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An Improved Method for the Determination of Glycosyltransferases Using *para*-Nitrophenyl-Glycosides as Substrates

Christian G. Haselberger, Diethelm J. Gauster, and **Helmut Schenkel-Brunner***

Institut für Biochemie, Universität Wien, A-1090 Vienna, Austria

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Due to their easy purification nitrophenyl-glycosides have become of increasing interest as acceptor substrates for the assay of glycosyltransferase activity. In this communication reversed-phase chromatography is shown to provide a convenient means for the isolation of the radioactively labelled nitrophenylglycoside(s) formed in this reaction, superior to the methods hitherto used with regard to speed and specifity.

(Keywords: Glycosyltransferase assay; Reversed-phase chromatography)

Eine verbesserte Methode zur Bestimmung von Glykosyltransferase-Aktivitäten unter Verwendung von Nitrophenyl-Glykosiden als Substrate

Nitrophenylglykoside werden immer häufiger als Akzeptor-Substrate bei der Bestimmung von Glykosyltransferase-Aktivitäten verwendet, da sie leicht aus dem Reaktionsgemisch zu isolieren sind. In der vorliegenden Mitteilung wird gezeigt, daß die „reversed-phase"-Chromatographie eine bequeme Möglichkeit darstellt, die bei der Reaktion entstehenden radioaktiv markierten Nitrophenylglykoside zu reinigen. Sie ist den bisher verwendeten Trennmethoden an Geschwindigkeit und Spezifität überlegen.

Introduction

Glycosyltransferase activity is determined usually by testing the amount of monosaccharide transferred from the respective sugar nucleotide onto suitable precursor substances. Oligosaccharides and glycoproteins are commonly used as sugar acceptors (see e.g.[1–5]), only in a few cases monosaccharides are applicable. Recently it was found[6] that also phenyl- and nitrophenylglycosides can act as acceptor substrates in glycosyltransferase assays. As they are easier available and well defined in their composition and structure, these substrates

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proved to be far more comfortable glycosyl acceptors, and so they have replaced to a high degree most of the other substances mentioned above (e.g.[6–11]).

In the investigations presented in this communication, reversed-phase chromatography was tested for its usefulness in glycosyltransferase assays. In contrast to the usual silica gel chromatography, the hydrophilic components of an aqueous solution are not adsorbed in „reversed-phase chromatography", whereas the lipophilic substances are retained on the column material. They can be eluted with dilute methanol.

This method promised to offer a simple means for isolating nitrophenyl-glycosides out of the incubation mixtures.

Materials and Methods

The preparation of the following materials has been described previously: β-galactosyltransferase from human milk[12], hog gastric mucosal microsomes[4], *N*-acetyl-galactosaminyl-1-phosphate[13], and *GDP*-fucose[14].

p-Nitrophenyl β-*D*-galactosyl-(2-*N*-acetyl)-β-*D*-glucosaminide was synthesized by enzymatic transfer of ^{14}C-galactose from *UDP*-galactose onto *p*-nitrophenyl *N*-acetyl-β-*D*-glucosaminide (see below) using paper chromatography for purification of the reaction product. *UDP*-galactose, *UDP*-*N*-acetylglucosamine as well as *p*-nitrophenyl β-*D*-galactoside, *p*-nitrophenyl *N*-acetyl-β-*D*-glucosaminide, *D*-galactose, *L*-fucose, and *N*-acetyl-*D*-glucosamine were purchased from Sigma Chemical Company, St. Louis, MO, USA; *UDP*-1-(^{14}C)galactose was supplied by the Radiochemical Centre, Amersham.

SEP-PAK-C_{18} cartridges (columns containing about 0.8 ml of conventional silica gel particles coated with C_{18}-hydrocarbon compounds) were obtained from Waters Associates Inc., Milford, MA, USA.

Concentrations of galactose and fucose were determined by the cysteine-sulphuric acid method[15], acetylaminohexoses according to *Reissig* et al.[16], *N*-acetylgalactosaminyl-1-phosphate being previously hydrolysed in 0.01 *M* hydrochloric acid (15 minutes at 100 °C). Sugar nucleotides and *p*-nitrophenyl-glycosides were assayed by measuring their light absorption at 280 and 300 nm, respectively.

Galactosyltransferase action was measured by incubating the respective enzyme preparation (about 10^{-3} I.U.) with *UDP*-(^{14}C)galactose (30 nmol, 10 mCi/mmol), $MnCl_2$ (1 μmol), and *p*-nitrophenyl *N*-acetyl-β-*D*-glucosaminide (200 nmol) in an imidazole—HCl buffer (0.02 mol/l, *pH* 7.0) at 37 °C; the final volume was 85 μl. The incubation was terminated by the addition of an equal volume of 96% ethanol; protein thereby denatured was removed by centrifugation and the supernatant tested for the presence of *p*-nitrophenyl-disaccharide

a) by descending paper chromatography[6] on Whatman 3 MM using ethylacetate/pyridin/water (10:4:3) as the solvent. Sugar nucleotides and sugar phosphates remained near the origin, free sugars showed R_f values between 0.25 and 0.40, and the *p*-nitrophenyl-disacharides migrated with R_f values between 0.5 and 0.7. Radioactivity peaks were localized with the aid of a

Berthold LB 280 paper chromatogram scanner, the radioactive zones were cut out and counted in toluene scintillation liquid[4] in an Isocap 300 liquid scintillation counter.

b) by chromatography on C_{18}-silica gel: The protein-free supernatant containing the radiolabelled nitrophenyl-glycoside was diluted with 10 volumes of water and loaded on a SEP-PAK-C_{18} column. The column was washed with another 9 ml of water under slight pressure, and the nitrophenyl-sugar was subsequently eluted with 3 ml 50% methanol and counted directly in 15 ml of a scintillation liquid[17] containing Triton X-100.

Results and Discussion

In order to determine the optimum conditions for reversed-phase chromatography in glycosyltransferase assays, the reactants and products as well as the expected by-products of such reactions were tested for their adsorption behaviour on SEP-PAK-C_{18} columns. The substances were applied to the columns in aqueous solutions and eluted with water and increasing concentrations of methanol at flow rates of about 1 drop/second. It is shown in Table 1 that sugar nucleotides, sugar phosphates, mono-, and disaccharides were not adsorbed, whereas the *p*-nitrophenyl glycosides were retained on the SEP-PAK-C_{18} material and could be eluted only with dilute methanol.

Table 1. *Elution behaviour of mono- and disaccharides, sugar nucleotides, sugar phosphates and p-nitrophenyl-glycosides on SEP-PAK-C_{18} cartridges.* 20 µmols of the substances dissolved in 1 ml of distilled water were applied to the column. The column was washed with 5 ml of distilled water and eluted with 2 ml portions of methanol of increasing concentration

	% of total, eluted by									
	water	5%	10%	15%	20%	25%	30%	40%	50%	methanol
]DP-Gal	100	0	0	0	0	0	0	0	0	0
]DP-GlcNAc	100	0	0	0	0	0	0	0	0	0
!DP-Fuc	100	0	0	0	0	0	0	0	0	0
!alNAc-1-Phosphate	100	0	0	0	0	0	0	0	0	0
!alactose	100	0	0	0	0	0	0	0	0	0
V-Acetyl-glucosamine	100	0	0	0	0	0	0	0	0	0
'ucose	100	0	0	0	0	0	0	0	0	0
,actose	100	0	0	0	0	0	0	0	0	0
)NP β-*Gal*	0	1	12	65	22	0	0	0	0	0
)NP β-*GlcNAc*	0	0	0	3	68	29	0	0	0	0
)NP (*Gal*-) *GlcNAc*	0	0	0	0	6	73	20	1	0	0

Abbreviations used: *Gal* = galactose; *Fuc* = fucose; *GlcNAc* = *N*-acetyl-*D*-glucosamine; *GlcNAc-1-P* = *N*-acetyl-*D*-glucosamine-1-phosphate; *pNP* = 1-0-*p*-nitrophenyl.

Based on these results a method was worked out to separate the products of transferase reactions, i.e. nitrophenyl di- and oligosaccharides, from the radiolabelled substrates (sugar nucleotides) and their degradation products in a one-step procedure. The enzymatic synthesis of *p*-nitrophenyl β-*D*-galactosyl-*N*-acetyl-β-*D*-glucosaminide by transfer of galactose onto *p*-nitrophenyl *N*-acetyl-β-*D*-glucosaminide mediated by a β-galactosyltransferase isolated from human milk was chosen as a model reaction.

The activity of a pure galactosyltransferase preparation was measured using reversed-phase chromatography and, for comparison, the usual paper chromatography as separation procedures. The amounts of radioactivity transferred to the acceptor substrate are plotted in Fig. 1. It can be seen that both methods yielded comparable

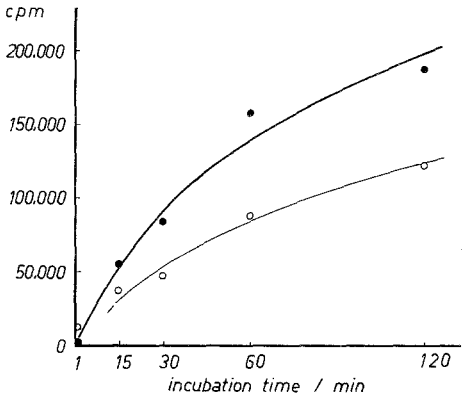

Fig. 1. Incorporation of labelled galactose into *p*-nitrophenyl-*N*-acetyl-β-*D*-glucosaminide by the β-galactosyltransferase from human milk as measured by SEP-PAK-C_{18} (●) and paper chromatography (○)

results, the cpm-values obtained by the C_{18}-column procedure, however, being higher than those obtained by paper chromatography by a factor of 1.8, presumingly because counting is more efficient when the radioactivity is dissolved homogeneously.

Assays of β-galactosyltransferase in human serum and in microsomes from hog gastric mucosa showed that the new method is also applicable to transferase tests with crude enzyme preparations (Table 2).

These results show clearly that reversed-phase chromatography is superior to the glycosyltransferase-assay methods hitherto used:

Table 2. *Assay of β-galactosyltransferase in crude enzyme preparations.* Procedure as described in the text, incubation time 2 hours

Enzyme source	*p*-nitrophenyl-*N*-acetyl-glucosamine added	cpm recovered using paper chromatography	cpm recovered using SEP-PAK-C_{18}
Human serum	yes	28000	34000
	no	130	150
Hog gastric mucosal microsomes	yes	2900	3350
	no	130	195

a) It is much less time-consuming than paper chromatography: Even a large number of SEP-PAK-cartridges is ready for use in as few as one or two minutes, and the separation procedure itself takes about 30 minutes for a whole series of samples when they are analysed simultaneously. Thus, 10 or more samples are easily prepared for scintillation counting in less than one hour. In contrast, spotting of the samples and development of the paper chromatogram would take at least 5 hours when using the original method.

b) Scintillation counting is more efficient when the radioactive material is assayed in homogeneous solution rather than fixed to a paper strip.

c) The columns are ready for re-use after a simple wash with one milliliter each of methanol and water; each column can be used for at least 30 times.

d) A number of investigators used ion exchange chromatography for the absorption of the sugar nucleotide out of the incubation mixture[18]. During most assays, however, a substantial part of the sugar nucleotide is degraded to the free sugar, which is not removed by ion-exchange chromatography and makes it therefore inevitable to set up a separate blank for each sample. In addition, the high background activity thus produced makes the test less sensitive.

In contrast, free sugars can be removed quantitatively by SEP-PAK chromatography, and the only remaining background is the transfer of the labelled sugar to the usually few glycolipid acceptors present in the enzyme preparation.

Since, in addition, the use of reversed-phase chromatography is not connected with any loss of accuracy or reliability, this method is likely to become an advantageous alternative for glycosyltransferase assays wherever lipophilic sugar derivates are applicable.

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