A Modified Assay System for Collagen Glucosyltransferase

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Summary. A simplified assay procedure has been developed for the determination of collagen glucosyltransferase activity in tissue extracts. Using degraded gelatine as acceptor it was possible to isolate the reaction product by precipitation on to a glass fibre disc. Under our conditions degraded gelatine is glucosylated with a reaction rate which is 3–4 times lower compared with the glucosylation of basement membrane derived glycopeptides. Good reproducibility is demonstrated by the coefficient of variation of 4% in the same assay and an interassay variation coefficient below 8%. As the assay allows the testing of large numbers of samples in a few hours, it should prove a useful tool to determine the enzyme level in the tissue of diabetic animals. In humans the activity of the glucosyltransferase could provide a biochemical parameter related to diabetic microangiopathy.

Key words: Glucosyltransferase assay, rat kidney, diabetic microangiopathy, collagen, glycoproteins.

Long standing diabetes results in biochemical and morphological alterations in the capillary system of all organs so far studied. Spiro and Spiro [1] find in the kidney cortices of diabetic rats an increase of an enzyme which is involved in the posttranslational modification of BM-collagen, namely the UDP¹-Glucose: Galactosylhydroxylysine-Glucosyltransferase (E. C. 2.4.1.66). Other authors [2] [3] have ques-

tioned these findings. Therefore further investigations are necessary to clarify this question. Assuming that an increased glucosylation of BM-collagen is related to BM-thickening, the determination of glucosyltransferase activities in human tissue or blood could provide a valuable diagnostic tool to follow the pathological changes in diabetic microangiopathy.

In most of the earlier studies a tedious and time consuming assay for the glucosyltransferase was used. The purpose of our study was to modify the assay system to avoid complicated manipulations and to achieve good reproducibility.

Methods

Kidney Extractions

The glucosyltransferase was extracted from the kidneys of male albino rats of the strain Hoe: WISKf (SPF 71) (Wistar), which were fed a normal diet ad libitum prior to sacrifice.

After extirpation the kidneys were frozen in liquid nitrogen and stored in liquid nitrogen up to several months without any change in enzyme activity.

All steps were carried out at 0°C. Kidneys were homogenized in a glass Potter homogenizer with a teflon pestle in 3.5 volumes of 0.15 mol/l tris acetate, pH 6.8, containing 0.002 mol/l 2-mercaptoethanol and 0.25 ml/100 ml Triton X 100. After stirring for one hour the homogenate was centrifuged at 100 000 × g for one hour.

To the supernatant polyethyleneglycol 6000 was added to a final concentration of 7% (w/v). After stirring for 15 min the suspension was centrifuged at 30 000 × g for 20 min. The precipitate was redissolved in the above mentioned buffer. The enzyme solutions were stored at –20°C; no change of enzyme activity was observed under those conditions within two months.

Enzyme Assay

In our modified assay the glucosyltransferase was determined using degraded gelatine as the acceptor. The reaction product was isolated in a disc precipitation procedure similar to that of Mans

¹ Abbreviations

UDP = uridine diphosphate
glc-gal-hyl = glucosyl-galactosyl-hydroxylysine
BM = basement membrane

Fig. 1. Amino acid analysis of the glucosyltransferase reaction product after alkaline hydrolysis. The broken line indicates the position of glc-gal-hyl. **A** = degraded gelatine (precipitate); **B** = degraded gelatine (incubation mixture); **C** = basement membrane peptide (incubation mixture)

Fig. 2. Effect of enzyme concentration on the incorporation of glucose-¹⁴C into degraded gelatine ($n = 5$, mean $\pm$ SEM)

and Novelli [4]. The incubations were carried out at 37°C in a total volume of 0.15 ml, containing 22.5 μ mol tris acetate, pH 6.5, 3.0 μ mol manganese acetate, 0.3 μ mol 2-mercaptoethanol, 22.5 μ mol sodium chloride and 6 mg partially degraded gelatine with a content of 5 nmol non-glucosylated galactosyl residues per mg, 5 residues hydroxylysine per 1000 amino acids and a molecular weight of 10–15 000 (Behringwerke AG, Marburg). The assay system for glucosyltransferase contained 10 nmol of UDP-glucose-¹⁴C (0.76 μ Ci) (Radiochemical Centre, Amersham). The reaction was started by adding 5 to 50 μ l of the enzyme solution and stopped after 40 min by transferring an 100 μ l aliquot of the reaction mixture on to a glass fibre disc (Whatman GF/C, 2.3 cms size). The disc was brought to near dryness by a stream of warm air and then immersed in a solution of 1% (w/v) phosphotungstic acid in 0.5 mol/l HCl for 15 minutes. Finally it was washed successively for 15 min each: four times in 1 mol/l trichloroacetic acid, twice in ether/ethanol (1:1, v/v) and once in ether. After drying the disc was transferred to a toluene scintillation solution and counted. To characterize the reaction product some of the discs were subjected to alkaline hydrolysis and analyzed as described later.

For comparative purposes the enzyme was tested for activity by the method of Spiro et al. [5], using glycopeptide from bovine glomerular basement membrane as acceptor. Following alkaline hydrolysis the C-14-labelled degradation product glc-gal-hyl was isolated by paper chromatography or by means of an automated amino acid analyzer.

Amino acid analysis was carried out following hydrolysis in 2 mol/l NaOH at 105°C in capped polypropylene tubes for 20 hr. The hydrolysates were acidified, desalted on columns of Dowex 50 WX8, according to Spiro [5], and analyzed on a Beckman Multi-chrom 4255 automated amino acid analyzer. The effluent was fractionated and radioactivity determined in a liquid scintillation counter. Automatic external standardization as well as internal standards were used for determining counting efficiencies. Protein was determined by the method of Lowry et al. [6] using bovine serum albumin as a standard.

Results

In order to facilitate the test procedure for the glucosyltransferase we looked for a suitable acceptor which is precipitable under conditions where the cosubstrate UDP-glucose remains in solution. We checked the glycopeptide fraction derived from glomerular basement membrane [5], under-glucosylated collagen (where glucose was partly removed by an acid hydrolysis under mild conditions), and degraded gelatine. To achieve a rapid and specific separation, the labelled reaction product was precipitated on a glass fibre disc. The most effective precipitant was phosphotungstic acid [7], and subsequent washes with trichloroacetic acid, ethanol and ether. Under these conditions the best substrate with respect to availability, handling properties, quantitative and specific precipitation, was degraded gelatine. As shown by amino acid analysis the only C-14-labelled product in the hydrolysate of the precipitate was the glc-gal-hyl residue (Fig. 1 A). When the incubation mixture including the excess of UDP-glucose was subjected to alkaline hydrolysis, peaks not corresponding to glc-gal-hyl were seen and glc-gal-hyl

represented a minute portion of the radioactive components in the hydrolysates (about 1% of the total radioactivity) (Fig. 1B). A similar pattern in the amino acid analysis was obtained when the substrate was BM-glycopeptide and the alkaline hydrolysis was carried out according to [5] (Fig. 1C).

Figure 2 shows the effect of enzyme concentration on the incorporation of C-14-glucose into degraded gelatine. In the same assay four enzyme concentrations were determined five times. Under these conditions good reproducibility is demonstrated by the coefficient of variation of 4%.

In two enzyme solutions glucosyltransferase activity was determined six times each within a period of two months. The interassay coefficients of variation in these series, representing high (mean 597 cpm/mg kidney) and low (mean 459 cpm/mg kidney) enzyme activities were 7.4% and 7.5% respectively. The reaction rate with degraded gelatine in our assay is 7 pmol/mg min, which is 3–4 times lower than the rate of glucosylation of BM-glycopeptides determined according to [5]. Double reciprocal plots of initial velocity and substrate concentrations were linear. The K_m obtained for UDP-glucose was about 70 $\mu\text{mol/l}$ and that for gelatine about 22 g/l (Fig. 3).

The activity of kidney glucosyltransferase shows a marked variation with age [8] with highest values in animals of about nine days of age. Table 1 shows that values are stable in adult rats of two different weights and also gives the normal range for our strain of rats.

Discussion

Spiro's method for the determination of glucosyltransferase activities [5] includes several tedious and time consuming steps: the preparation of the substrate from glomerular basement membrane and the steps following the enzyme reaction involving alkaline hydrolysis, ion exchange chromatography and a paper chromatographic technique taking six days. The test procedure of Myllylä [9], using gelatinized calf skin collagen as acceptor, includes a precipitation step, alkaline hydrolysis, ion exchange chromatography and paper electrophoresis.

These manipulations which are necessary to achieve a high specificity of the test imply many potential sources of error. Both authors claim that several purification steps are necessary because the separation efficiency of one step is not sufficient.

We think that the intensive purification procedures are mainly necessary to eliminate labelled products derived from the UDP-glucoside, which were removed either incompletely or not at all before

Fig. 3. Lineweaver-Burk plot for glucosyltransferase with degraded gelatine **A** and UDP-glucose **B** as the variable substrates. Standard assay conditions were employed with substrate concentrations as indicated and 0.27 mg enzyme protein

Table 1

Number of rats (n)	Body weight ^a (g)	Total weight of both kidneys ^a (g)	Glucosyltransferase activity ^a (cpm per mg kidney)	Range of glucosyl- transferase activity (cpm per mg kidney)
15	372 ± 11	2,2 ± 0,1	524 ± 27	440–580
20	497 ± 9	2,8 ± 0,1	517 ± 46	423–675

^a The values are given as the mean ± SEM

alkaline hydrolysis. We used a commercially available degraded gelatine, with a molecular weight of 10–15000, as acceptor. After quantitative precipitation on a glass fibre disc the excess of labelled UDP-glucose was washed out completely. When this precipitate was subjected to alkaline hydrolysis the only labelled product found in the hydrolysate was glc-gal-hyl, as shown by amino acid analysis. Obviously there is no need for any further processing of the precipitate to achieve a high specificity of the assay.

The reaction rate with degraded gelatine as an acceptor is lower than with BM-glycopeptides. This is probably due to the specificity of the enzyme and to the lower content of acceptor sites in the degraded gelatine and does not conflict with the requirements for such an assay.

The main asset of this procedure is that it allows the testing of large numbers of samples in a few hours, using special devices for the washing steps.

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