to be sensitive, inexpensive and reproducible, thus constituting a viable alternative to radiolabelling-based procedures. In this report, a further

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improvement of the assay is described which involves a substrate which is already tagged with dansyl chloride, thereby eliminating time-consuming, post-reaction derivatizations. Synthesis and fractionation of this adduct were simple and highly efficient and this approach should prove versatile for generating fluorescent oligosaccharide substrates for a variety of glycosyltransferase activity determinations.

Materials and methods

Chemicals

Galβ1,3GalNAcα-pNitrophenyl, Galβ1,3[GlcNAcβ1,6]-GalNAcα-pNitrophenyl and GalNAcα-pNitrophenyl were from Toronto Research Chemicals (Canada); dansyl chloride, UDP-GlcNAc, UDP-Gal, GlcNAc, buffer salts, Triton X-100 and Brij-35 were purchased from Sigma (USA); HPLC-grade acetonitrile was obtained from BDH (UK); C_{18} Sep-Pak cartridges were from Millipore-Waters (USA). UDP-[6-^{3}H] *N*-acetylglucosamine (26.8 Ci mmol^{-1}) was provided by Amersham (UK). All other reagents were of the highest grade available.

Cell cultures

MDAY-D2 [16], PYS-2 [17] and CHO [18] cell lines were cultured in α-modified Eagle's minimum essential medium supplemented with 10% fetal calf serum and maintained at 37 °C in a 95% O_2:5% CO_2 humidified atmosphere.

Synthesis of Galβ1,3GalNAcα-pNHDansylphenyl

Commercially available Galβ1,3GalNAcα-pNitrophenyl was completely converted to Galβ1,3GalNAcα-pAminophenyl as previously described [15]. Galβ1,3GalNAcα-pAminophenyl (0.5 mg in 90 μl of water) was derivatized in a sealed tube containing 100 μl of dansyl chloride (5 mg ml^{-1} stock solution in acetone) and 10 μl of 0.5 M TES, pH 7.0. The reaction was carried out at 65 °C for 1 h after which the tube was opened and maintained in the 65 °C water bath for a few seconds to allow evaporation of the acetone. Three ml of water were added to the contents of the tube, followed by sample application to a C_{18} Sep-Pak cartridge. The column was initially washed with 7 ml of water, then with 10 ml of 30% methanol and finally eluted with 15 ml of 100% methanol. Solvent was evaporated in a vacuum concentrator and the residue redissolved in 100 μl water. The dansylated compound was stored at −20 °C indefinitely.

^{1}H NMR analysis

All ^{1}H NMR spectra were run on dilute CD_3OD solutions (internal Me_4Si) at room temperature, using a Bruker AC-200 instrument.

Fluorometric core 2 GlcNAc-T assay

Cell extracts employed as sources of enzyme activity were prepared as previously described [15]. The assay mixture (total volume 50 μl) contained 0.1 M TES buffer, pH 7.0, 0.1 M GlcNAc, 2 mM UDP-GlcNAc, 20 μl cell lysate (5-500 μg protein) and 1 mM Galβ1,3GalNAcα-pNHDansylphenyl as the acceptor substrate. After a 2 h incubation at 37 °C, the reaction was stopped by heating to 100 °C for 3 min in order to coagulate proteins. The volume of each tube was brought to 100 μl with water and samples were microfuged at top speed for 10 min. Supernatant was collected and analysed immediately by HPLC or stored at −20 °C until used. To separate core 2 GlcNAc-T reaction product, 10–100 μl of sample was applied to a 4.6 × 250 mm Spherisorb ODS2 HPLC column which was run isocratically at 1.2 ml min^{-1} in 25% acetonitrile containing 0.1% Brij-35. Eluate was continuously monitored for fluorescence at 340 nM excitation/525 nM emission ('standard protocol'). Alternatively, in order to perform assays with enhanced sensitivity ('enhanced protocol'), the HPLC eluant corresponding to Galβ1,3[GlcNAcβ1,6] GalNAcα-pNp was collected, dried and resuspended in a 25:50:25 acetonitrile:DMSO:water solution. Sample (20 μl) was then reinjected into the automated circuit and run in the 25:50:25 acetonitrile:DMSO:water solution bypassing the chromatographic step. Fluorescence of the core 2 GlcNAc-T reaction product was read at 340 nM excitation/525 nM emission. All measurements were performed on a Jasco fluorometer Model 821-FP connected to a Hewlett-Packard integrator Model HP 3395.

Conventional core 2 GlcNAc-T assay

The reaction contained 0.1 M TES buffer, pH 7.0, 0.125% Triton X-100, 0.1 M GlcNAc, 2 mM UDP-GlcNAc, 0.5 μCi of UDP-[6-^{3}H] *N*-acetylglucosamine, 1 mM Galβ1,3GalNAcα-pNp as acceptor and 20 μl of cell lysate. Product was separated using Sep-Pak C_{18} cartridges and counted in a beta scintillator counter as previously described [9, 19].

Kinetic studies

Apparent K_m and V_{max} values were determined by double reciprocal Lineweaver-Burk plots using 2 mM UDP-GlcNAc for both conventional and fluorometric core 2 GlcNAc-T assays and five different concentrations ranging between 0.0625 and 1 mM of either Galβ1,3GalNAcα-pNp or Galβ1,3GalNAcα-pNHDansylphenyl.

Results and discussion

Dansyl chloride is reactive toward primary and secondary amines, and has been extensively used to fluorescently tag peptides and proteins for biochemical analyses [20–22].

We have employed this compound for labelling the reduced form (pNH_2phenyl) of a hydrophobic, aglycon moiety covalently linked to a number of substrate acceptors of glycosyltransferases (pNO_2phenyl) [23]. The disaccharide Galβ1,3GalNAcα-pNHDansylphenyl was chosen as a representative in order to explore the general utility and applicability of dansylated substrate acceptors for enzyme assays.

The structure of this compound, which is shown in Fig. 1, was produced by reacting Galβ1,3GalNAcα-pAminophenyl, obtained as previously described [15], with dansyl chloride. The product was fractionated by using a C_{18} Sep-Pak column and then stored indefinitely at −20 °C in water at a 10 mM concentration. The recovery of product after Sep-Pak chromatography was complete based on pilot studies showing that unreacted dansyl chloride was released during column washings whereas Galβ1,3GalNAcα-pNHDansylphenyl was retained (not shown). When diluted in water, the dansyl derivative gave maximum excitation and emission at 340 and 500 nM, respectively.

Purity and identity of Galβ1,3GalNAcα-pNHDansylphenyl were verified by ^{1}H NMR analysis. The NMR spectrum of dansylated disaccharide showed the aromatic protons as multiplets at 7.0–8.5 ppm (versus 7.0–8.8 ppm for dansyl chloride) and the $-N(CH_3)_2$ at 2.7 ppm (Fig. 2).

Conditions for the derivatization procedure were optimized with regard to the reactivity of dansyl chloride toward amine groups. In fact, the extent of dansylation is susceptible to change depending upon: i) absolute concentration of dansyl chloride; ii) pH, which should balance the need for having the amino group in the NH_2 form with limiting hydrolysis of dansyl chloride by hydroxyl ions; and iii) time of incubation and temperature [21, 24, 25]. Initially, the same labelling conditions adopted for amino groups of peptides were employed (5 mg ml^{-1} dansyl chloride, pH 9.5, 37 °C, 1 h incubation) [20, 21, 24, 25]. Optimal labelling was achieved at this concentration of dansyl chloride, in the pH range of 7–10 (Fig. 3). The latter observation indicated that at neutral pH the amino group is essentially present as a free base, with a probable pK of approximately 6.5. Therefore, pH 7.0 seemed optimal in rendering the highest reactivity with the lowest rate of hydrolysis by OH^- ions. In addition, it was observed that when the dansylation process was performed at 65 °C, presence of contaminating products (i.e. dansyl-OH, dansyl-NH_2) was significantly reduced, probably because of degradation (not shown).

The dansylated disaccharide was used in core 2 GlcNAc-T reactions to evaluate its suitability as an acceptor substrate. By employing crude lysates from MDAY-D2 and PYS-2 cells as sources of enzyme activity (Fig. 4 shows a representative HPLC elution pattern of

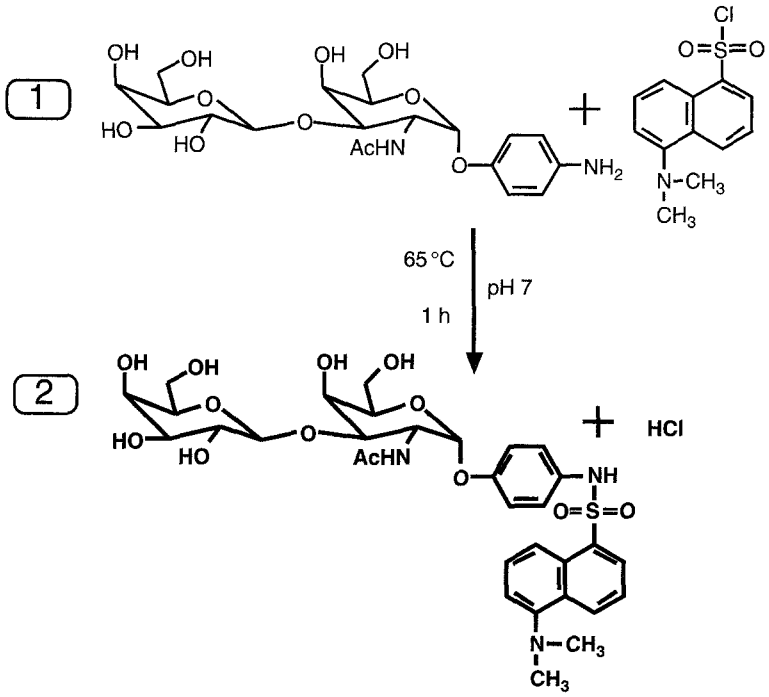

Figure 1. Structure of Galβ1,3GalNAcα-pNHDansylphenyl. Details of the reaction are reported in the text. The fluorescent substrate for core 2 GlcNAc-T (structure no. 2) was separated from unreacted dansyl chloride and salts by reverse-phase chromatography using Sep-Pak C_{18} columns.

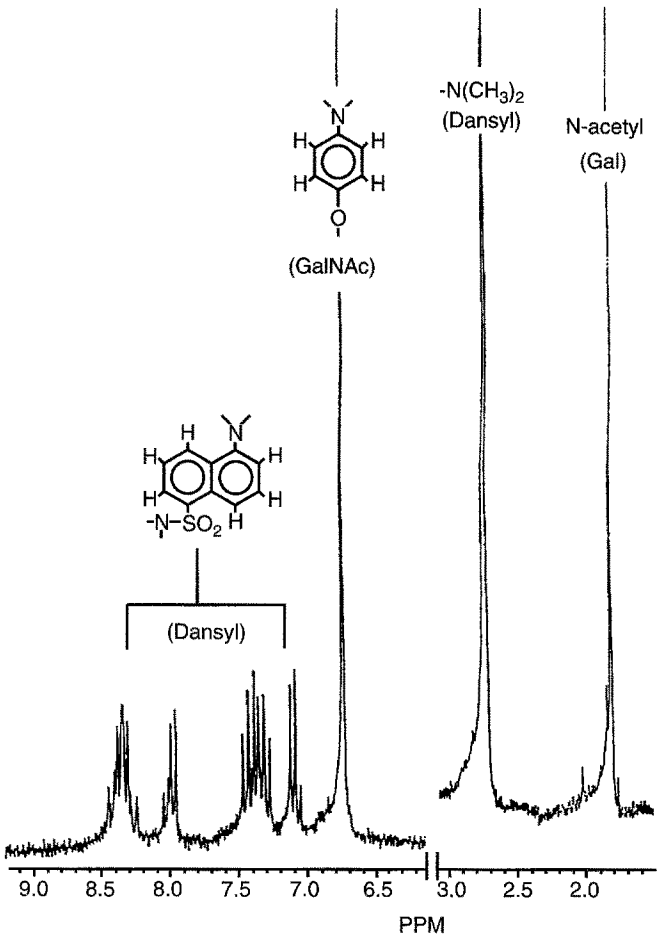

Figure 2. 200 MHz partial proton NMR spectrum at room temperature of the dansylation reaction product Galβ1,3GalNAcα-pNHDansylphenyl. Experimental details are reported under Materials and methods.

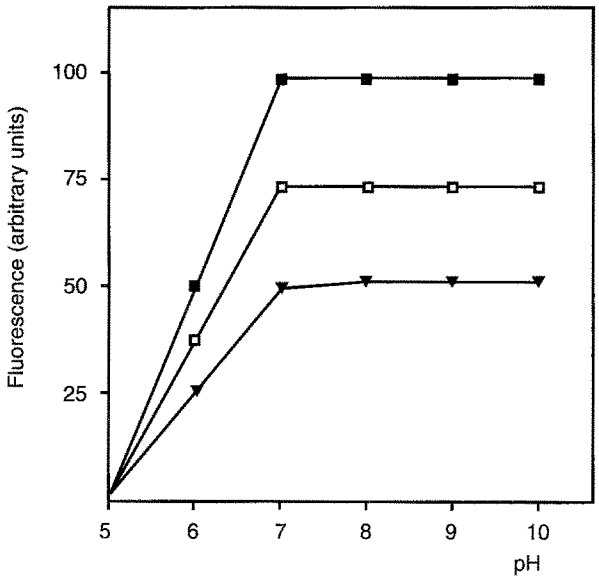

Figure 3. Labelling efficiency as a function of pH and dansyl chloride concentration for Galβ1,3GalNAcα-pAminophenyl. Dansylation reactions were carried out at 65 °C for 1 h. Products were separated by Sep-Pak C_{18} chromatography and subjected to HPLC and fluorescence measurements as described under Materials and methods. Concentrations of dansyl chloride were: 1 mg ml^{-1} (▼—▼), 3 mg ml^{-1} (□—□) and 5 mg ml^{-1} (■—■). Buffers were: 0.1 M sodium acetate (pH 5.0), 0.1 M sodium phosphate (pH 6.0), 0.1 M TES (pH 7–8) and 0.1 M sodium bicarbonate (pH 9–10). Concentrations of dansyl chloride higher than 5 mg ml^{-1} did not increase labelling efficiencies, likely because of solubility problems.

the reaction products), it became apparent that conditions for enzyme catalysis were the same as for the conventional assay. For example, the rate of the reaction as a function of incubation time proved linear at both 30 and 37 °C for up to 2 h (not shown). Interestingly, the apparent K_m for Galβ1,3GalNAcα-pNHDansylphenyl compared well with the K_m value determined by employing the substrate Galβ1,3GalNAcα-pNitrophenyl in the conventional radioactive-based procedure (Table 1). This observation indicated that the addition of a bulky fluorescent group to the hydrophobic aglycon linker did not cause steric hindrance and/or reduced enzyme affinity. Similarly, V_{max} values determined with the fluorometric assay were essentially coincident with those calculated by employing the radiochemical method (Table 1).

As previously reported [15], proportionality between dansylated trisaccharide and fluorescence intensity was in the range 1–300 pmol. The amount of enzymatically-formed product (i.e. Galβ1,3[GlcNAcβ1,6]GalNAcα-pNHDansylphenyl) was linear with increasing protein up to 500 μg of MDAY-D2 or PYS-2 cell lysate per reaction. Controls showed no fluorescence background when Galβ1,3GalNAcα-pNHDansylphenyl was replaced by water in the core 2 GlcNAc-T reaction mixture. Additionally, the fluorometric assay method was found to be highly reproducible, in that five determinations of core 2 GlcNAc-T specific activity, which were performed at different times by employing different amounts of MDAY-D2 cell lysates, fell within 10% of average values (data not shown).

It was also investigated whether or not the spectral properties and the fluorescence intensities of the dansyl derivatives were altered by different solvents. Indeed, it has been previously shown that, for some dansyl compounds, higher fluorometric efficiencies as well as shifts of emission maxima were associated with decreasing dielectric constants of the solvents used [26]. It was observed that the dansyl group of the core 2 GlcNAc-T product (i.e. Galβ1,3[GlcNAcβ1,6]GalNAcα-pNHDansylphenyl) in 25% acetonitrile-water (after elution from a Spherisorb ODS 2 HPLC column) and the core 2 GlcNAc-T substrate acceptor in water had slightly

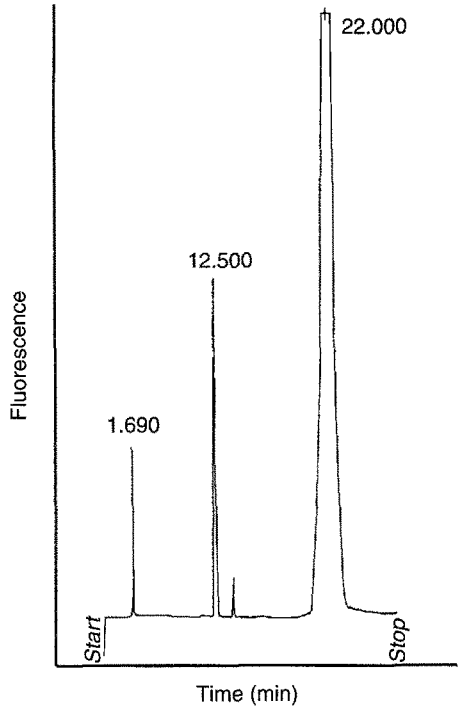

Figure 4. A typical HPLC elution profile of core 2 GlcNAc-T reaction products. The fluorometric assay for core 2 GlcNAc-T was performed using 15 μg of MDAY-D2 cell lysate and 1 mM Galβ1,3GalNAcα-pNHDansylphenyl as the acceptor substrate. Additionally, the reaction mixtures contained, in a final volume of 50 μl, 0.1 M TES, pH 7.0, 0.1 M GlcNAc and 2 mM UDP-GlcNAc. Incubation was carried out for 2 h at 37 °C after which samples were processed as described under Materials and methods. A Galβ1,3[GlcNAcβ1,6]GalNAcα-pNHDansylphenyl standard [15] comigrated with the peak eluting at 12.5 min.

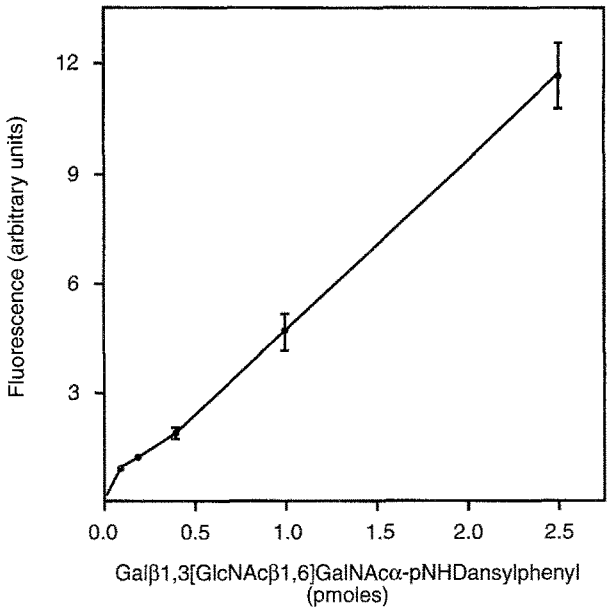

Figure 5. Proportionality between the fluorescence of Gal-β1,3[GlcNAcβ1,6]GalNAcα-pNHDansylphenyl in the presence of 50% DMSO and its concentration. Commercially available Galβ1,3[GlcNAcβ1,6]GalNAcα-pNitrophenyl was converted to the pAminophenyl form as previously described [15]. The trisaccharide was dansylated and subsequently fractionated via Sep-Pak C_{18} chromatography as reported under Materials and methods for Galβ1,3GalNAcα-pAminophenyl. Galβ1,3[GlcNAcβ1,6]GalNAcα-pNHDansylphenyl was separated by HPLC in 25% acetonitrile containing 0.1% Brij-35. The corresponding peak was collected, dried and resuspended in a 25:50:25 acetonitrile:DMSO:water solution. Sample was injected into the automated circuit bypassing the column and run in the above solution. Fluorescence measurements were performed at 340 nM excitation/525 nM emission. Intensities have been reported as integrated peak areas. Each point is the mean ± the range of triplicate determinations.

different spectral features, the former with a λ_{em} peak shifted to 525 nM. More importantly, we found that lowering the dielectric constant of the eluant by including 50% DMSO in the running solution, increased the intensity of emission 3.4-fold (not shown). This observation was therefore exploited to improve the detection method, described as the 'enhanced protocol' under Materials and methods. As shown in Fig. 5, fluorescence intensity of core 2 GlcNAc-T reaction product, in the presence of 50% DMSO, allowed the detection of 0.4 pmol, thus rendering this method the most sensitive core 2 GlcNAc-T assay to date.

The 'enhanced protocol' was used to perform core 2 GlcNAc-T assay in CHO cells, which have been previously shown to exhibit very low levels of enzyme

Table 1. Kinetic parameters determined by using the conventional (acceptor Galβ1,3GalNAcα-pNp) and fluorometric (acceptor Galβ1,3GalNAcα-pNHDansylphenyl) core 2 GlcNAc-T assay procedures.

Cells	*Acceptor*	K_m (*mM*)	V_{max} (*nmol mg^{-1} h^{-1}*)
MDAY-D2	Galβ1,3GalNAcα-pNp	0.150	12.45
	Galβ1,3GalNAcα-pNHDansylphenyl	0.190	11.62
PYS-2	Galβ1,3GalNAcα-pNp	0.420	16.20
	Galβ1,3GalNAcα-pNHDansylphenyl	0.500	16.66

Note: Conventional and fluorometric ('standard protocol') core 2 GlcNAc-T assays were carried out as described under Materials and methods using MDAY-D2 and PYS-2 cells as sources of enzyme activity. K_m and V_{max} values were determined by double reciprocal Lineweaver-Burk plots at five different substrate concentrations ranging between 0.0625 and 1 mM.

activity (i.e. 8–10 pmol $mg^{-1} h^{-1}$) [9, 15, 27]. The mean activity was 10.7 ± 1.0 pmol $mg^{-1} h^{-1}$ ($n = 5$), revealing the high sensitivity of this method. In this regard, quantification of enzymatically-formed product was determined in as little as 20 μg of CHO cell lysate (Fig. 6). Considering that expression of core 2 GlcNAc-T in CHO cells cannot be detected using the conventional assay, these data confirm the usefulness of the fluorometric procedure for studies where biological material is limiting or enzyme activity is low.

In summary, we have developed a strategy for the fluorometric determination of glycosyltransferase activities, based upon the dansylation of pAminophenyl acceptors. Indeed, the method could be widely adapted, given that the only prerequisite is the availability of acceptor substrates linked to hydrophobic aglycon groups (e.g. phenyl or benzyl) substituted by nitro or amino moieties. For example, the approach should prove suitable for measuring core 1 β1,3 Gal-T and β1,3 GlcNAc-T (elongation) activities, which are generally determined by radioactive-based procedures employing GalNAcα-pNp [27] and Galβ1,3(GlcNAcβ1,6)GalNAcα-benzyl(phenyl) [28], respectively, as acceptors.

Synthesis and fractionation of the derivatized compound have been shown to be rapid, easy and inexpensive. Given its sensitivity and reproducibility, the new assay should prove useful for investigating enzymology of glycosyltransferases. Interesting applications may concern, for example, large-scale surveys aimed towards the elucidation of an enzyme's diagnostic potential or studies regarding differential expression of glycosyltransferases during oncodevelopmental processes.

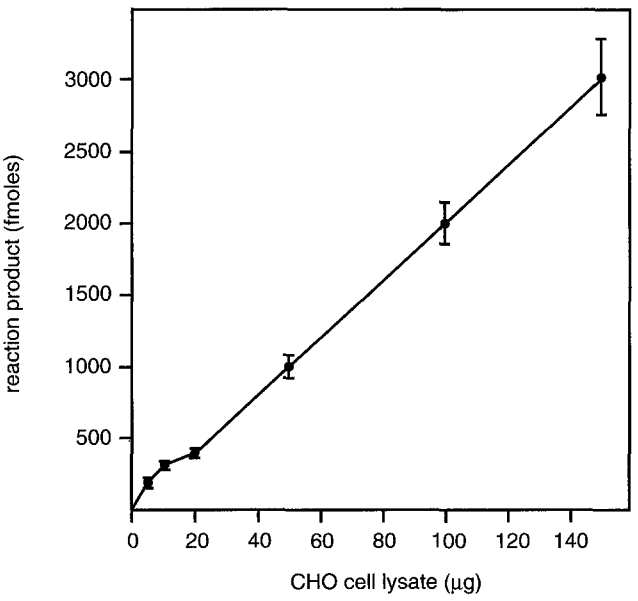

Figure 6. Relationship between core 2 GlcNAc-T activity and CHO cell lysate concentrations. Enzyme assays were performed using the 'enhanced protocol' as described under Materials and methods. Reaction products were quantitated as shown in Fig. 5. Each point is mean ± the range of duplicate determinations.

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