Amino Acids (1993) 4: 63–72

Amino
Acids

Studies on the catabolism of glycated haemoglobin: Glycated globin is not a substrate for the glucosyl-galactosyl-hydroxylysine glucosidase but for a peptidase activity present in rat kidney and spleen

A. M. Grigorova-Borsos[1], F. Tacnet[1], R. W. Fischer[2], K. H. Winterhalter[2], and M. Sternberg[1]

[1] Equipe de Recherches sur la Biochimie et la Pharmacologie des Vaisseaux et du Rein, Faculté de Médecine des Saints-Pères, et Faculté de Pharmacie, Université de Paris V et VI, France
[2] Laboratorium für Biochemie, Eidgenössische Technische Hochschule, Zürich, Switzerland

Accepted May 21, 1992

Summary. Rat kidney and spleen glucosyl-galactosyl-hydroxylysine glucosidase (EC.3.2.1.107) whose specificity for the hydroxylysine-linked disaccharide units present in collagens depends upon the substrate's free amino group was tested for its glycosidase activity on the ketoamine form of glycated [^{14}C]Glc-Globin. The most stable preparations of the enzyme from normal and diabetic rat tissues, partially purified by ultracentrifugation and ammonium sulphate fractionation, were used. These glucosidase preparations did not release any significant amount of radioactive neutral hexose. But a radioactive glycopeptide of about 1,400 Da was released from [^{14}C]Glc-Globin at a pH optimum of 4.0. It appears to be released by a peptidase activity present in the kidney and spleen of normal and diabetic rats.

Keywords: Amino acids – Hydroxylysine-linked disaccharide units – Glycated haemoglobin – Glucosidase – Diabetic rat – Peptidase – Kidney – Spleen

Introduction

2-O-α-D-glucopyranosyl-O-β-D-galactopyranosyl-L-hydroxylysine glucosidase (EC.3.2.1.107) (GGHG) has been described in rat kidney (Sternberg and Spiro, 1979), spleen (Hamazaki and Hotta, 1980) and chick embryo (Hamazaki and Hotta, 1979). This acid α-glucosidase is highly specific for the hydroxylysine-linked disaccharide units characteristic of collagens. The rat kidney cortex enzyme (70,000 Da) (Grigorova-Borsos et al., 1987) is able to bring about complete glucose removal from disaccharide units of peptides as well as of whole basement membrane and is believed to be involved in their catabolism. The

enzyme specificity depends on the free ε-amino group of hydroxylysine to which carbohydrates are bound O-glycosidically. Therefore it was of interest to test its activity towards the ketoamine form of glycated Hb which however has the carbohydrate bound N-glycosically. The pathological consequences of non-enzymatic glucosylation of proteins in diabetes mellitus have been investigated (Bunn et al., 1978), but little information is available about the enzymes which play a role in the catabolism of glycated proteins. The aim of this study was to answer the question whether the Glc-Gal-Hyl glucosidase was able to release glucose or fructose from glycated Hb. A glycosidase activity towards this substrate has been reported in human diabetic lymphocytes (Lubec et al., 1982). Since the activity of the enzyme is high in kidney and spleen of normal and diabetic rats, this investigation was carried out with enzyme isolated from these organs; in addition, the spleen is a physiological organ for Hb catabolism and the kidney is able to filter, reabsorb and catabolize circulating free Hb after acute pathological hemolysis. The most stable preparations of the enzyme, partially purified by ultracentrifugation and ammonium sulphate fractionation, were used (Sternberg and Spiro, 1979).

Materials and methods

Animals

For the enzyme preparations, normal male Wistar rats from 260 to 320 g were used. After decapitation the kidneys and spleen were immediately removed and frozen at −80°C until homogenization. For the study of enzyme activities in diabetes, two months old normal male Wistar rats, from 140 to 160 g, were injected intramuscularly with streptozotocin (Sigma, St. Louis) 55 mg per kg body weight, in 100 mM NaCl, 1 mM citrate buffer pH = 4.5. The injection was repeated one week later. Age-matched control groups were injected with the buffer alone. Tail capillary blood glucose was checked every week and carotid plasma glucose at sacrifice. The rats were killed three months after the streptozotocin injections. Spontaneously diabetic BB Wistar rats were donated by the Animal Resources Division Health Protection Branch, Health and Welfare, Ottawa, Canada: diabetes was detected at about 2 months of age by checking daily urinary glucose and treated from then on with a minimal dose of insulin (Bovine Insuline Protamine Zinc Choay), usually at 10 units per kg daily. The animals were followed until the age of 5 months and killed alternately with the streptozotocin diabetic and age-matched control rats. After decapitation kidneys and spleens were taken immediately and frozen in liquid nitrogen until used for individual rat enzyme assay.

Substrates and effectors

Glucose-galactose-hydroxylysine (Glc-Gal-Hyl) was prepared from bovine kidney basement membranes as in Sternberg and Spiro, 1979. Chromatographically purified HbAo was incubated at 37°C with freshly purified (Vaughan et al., 1984) [^{14}C]Glucose (CFB2, 2–4 mCi/mmol, Amersham Bucks, UK.). After a 720 h incubation period performed in a CO atmosphere at a 100 mM glucose concentration, the incubation mixture was exhaustively dialyzed against phosphate buffer (100 mM, pH = 6, 8). The Hb was further purified by ion exchange chromatography. From the resulting glycated Hb (HbAIc and HbAoGlc) globin was prepared by the acid-acetone method (Fluckiger and Winterhalter, 1976). The incorporated radioactivity was determined as 32,000 dpm per mg protein. All chemicals used as inhibitors were purchased from Sigma.

The *different enzyme preparations* were obtained as described in Sternberg and Spiro, 1979.

Enzyme assays

Glc-Gal-Hyl glucohydrolase activity was measured according to Sternberg and Spiro, 1979. [^{14}C]Glc-Globin (15,000 dpm) was incubated for 1 h at 37°C with the enzyme in 0.2 M sodium phosphate buffer, pH = 4.0. The reaction was terminated by the addition of 1 ml cold distilled water containing 1 mg/ml of bovine serum albumin (Sigma), followed by 0.5 ml of 0.12 M $ZnSO_4$ and 0.5 ml of 0.12 M $Ba(OH)_2$ (Somogyi, 1945). After centrifugation, a 0.5 ml aliquot of supernatant was counted for radioactivity and a 1 ml aliquot was applied to a small column (1 cm diameter) containing 1 ml of Dowex 50 W × 4 (H^+ form, 200–400 mesh) on top of which 1 ml of Dowex 1 × 2 (formate form, 200–400 mesh) was packed. The column was then washed with 4 ml distilled water. The 5 ml eluted from the column were lyophilized and the dried material was redissolved in 0.5 ml distilled water and counted for neutral product radioactivity. The supernatant obtained from tubes with substrate alone was submitted to the same procedure and used as blank.

For the *characterization of radioactive products* released from [^{14}C]Glc-Globin, a kidney enzyme preparation C (47.9 mg protein, see Table 1) was incubated for 24 h at 37°C with the [^{14}C]Glc-Globin (110,000 dpm) at pH = 4.0. The supernatant obtained after precipitation by $ZnSO_4$ and $Ba(OH)_2$ (150 ml) was applied to a Dowex 50 W × 4 (H^+ form, 200–400 mesh) column (8.5 × 1.6 cm). After washing by 150 ml distilled water, the effluent (300 ml) was dried and redissolved in 150 ml of water. An aliquot (0.8 ml) was counted for radioactivity and the rest was applied to a Dowex 1 × 2 (formate form, 200–400 mesh) column (8.5 × 1.6 cm) and washed with the same volume of distilled water. An aliquot of the 300 ml effluent was counted for radioactivity. The fraction bound to Dowex 50 W × 4 (fraction I) was eluted with 150 ml of 1.5 N ammonium hydroxide. The fraction bound to Dowex 1 × 2 (fraction II) was eluted by 30 ml of pyridine acetate 0.5 M, pH = 5.0. The eluates were evaporated, redissolved in distilled water and an aliquot was counted for radioactivity. The *positively charged glycopeptide* eluted from Dowex 50 W × 4 (H^+ form) was submitted to filtration on a Biogel P10 column (2.0 × 27 cm) in pyridine acetate 0.5 M, pH = 5.0. A flow rate of 4 ml/hour was maintained and 1 ml fractions were collected. Absorbance at 280 nm and radioactivity were measured. The Biogel P10 column was calibrated with Dextran Blue and [^{125}I] Angiotensin I (ORIS, Saclay, France).

Other methods

Proteins were estimated according to Lowry et al., 1957. Glucose was measured by a micromethod using glucose oxidase as in Sternberg and Spiro, 1979. The statistical methods are discribed in Sternberg et al., 1982. [^{14}C]radioactivity was counted by liquid scintillation in Picofluor 30 (Packard Tri-Carb 3255 spectrometer). Efficiency corrections were made by the external standard method. [^{125}I] radioactivity was determined on a Beckman Radioimmuno Analyzer.

Results

Characteristics of the enzyme activities on Glc-Gal-Hyl and [^{14}C]Glc-Globin, in the different dialyzed fractions of normal rat kidney cortex and spleen, are given in Table 1. No significant release of neutral radioactivity was detected from [^{14}C]Glc-Globin even after 24 h incubation (Table 2). However in the various preparations we have found an enzyme activity which was able to release a radioactive glycopeptide from [^{14}C]Glc-Globin. This activity had a pH optimum of 4.0 (Fig. 1A), suggesting a lysosomal origin, and increased linearly with

Table 1. Preparation of normal rat kidney cortex and spleen Glc-Gal-Hyl Glucohydrolase

Fraction	Proteins	Glucohydrolase activity on Glc-Gal-Hyl				"Glycopeptide releasing activity" on [^{14}C]Glc-Globin[a]			
Identification	*(mg/g tissue)*	Specific activity *(nmol/mg protein)*	Purification factor	Total activity *(nmol/g tissue)*	Yield %	Specific activity *(dpm/mg protein)*	Purification factor	Total activity *(dpm/g tissue)*	Yield %
Kidney									
– Homogenate (AK)	187	6.6	1	1,240	100	859	1	160,595	100
– Supernatant 7×10^6 g.min (BK)	72.6	20.4	3.1	1,484	119.6	1,932	2.25	140,263	87.3
– $(NH_4)_2SO_4$ 1.56 M precipitate (CK)	17.3	50.8	7.6	881	71.1	3,352	3.9	57,996	36.1
– $(NH_4)_2SO_4$ 1.56 M supernatant (DK)	38.3	8.56	1.3	327	26.4	235	0.27	9,000	5.6
Spleen									
– Homogenate (AS)	147	28.9	1	4,254	100	925	1	135,945	100
– Supernatant 7×10^6 g.min (BS)	65.0	68.0	2.35	4,426	104	1,118	1.2	72,650	53.4
– $(NH_4)_2SO_4$ 1.56 M precipitate (CS)	18.38	99.8	3.45	1,838	43.2	759	0.82	13,943	10.25

[a] "Glycopeptide releasing activity" refers to [^{14}C]-Glc-peptide released from [^{14}C]-Glc-Globin under the standard assay conditions (see Material and methods).

Table 2. Characterization of the radioactive product liberated from [^{14}C]Glc-Globin by the kidney cortex enzyme preparation (CK)[a] after 24 hours incubation

Fraction	dpm	Yield %
Total Product	81,000[b]	100
I – Positively charged product[c]	75,337	93.0
II – Negatively charged product	496	0.6
III – Neutral product	1,305	1.6

[a] See Table 1;
[b] 73.6% of the substrate radioactivity (110.000 dpm);
[c] see Material and methods.

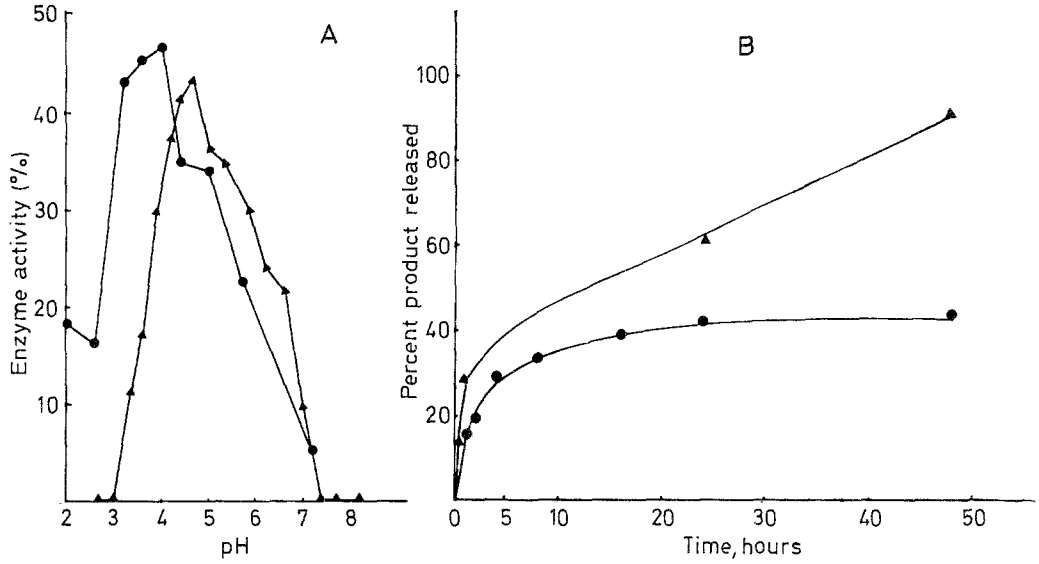

Fig. 1. A Effect of pH on normal rat kidney cortex enzyme activities towards [^{14}C]Glc-Globin (●) (24 hours incubation) and towards Glc-Gal-Hyl (▲) (2 hours incubation). Incubations were performed respectively with CK enzyme preparation containing 0.648 mg protein and BK enzyme preparation containing 0.664 mg protein, by the standard assay procedures. Enzyme activities were expressed as percent of total substrate hydrolyzed. **B** Effect of varying incubation time on enzyme activities of normal rat kidney cortex fraction C 0.6 mg towards [^{14}C]Glc-Globin (●) and towards Glc-Gal-Hyl (▲)

time up to 2 h; 43% of radioactive product could be liberated after 48 h, whereas 90% glucose was released from Glc-Gal-Hyl (Fig. 1B). 93% of the radioactive product obtained after 24 h incubation of a normal rat kidney cortex enzyme fraction C with [^{14}C]Glc-Globin corresponded to a positively charged glycopeptide fraction (I, Table 2). After application on a Biogel P2 gel filtration column, 89% of the cleaved glycopeptide eluted as a single peak with the same elution volume as determined for [^{125}I] Angiotensin I (1,400 Da) (Fig. 2). The glycopeptide releasing activity was unaffected by the presence of D-gluconolactone and D-glucosamine, both effective inhibitors of GGHG (Sternberg and Spiro, 1979; Hamazaki and Hotta, 1980). The reaction does not require

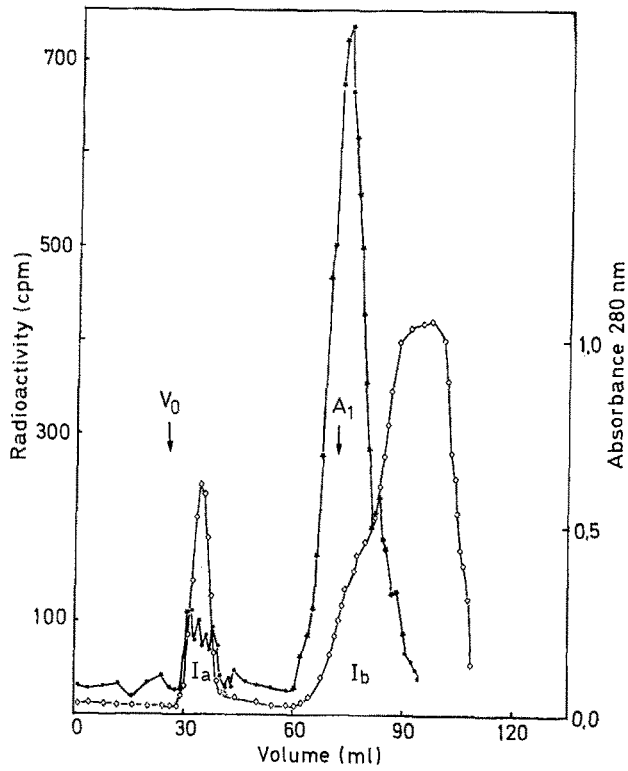

Fig. 2. Gel filtration of the radioactive positively charged glycopeptide (fraction 1, see Table 2) on Biogel P10 column. The fraction was applied to a column (2 × 27 cm) of Biogel P10 equilibrated in 0.1 M pyridine acetate pH = 5.0. The symbols refer to radioactivity (▲) and to a peptide concentration (◇) monitored by absorbance at 280 nm. VO refers to the void volume determined by Dextran Blue. AI indicates the elution volume of [^{125}I] Angiotensin I. Peaks Ia and Ib correspond to 10% and 89% total radioactivity of fraction I

divalent cation but it appears that free sulfhydryl groups are important for the catalytic activity (88% of inhibition in presence of paramercuribenzoate, Table 3). But other protease inhibitors also had an effect and 100% inhibition was obtained with a mixture of protease inhibitors (Table 3).

Comparison of the activities in the dialyzed kidney and spleen supernatants of diabetic and control rats is shown in Table 4. An increase in GGHG specific activity was observed in the kidney but not in the spleen of the STZ-diabetic and the spontaneously diabetic rats. No neutral radioactive product was released from [^{14}C] globin by kidney or spleen enzyme preparations from STZ-diabetic and normoglycemic control rats. The radioactive glycopeptide releasing activity was slightly decreased, but without statistical significance ($p < 0.10$), in the kidney of STZ-diabetic rats when compared with their normoglycemic controls.

Discussion

In contrast with a previous report of enzymatic glucose release from HbA_{1c} by lysosomal preparations from lymphocytes of diabetic patients (Lubec et al.,

Table 3. Effect of various specific inhibitors on Glc-Gal-Hyl glucohydrolase and on "Glycopeptide releasing activity" from kidney enzyme preparation (CK)

Inhibitors		"Glycopeptide releasing activity" on [^{14}C] Glc-Globin[a]		Glc-Gal-Hyl glucohydrolase activity on Glc-Gal-Hyl	
Identification	(mM)	Incubation time *(hours)*	Inhibition *(%)*	Incubation time *(hours)*	Inhibition *(%)*
D-Gluconolactone, 1–5	0.6	4	0	1	62.3
	10	4	0	1	94
D-Glucosamine	4	24	0	24	82.4
	16	24	0	N.D.	N.D.
p-Mercuribenzoate (PCMB)	1.2	4	88	N.D.	N.D.
Pepstatin (PEP)[b]	1.4×10^{-3}	4	32.1	N.D.	N.D.
Ethyldiaminetetraacetic acid (EDTA)	20	4	19.2	2	16.4
N-Ethylmaleimide (NEM)	2	4	31	N.D.	N.D.
Phenylmethanesulfonyl fluoride (PMSF)	1	4	28.5	N.D.	N.D.
PCMB + PEP + PMSF + EDTA		4	100	1	77
NEM + PEP + PMSF + EDTA		4	80.8	1	65.2
Ethanol	150	4	8.6	2	12

[a] Incubations were performed with the CK enzyme preparation containing 0.648 mg protein (Table 1) and the 1.337 μM substrate concentration under the standard assay conditions.
[b] PMSF and pepstatin dissolved in ethanol.
N.D. not determined.

Table 4. Glucosyl-galactosyl-hydroxylysine glucohydrolase and radioactive glycopeptide or neutral product releasing activities from [^{14}C]Glc-Globin in spleen and kidney cortices[c] of diabetic and normal age-matched control rats

Animal groups	Age *(months)*	Duration of diabetes *(months)*	Body weight *(g)*	Blood glucose level *(mmol/L)*	Total protein content[a] *(mg)*	Glc-Gal-Hyl glucohydrolase activity[a] *(%)*	Radioactive glycopeptide releasing activity[a] *(%)*	Neutral radioactive product released from [^{14}C]-Glc-Globin *(%)*
Spleen								
Control ($n = 6$)	5	—	427 ±8	8.69[b] ±0.35	36.7 ±5.5	100 ±6	100 ±7	0
STZ Diabetic ($n = 8$)	5	3	279*** ±12	35.7***[b] ±2.1	32.3 ±5.8	98.6 ±5.5	89.6 ±8.7	0
BB Diabetic ($n = 7$)	5	3	428 ±16	25.1***[b] ±7	37.9 ±7.1	75.3*** ±3.3	88.6 ±7.5	N.D.
Kidney								
Controls ($n = 7$)	5	—	427 ±8	8.69[b] ±0.35	162.8 ±5.8	100 ±9.6	100 ±13	0
STZ Diabetic ($n = 9$)	5	3	279*** ±12	35.7***[b] ±2.1	186.2* ±7.4	143** ±8	80.2 ±7.3	0
BB Diabetic ($n = 7$)	5	3	428 ±16	25.1***[b] ±7	213.2 ±16	124* ±9	89 ±6	N.D.

Values expressed as mean ± SEM. Comparisons between diabetic and age-matched normal control were effected by Student's t test or Mann-Witney's U test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. 0, undetected; *N.D.* not determined.

[a] Activity determined per mg protein and expressed relative to the age-matched normal control groups;

[b] Measured on carotid plasma taken during decapitation in non fasting animals.

[c] Determined in fractions B. (See Table 1).

1982), no release of neutral radioactive hexose could be detected from [^{14}C]Glc-Globin in our experiments, using normal or diabetic kidney and spleen GGHG preparations isolated by ammonium sulphate fractionation of the high speed supernatant. However other lysosomal enzymes could have been precipitated by ultracentrifugation if they were membrane-bound.

The glycopeptide releasing activity observed with our preparations may be related to the presence of several acidic proteases such as cathepsin D or L (Takahashi and Tang, 1981; Barrett and Kirschke, 1981). This hypothesis is supported by a biphasic pH curve. This protease activity does not affect the GGHG activity which is most stable in the 7 × 10 g.min supernatant and in the 1.56 M ammonium sulphate precipitate. Further purification of the GGHG decreases its stability (Sternberg and Spiro, 1979).

An increase of GGHG specific activity in dialyzed kidney cortex fractions of streptozotocin-diabetic rats has been reported, after 19, 23 and 28 weeks of diabetes (Sternberg et al., 1982). The results obtained in this work for a 13 weeks period after streptozotocin treatement are similar. In addition, an increased enzyme activity in the kidney of spontaneously diabetic rats could be demonstrated (Table 4). This increase might be due to increased biosynthesis of the enzyme consecutive to the basement membrane thickening which represents an increase in the enzyme's substrate. Renal cells are freely permeable to glucose (Wahl et al., 1973) and glucose concentration appears to be an important regulatory factor in the catabolism of basement membrane collagen disaccharide units especially in the kidney (Sternberg et al., 1983). The spleen GGHG activity was not altered after one and three months of streptozotocin-diabetes, as we found in previous studies after 23 weeeks of disease (Sternberg et al., 1982). Besides, the spleen GGHG activity was decreased in the spontaneously diabetic rats: this may be related to the minimal insulin treatment of these animals. The difference between kidney and spleen responses to diabetes and insulin may be due to different glucose transporters and insulin receptors. The glycopeptide releasing activity was not modified significantly in the kidney and spleen of streptozotocin- and spontaneously diabetic rats. Nevertheless a decreasing trend was observed.

In summary, a specific enzymatic activity for the removal of hexose from nonenzymatically glycated proteins could not be found in any kidney or spleen fractions of normal and diabetic rats containing glucosyl-galactosyl-hydroxylysine glucohydrolase.

Acknowledgements

We thank G. Mozère, J. André, O. M' Boudou, S. Picard, A. Bessala, and J. Bourlier for excellent assistance; the Animal Resources Division, Ottawa, and Dr. R. Guillot for the BB rats; L. Vaughan for the [^{14}C]Glc-Globin.

References

Barrett AJ, Kirschke H (1981) Cathepsin B, H and L. Methods Enzymol 80: 535–561

Bunn FH, Gabbay KH, Gallop PM (1978) The glycosylation of hemoglobin: relevance to diabetes mellitus. Science 200: 21–27

Fluckiger R, Winterhalter KH (1976) In vitro synthesis of hemoglobin AIc. FEBS Letters 71 2: 356–360

Grigorova-Borsos AM, Cohen-Forterre L, Sternberg M (1987) Exoglycosidases degrading glomerular basement membrane carbohydrates: Glc-Gal-Hyl glucohydrolase and sialidase from rat kidney. In: Price RG, Hudson BG (eds) Renal basement membranes in health and disease. Academic Press, London, pp 209–218

Hamazaki H, Hotta K (1979) Purification and characterization of an alpha-glucosidase specific for hydroxylysine-linked disaccharide of collagen. J Biol Chem 254: 9682–9687

Hamazaki H, Hotta K (1980) Enzymatic hydrolysis of disaccharide unit of collagen. Isolation of 2-O-alpha-D-glucopyranosyl-O-beta-D-glactopyranosyl-hydroxylysine glucohydrolase from rat spleen. Eur J Biochem 111: 587–591

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193: 265–276

Lubec G, Vykudilik W, Frisch H, Coradello H, Pollak A (1982) Enzymatic reversibility of nonenzymatic glycosylation: a glucosidase activity in diabetic lysosomes cleaving a monosaccharide from HbAIc. IRCS Med Sci 10: 50–51

Somogyi M (1945) A new reagent for the determination of sugars. J Biol Chem 160: 61–68

Sternberg M, Spiro RG (1979) Studies on the catabolism of the hydroxylysine-linked disaccharide units of basement membranes and collagens. Isolation and characterization of a rat kidney alpha-glucosidase of high specificity. J Biol Chem 254: 10329–10336

Sternberg M, Grochulski A, Peyroux J, Hirbec G, Poirier J (1982) Studies of the alpha-glucosidase specific for collagen disaccharide units: variations associated with capillary basement membrane thickening in kidney and brain of diabetic and aged rats. Collagen Rel Res 2: 495–506

Sternberg M, André J, Peyroux J (1983) Inhibition of the alpha-glucosidase specific for collagen disaccharide units in diabetic rat kidney by in vivo glucose levels: possible contribution to basement membrane thickening. Diabetologia 24: 286–289

Takahashi T, Tang J (1981) Cathepsin B from porcine and bovine spleen. Methods Enzymol 80: 565–581

Vaughan L, Fischer RW, Zimmermann D, Winterhalter KH (1984) Nonenzymatic glucosylation of proteins: a new and rapid solution for in vitro investigation. FEBS Letters 173: 173–178

Wahl P, Deppermann D, Deschner W, Fuchs E, Rexroth W (1973) The metabolism of the isolated renal glomerulus and its basement membranes. In: Camerini-Davalos RA, Cole HS (eds) Vascular and neurological changes in early diabetes. Academic Press, New York, pp 147–153

Authors' address: Dr. M. Sternberg, Département de Biochimie, Faculté de Médecine des Saints-Pères, 45 rue des Saints-Pères, F-75270 Paris Cedex 06, France.

Received April 22, 1992