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Note

Thin-layer chromatographic assay for *endo*-β-N-acetylglucosaminidase activity in rat tissues

N. J. COOK, J. P. ZANETTA* and G. VINCENDON
Centre de Neurochimie du CNRS, 5 Rue Blaise Pascal, 67084 Strasbourg Cedex (France)
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endo-β-N-Acetylglucosaminidase (E.C. 3.2.1.96) hydrolytically cleaves the core N,N'-diacetylchitobiose moiety of the asparagine-linked glycans of glycoproteins. Previously described assays of enzyme activity in bacterial or mammalian extracts generally utilize asparagine-linked oligosaccharides derived from exhaustive glycoprotein digestion and labelled isotopically in the asparaginyl moiety using either [^{3}H]1-dimethylaminonaphthalene-5-sulphonyl (dansyl) chloride[1–3] or [^{14}C]acetic anhydride[4,5] as substrates. The action of the enzyme on these substrates produces radioactive products which are separated from unhydrolysed glycopeptide using either paper chromatography[1–3] or high-voltage paper electrophoresis[4–6] and then measured for radioactivity.

In this paper, we describe a rapid, simple assay which uses a heterogeneous mixture of dansylated ovalbumin glycopeptides as the substrate. After incubation, an internal standard (dansyl-L-leucine) is added and then the reaction product is separated and quantitated using thin-layer chromatography (TLC) and fluorescence scanning.

MATERIALS AND METHODS

Chemicals and reagents

Ovalbumin (grade V), pronase, asparaginyl-2-deoxy-2-N-acetamidoglucose (Asn-GlcNAc) and dansyl-L-leucine were supplied by Sigma (St. Louis, MO, U.S.A.), 1-dimethylamino-naphtalene-5-sulphonyl chloride (Dns-Cl) and 2-mercaptoethanol by Fluka (Buchs, Switzerland). Triton X-100 was from Serva (Heidelberg, F.R.G.) and ethylenediaminetetraacetic acid (EDTA) from Aldrich Europe (Beerse, Belgium). High-performance thin-layer chromatographic (HPTLC) silica gel 660 plates were from Merck (Darmstadt, F.R.G.). All other reagents and chemicals were of analytical grade.

Substrate preparation and characterization of the reaction product

Ovalbumin glycopeptides were obtained by extensive pronase digestion followed by successive gel filtrations on Sephadex G-25 and Bio-Gel P-30 as previously described[7]. After lyophilization, the glycopeptides were solubilized in 0.25 *M* $NaHCO_3$–Na_2CO_3 buffer, pH 9.5 at a concentration of 3 m*M*. For each ml of gly-

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copeptide solution, 1 ml of Dns-Cl (6%, w/v in acetone) was added and the mixture allowed to react for 3 h in the dark at room temperature with slow agitation. The acetone was then removed under a stream of air and excess of Dns-Cl removed by repeated extraction with benzene. The residue was redissolved in water and Dns hydroxide removed by gel filtration on Sephadex G-25 in the dark. The Dns-derivatized glycopeptide fraction was collected, five times its volume of acetone was added and the mixture left overnight at −20°C. The resulting suspension was centrifuged at 2000 *g* for 1 h, and the pellet washed three times with acetone. The acetone-precipitation step separates Dns-peptides which interfere with the HPTLC separation from the Dns-glycopeptide fraction. The Dns-glycopeptides were dissolved in water at a concentration of 0.5 m*M*. The solution was stored at −20°C in 1-ml aliquots until use.

The N-terminal residue and amino acid composition of the substrate was analysed as previously described[8]. Dns-Aspartate was the only compound found in both cases, indicating that asparagine is the only amino acid bound to the glycopeptide. The carbohydrate composition is still heterogeneous, as revealed by chromatography on HPTLC plates in the solvent chloroform–methanol–methyl acetate–*n*-propanol–0.25% (w/v) aqueous potassium chloride (15:20:20:20:25, v/v).

Assay conditions

Tissues from adult male Wistar albino rats were homogenized (15%, w/v) in 20 m*M* Tris–HCl buffer pH 7.2, containing 30 m*M* 2-mercaptoethanol and 0.4 *M* NaCl, using a Potter–Elvehjem homogeniser with a tight-fitting pestle at 4°C. A 20-μl volume of crude homogenate was added to 20 μl of 0.2 *M* Na_2HPO_4–citric acid buffer pH 6.5 containing 0.3% (v/v) Triton X-100 and 3 m*M* EDTA, in a plastic conical capped tube. After the addition of 20 μl of Dns-derivatized glycopeptide solution, the mixture was incubated in the dark at 37°C. The reaction was stopped by instantaneous freezing, and the samples stored at −20°C until chromatography. All incubations were performed in triplicate. The protein concentrations were determined by the method of Lowry *et al.*[9] using bovine serum albumin as the standard.

Thin-layer chromatography

A 1-nmol amount of internal standard [10 μl of 0.1 m*M* dansyl-L-leucine in methanol–water (1:9, v/v)] was added to the sample, which then thawed, mixed and centrifuged. Approximately 2 μl were applied as a thin line (1 × 5 mm) 1 cm from the edge of a HPTLC silica gel G-60 plate. Chromatography was then performed in the dark (30 min) using the solvent methyl acetate–chloroform–*n*-propanol–methanol–0.25% (w/v) aqueous potassium chloride (25:20:20:20:17, v/v)[10] in standard chromatographic tanks lined with filter-paper. In this chromatographic system, Dns-Leu has a R_F of 0.64. The reaction product (R_F 0.43) was identified as Dns-Asn-Glc-NAc (i) by comparison of the HPTLC migration with authentic Dns-derivatized, commercially available, Dns-Asn-GlcNAc and (ii) from the N-terminal and amino acid composition[8] and carbohydrate analysis[11]. Dns-Asp was the only amino acid, and N-acetylglucosamine the only carbohydrate, found in the reaction product isolated from HPTLC plates. The developed plates were scanned using a Camag TLC scanner (excitation wavelength 366 nm, cut-off filter 400 nm) coupled to a 3390 A Hewlett-Packard integrator. Quantitation of the reaction product (Dns-Asn-Glc-

NAc) peak was achieved by comparison of its area to that of the internal standard peak.

RESULTS AND DISCUSSION

The method described utilizes a Dns-derivatized mixture of ovalbumin glycopeptides as the substrate. This was chosen for two reasons: (i) the recently demonstrated substrate specificity of rat liver *endo*-β-N-acetylglucosaminidase[12] showed that the enzyme would be active, albeit to different extents, against all of the glycopeptide species known to exist in ovalbumin and (ii) this allowed the simplification, and increased the yield of, the substrate preparation.

Under the TLC conditions described, the reaction product Dns-Asn-GlcNAc and the internal standard (R_F 0.43 and 0.64 respectively) can easily be separated, whereas the unhydrolysed substrate remains close to the origin (Fig. 1A and B). This good and rapid separation allows a good peak integration after scanning. Furthermore the extremely high fluorescence of Dns derivatives allows a very sensitive determination of the enzyme activity (as low as 2 pmol can be quantified and as low as 0.5 pmol can be visualized under a UV lamp).

In all tissues examined (brain, liver, spleen, lung, kidney), the optimum pH values were found to be of the same order (pH 6.4–6.7, Fig. 2A). No activity was found below pH 5.0 but the enzyme was still active up to pH 9.0. This indicates that the rat *endo*-β-N-acetylglucosaminidase is significantly different from classical lysosomal enzymes.

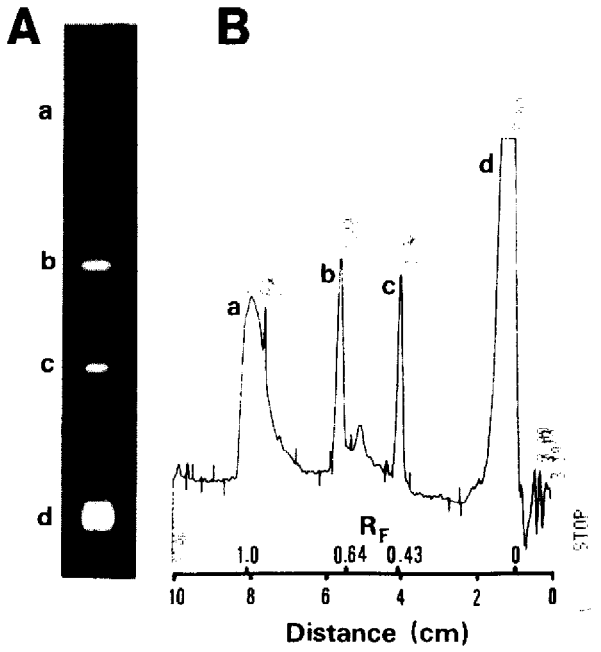

Fig. 1. A, Photograph of the HPTLC separation obtained after incubation of Dns-glycopeptide with an homogenate of rat brain: a, solvent front; b, internal standard (Dns-Leu); c, Dns-Asn-GlcNAc; d, unhydrolyzed Dns-glycopeptide. B, TLC-fluorescence scanning of the assay mixture (rat brain).

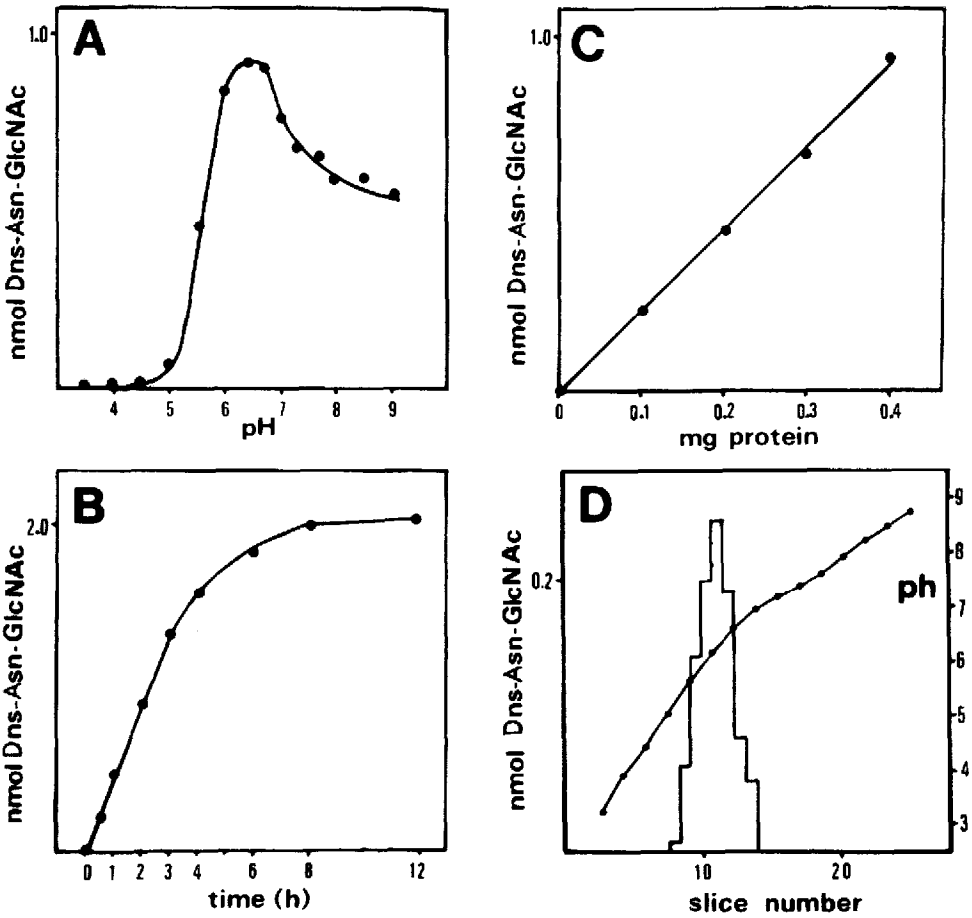

Fig. 2. Formation of Dns-Asn-GlcNAc (rat brain homogenate) as a function of pH (2 h incubation) (A), of time (pH 6.5) (B) and of the quantity of protein [different dilutions of homogenate (and therefore, of enzyme) added to the reaction mixture; 2 h incubation, pH 6.5] (C). D, Isoelectric focusing[21] of rat brain *endo-β*-N-acetylglycosaminidase: ——, nanomoles of Dns-Asn-GlcNAc in the reaction mixture after incubation (6 h); ●—●, pH. The crude homogenate, obtained as above, was centrifuged at 100,000 *g* for 60 min. Ammonium sulphate up to 40% saturation was added to the supernatant, and the precipitate resuspended in 20 m*M* Tris–HCl buffer pH 7.2 containing 10 m*M* 2-mercaptoethanol, then dialyzed overnight against the same buffer. Samples (100 μg of protein) were applied to the basic region of the pH gradient (about pH 8) and then submitted to isoelectric focusing[21] for 120 min under the same conditions. The gel was then sliced into 3-mm sections and analyzed for activity as described above. In A–D, the ordinates represent nanomoles of Dns-Asn-GlcNAc in the reaction mixture after incubation.

When assayed at optimal pH (6.5) the formation of the reaction product was found to be linear with time for a period of about 3 h (Fig. 2B). The subsequent decrease is most likely due to a progressive denaturation of the enzyme since, at this stage, there is still a 4–5 fold excess of substrate relative to the product. During the first 2 h, the quantity of reaction product formed was proportional to the quantity of enzyme in the incubation mixture (Fig. 2C). Under optimum conditions [stabilizing agents (see below), pH and time], the method showed good reproducibility for re-

TABLE I

ENDO-β-N-ACETYLGLUCOSAMINIDASE ACTIVITY IN RAT TISSUE HOMOGENATES

Each value represents six determinations.

Tissue	*Specific activity** *(± standard deviation)*	*Range*
Liver	1.39 ± 0.14	1.23–1.57
Kidney	1.38 ± 0.16	1.13–1.70
Spleen	2.06 ± 0.25	1.81–2.57
Lung	0.64 ± 0.17	0.41–0.82
Brain	1.06 ± 0.16	0.84–1.40

* Nanomoles of Dns-Asn-GlcNAc produced per hour per milligram of protein.

peated analysis of a single homogenate (rat brain), giving a coefficient of variation of 3.5% (n = 18, range 0.448–0.519 nmol h^{-1} per 20 μl, mean 0.487).

The determination of the enzyme activity in various tissues (Table I) showed that the tissues containing the highest specific activities were spleen, liver and kidney, whereas rat brain exhibited moderate and lung low activities. The relative specific activities determined in homogenates of liver, kidney and lung corresponded well with values previously reported[14,15]. However, contrary to those results, we found high activity in spleen. This is possibly due to the fact that in previous works[13,14] the enzyme activity was measured in a supernatant fraction obtained in the absence of thiol reagents and salts, and that the enzyme is poorly solubilized under these conditions. Our results are similar to those of Overdijk *et al.*[15] who found high activity in spleen when using total homogenates of human tissues and incubating in the presence of dithiothreitol.

In agreement with others[13,16], we found that rat *endo-β*-N-acetylglucosaminidase is a highly unstable and labile enzyme. However, 2-mercaptoethanol, Triton X-100, EDTA and NaCl in the assay were all found to be useful in stabilizing the enzyme, and necessary to ensure reproducible results.

It has previously been suggested[13,16] that the stepwise action of exoglycosidases would also be capable of producing *endo-β*-N-acetylglucosaminidase products. This was not the case using our method since in the presence of excess (4 mM) of *p*-nitrophenyl-α-D-mannoside and *p*-nitrophenyl-β-D-N-acetylglucosaminide no change in formation of the product was observed (not shown). This apparent absence of the exoglycosidase contribution is presumably due to our reaction conditions. The pH is above that required for maximum activity of lysosomal glycosidases, and the presence of 1 mM EDTA would destabilize cytosolic α-D-mannosidase, which requires bivalent cations for maximum stability[17].

It was shown previously[18,19] that aspartylglucosaminidase could not act on the reaction product, Dns-Asn-GlcNAc[20], to give a false low estimation of *endo-β*-N-acetylglucosaminidase activity. This was confirmed in the present study by incubating Dns-Asn-GlcNAc with tissue homogenates under the conditions described above. Prolonged incubation produced insignificant modification of Dns-Asn-GlcNAc to give Dns-aspartate. These two experiments indicate that this method allows the determination of the enzyme without any interferences from other glycosidases present in the reaction mixture.

This method can be used for studying the properties of the enzyme either in homogenates or in crude fractions, for example by determining the molecular weight by gel filtration (not shown) or the isoelectric point of the enzyme (Fig. 2D). Even the presence of agarose from the isoelectrofocusing gel does not perturb the enzyme activity or the HPTLC separation.

In conclusion, the method described has the advantages of being simple, rapid, extremely sensitive, reproducible and can be applied to most of the problems concerning the enzyme.

REFERENCES

1 A. L. Tarentino and F. Maley, *J. Biol. Chem.*, 249 (1974) 811.
2 A. L. Tarentino and F. Maley, *Methods Enzymol.*, 50 (1978) 580.
3 R. B. Trimble, A. L. Tarentino, G. E. U. Maley and F. Maley, *Methods Enzymol.*, 83 (1982) 603.
4 N. Koide and T. Maramatsu, *J. Biol. Chem.*, 249 (1974) 4897.
5 T. Maramatsu, *Methods Enzymol.*, 50 (1978) 555.
6 R. J. Pierce, G. Spik and J. Montreuil, *Biochem. J.*, 180 (1979) 673.
7 A. Reeber, J. P. Zanetta, I. G. Morgan and G. Gombos, *Méthodologie de la Structure et du Métabolisme des Glycoconjugués*, Éditions du CNRS, Paris, 1974, p. 815.
8 J. P. Zanetta, G. Vincendon, P. Mandel and G. Gombos, *J. Chromatogr.*, 51 (1970) 441.
9 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
10 J. P. Zanetta, F. Vitiello and G. Vincendon, *Lipids*, 15 (1980) 1055.
11 J. P. Zanetta, W. C. Breckenridge and G. Vincendon, *J. Chromatogr.*, 69 (1972) 291.
12 Y. Tachibana, K. Yamashita and A. Kobata, *Arch. Biochem. Biophys.*, 214 (1982) 199.
13 A. L. Tarentino and F. Maley, *Anal. Biochem.*, 77 (1977) 185.
14 N. Nishigaki, T. Muramatsu and A. Kobata, *Biochem. Biophys. Res. Commun.*, 59 (1974) 638.
15 B. Overdijk, W. M. J. van der Kroef, J. J. W. Lisman, R. J. Pierce, J. Montreuil and G. Spik, *FEBS Lett.*, 128 (1981) 364.
16 A. L. Tarentino and F. Maley, *J. Biol. Chem.*, 251 (1976) 6537.
17 V. A. Shoup and O. Touster, *J. Biol. Chem.*, 251 (1976) 3845.
18 M. Malino, T. Tojimo and I. Yamashina, *Biochem. Biophys. Res. Commun.*, 24 (1966) 961.
19 R. Kornfeld and J. Kornfeld, *Annu. Rev. Biochem.*, 45 (1976) 217.
20 T. H. Plummer, A. L. Tarentino and F. Maley, *J. Biol. Chem.*, 243 (1968) 5158.
21 P. Basset, C. Froissart, G. Vincendon and R. Massarelli, *Electrophoresis*, 1 (1980) 168.