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SUBFRACTIONATION OF THE DANSYLATED DERIVATIVES OF GLUCOSYL GALACTOSYL HYDROXYLYSINE BY LIQUID CHROMATOGRAPHY AND ITS APPLICATION TO A SPECIFIC α-1,2-GLUCOSIDASE ASSAY

HITOO IWASE*, IKUKO ISHII, YUKINOBU KATO, HIDEAKI HAMAZAKI and KYOKO HOTTA

Department of Biochemistry, School of Medicine, Kitasato University, Sagamihara, Kanagawa 228 (Japan)

and

AKIRA UMEZAWA and TAMOTSU KANZAKI

Department of Dermatology, School of Medicine, Kitasato University, Sagamihara, Kanagawa 228 (Japan)

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SUMMARY

The dansyl derivative of glucosyl galactosyl hydroxylysine (GGH) was separated into two components, as GP-I (monodansyl GGH) and GP-II (didansyl GGH) by paper chromatography. GP-I was further fractionated into four peaks (a, b, c and d) by reversed-phase liquid chromatography. These peaks corresponded to the dansyl derivatives at the α-amino (a and b) and ϵ-amino (c and d) groups of their hydroxylysine residues. There is the possibility that the fractions for b and d are diastereoisomers of a and c, respectively, since the monodansyl derivative from human urine consists of a and c. GP-II was fractionated into two peaks, e and f, which may possibly be diastereoisomers of each other. Treatment of the a, b, c and d fractions with crude chicken liver enzyme resulted in the preferential cleavage of a and b and the production of monodansyl galactosyl hydroxylysine. Components c and d were also cleaved slowly, resulting in the production of monodansyl hydroxylysine by the successive action of β-galactosidase on dansyl galactosyl hydroxylysine. The detected α-glucosidase activity was strongly inhibited by free mannosamine. The method developed using the monodansyl GGH fraction a (or b) and high-performance liquid chromatography facilitated the detection of α-1,2-glucosidase, which acts specifically toward GGH even in a crude enzyme preparation.

INTRODUCTION

Glucosyl galactosyl hydroxylysine (Glc-Gal-Hyl or GGH) is a well known glycopeptide derived from collagen by alkaline treatment [1]. This type of carbo-

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hydrate chain is distributed widely in vertebrates and invertebrates [2–4], and is also found in the complement, Clq [5]. In spite of its wide distribution, the biological significance of the disaccharide unit is still unknown.

Recently, Hamazaki and Hotta [6] reported on the degradation enzyme α-1,2-glucosidase, which acts specifically on GGH. This enzyme is important in the catabolism of collagen. Its action on the disaccharide prosthetic group of the collagen molecule possibly causes exposure of the galactose residue; this would be an indicator of the degradation of the protein in the case of serum glycoprotein [7].

Moro et al. [8] reported that the quantitative measurement of dansyl derivatives of glycosylated hydroxylysine components in urine makes it possible to evaluate the metabolic turnover of the collagen molecule.

This present paper describes the analysis of the dansyl derivatives of GGH, using the method developed for the N-glycosidic type glycopeptide [9–12]. The purpose of this work was to devise a method for rapid and easy separation of glycosylated hydroxylysine and/or relevant peptides, to find a good substrate for the enzyme assay and a means by which specific α-glucosidase in a crude enzyme preparation could more easily be detected.

EXPERIMENTAL

Materials

The following compounds and materials were obtained commercially: dansyl chloride (5-dimethylaminonaphthalene-1-sulphonyl chloride) from Pierce (U.S.A.); acetonitrile, 1-butanol and acetic acid, from Wako (Japan); Whatman 3MM paper from W&R Balston (U.K). All HPLC solvents were filtered through Toyo Roshi filters (pore size 0.45 μm). All other reagents were of the highest grade available and used without further purification.

High-performance liquid chromatography (HPLC)

The HPLC apparatus consisted of a Hitachi solvent-delivery system equipped with a Hitachi 655-71 Processor B, a proportioning valve, a Rheodyne 7125 sample injector, an Inertsil ODS column (5 μm; 25 cm$\times$4.6 mm I.D.) protected by a Brownlee Labs. MPLC guard column (30 mm$\times$4.6 mm I.D.) and a FP-210 (Japan Spectroscopic) fluorescence monitor. For the HPLC analysis, ca. 1 nmol of dansyl glycopeptides was injected into the column. Elution of the column was then carried out at a flow-rate of 0.5 ml/min using a mobile phase containing 20 or 17% acetonitrile in 25 m*M* sodium borate buffer (pH 7.0). The effluent was monitored fluorometrically (excitation at 325 nm, emission at 530 nm).

For the preparation of the monodansyl derivatives, ca. 0.2 μmol of sample was applied to the column followed by elution with 16% acetonitrile in distilled water. The fractionated dansyl derivatives were manually monitored by UV absorption at 210 nm.

Preparation of dansyl GGH

GGH purified from alkaline degradated edible medusae [6] was dansylated. Briefly, the glycopeptide was dissolved in 0.2 *M* sodium bicarbonate at a concen-

tation of 2.5 mg/ml. To this solution was added an equal volume of dansyl chloride solution (10 mg of dansyl chloride per ml of acetone). The reaction mixture set aside for 12 h at room temperature. The mixture was spotted on Whatmann 3MM paper and developed with 1-butanol–acetic acid–water (3:1:1, v/v/v). A broad yellowish fluorescence band near the origin was observed in addition to one moving faster than dansyl acid (Dns-OH). These bands were cut from the paper and eluted with water. The former was designated GP-I and the latter GP-II. Each purified dansylated glycopeptide fraction showed one band on thin-layer chromatography (TLC) under the above solvent conditions. The $R_{\text{Dns-OH}}$ values (relative mobility to Dns-OH) for GP-I and GP-II were 0.52 and 0.86, respectively. It should be noted that the dansyl derivative on the TLC plate (silica gel 60) decomposed more quickly than that on filter paper. The reaction product was detected fluorometrically, and diphenylamine aniline reagent was used for sugar detection on the TLC plate. Under the present experimental conditions, GP-I represented approximately the same amount of hexose as GP-II. Since GP-I could be converted into GP-II by redansylation, it was concluded that both spots were partially or fully dansylated GGH, i.e. the monodansyl derivative and didansyl derivative, respectively.

Preparation of monodansyl GGH from human urine

Fresh human urine (50 ml) was lyophilized and redissolved in 6 ml of 0.1 *M* acetic acid. The residue was removed by centrifugation at 9000 *g*. The clear yellowish supernatant was fractionated by chromatography of Sephadex G-25. A portion of the fraction eluted from the column was dansylated and then separated by TLC to detect dansyl GGH. GGH eluted immediately before the peak corresponding to the salt. The fractions containing GGH were collected and concentrated in a rotary evaporator. About half the amount of recovered glycopeptide was used for the dansylation reaction. Monodansyl GGH was purified by paper chromatography as described in the text, and the amount obtained was sufficient for ten HPLC analyses.

Preparation of the crude enzyme from chicken liver and assay of the enzyme activity

The crude enzyme of α-glucosidase from chicken liver was prepared by homogenizing the liver (20 g) in 50 m*M* phosphate buffer (pH 7.0) (80 ml) using a Polytoron homogenizer. The homogenate was centrifuged at 105 000 *g* for 60 min and the supernatant was stored as enzyme preparation at $-20\,^{\circ}$C. About 10 nmol of dansyl derivatives in 20 μl of 0.2 *M* acetate buffer (pH 5.0) was treated with 20 μl of the above crude enzyme. The insoluble material was removed by centrifugation at 8500 *g* followed by HPLC analysis of the supernatant of the reaction mixture.

RESULTS AND DISCUSSION

Fractionation of monodansyl GGH (GP-I) and didansyl GGH (GP-II) by reversed-phase liquid chromatography

GP-I and GP-II, purified by preparative paper chromatography as described in Experimental, were further analysed by reversed-phase liquid chromatography.

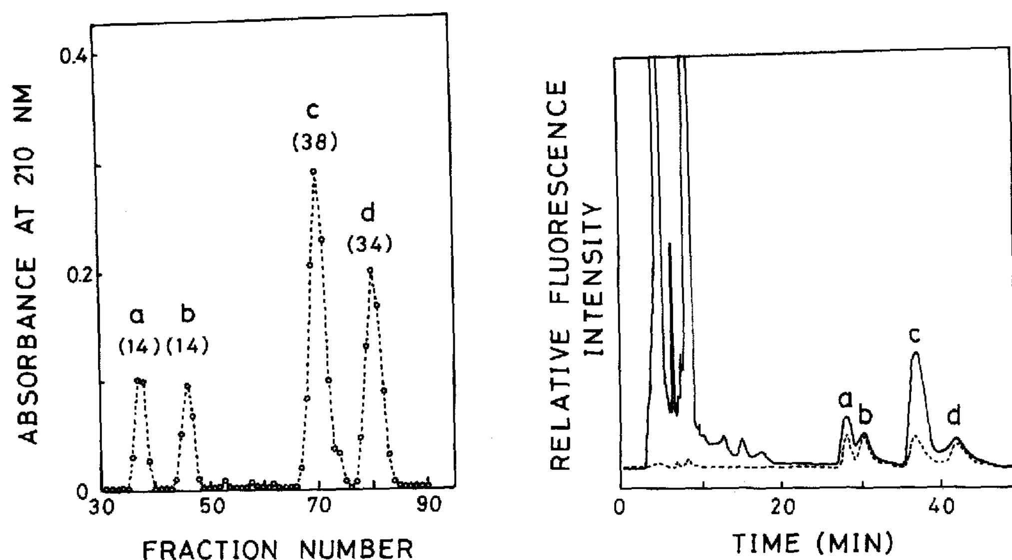

Fig. 1. Subfractionation of monodansyl GGH by reversed-phase liquid chromatography. GP-I (monodansyl GGH) was fractionated into peaks a, b, c and d by HPLC using 16% acetonitrile in water as the mobile phase (described in the text). Numbers in parentheses indicate the relative content (%) of each component based on UV absorption. Fractions were collected and used for further analyses.

Fig. 2. HPLC analysis of monodansyl GGH prepared from human urine. Elution profiles of GP-I (---) and the mixture of GP-I and monodansyl GGH prepared from human urine. The samples were eluted from the column with 17% acetonitrile in 25 m*M* borate buffer (pH 7.0). The peaks a, b, c and d are the same as in Fig. 1.

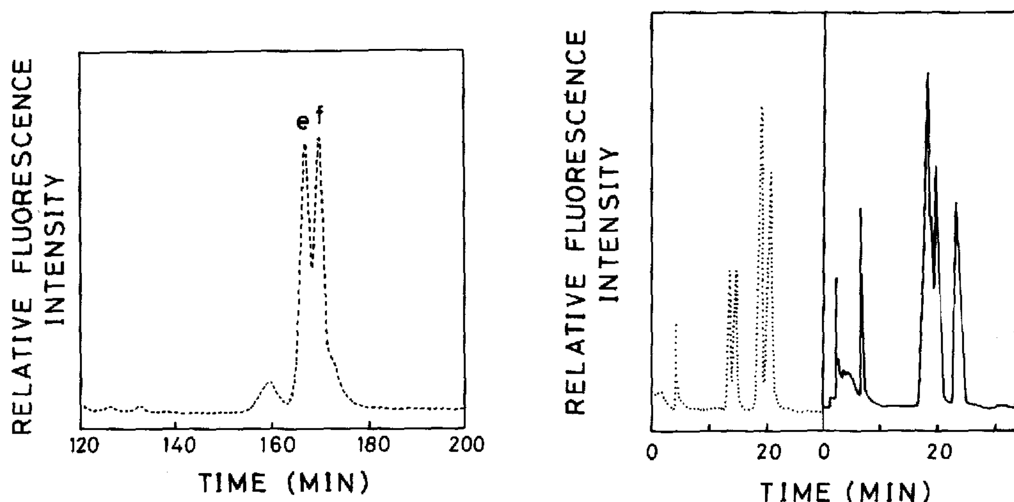

Fig. 3. HPLC analysis of the didansyl derivative from GGH. GP-II (didansyl GGH) purified by paper chromatography was eluted from the column using a linear gradient from 20 to 40% acetonitrile in 25 m*M* borate buffer (pH 7.0) in 200 min. Peaks e and f were diastereoisomers of each other, as described in the text.

Fig. 4. Treatment of monodansyl GGH with crude chicken liver enzyme. Chromatograms of (---) the control monodansyl GGH and (—) the monodansyl GGH treated with crude chicken liver enzyme; elution was carried out isocratically with 20% acetonitrile in 25 m*M* borate buffer (pH 7.0).

Fig. 1 shows the separation of GP-I into four peaks: a, b, c and d. The fractional ratio (a:b:c:d), based on their UV absorption at 210 nm, was 14:14:38:34. The fractionation of monodansyl glycopeptide prepared from human urine showed many peaks. Two components eluted at the same time as a and c among the four peaks shown in Fig. 1 (Fig. 2). This indicates that a and c correspond to the monodansyl derivative of the native GGH molecule. Possibly, the remaining peaks b and d are diastereoisomers of a and c, respectively, produced by racemization of the hydroxylysine residue during alkaline treatment of GGH.

GP-II could not be eluted under the above conditions. However, using a linear gradient of 20–40% acetonitrile in 25 mM borate buffer, pH 7.0 (Fig. 3) it was separated into two peaks (e and f). This shows that didansyl GGH is more hydrophobic than monodansyl GGH and that the two peaks are possibly diastereoisomers of each other.

Enzyme treatment of monodansyl GGH

Treatment of GP-I with the crude enzyme from chicken liver produced the complex chromatogram shown in Fig. 4. It was difficult to calculate the enzyme activity. Each component purified from GP-I could be treated separately with the same enzyme. Complete cleavage of a and b by the enzyme preparation in Fig. 5 indicated that these components are good substrates for the detection of a specific α-glucosidase.

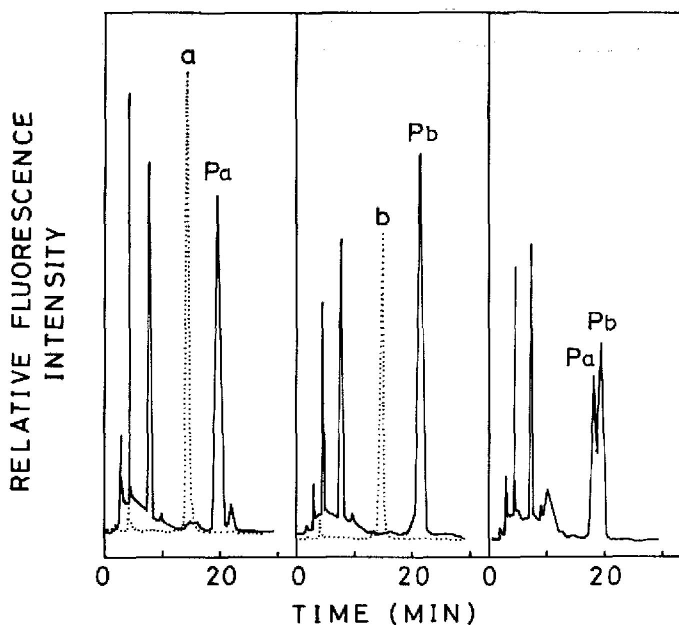

Fig. 5. Treatment of α-aminodansyl GGH (a and b) with chicken liver enzyme. The broken line shows the chromatogram of the substrate, α-aminodansyl GGH (a and b). Each substrate was treated for 16 h with chicken liver enzyme and analysed by HPLC. Pa and Pb represent the deglucosylated products, monodansyl GH from a and b, respectively. The right-hand panel shows the chromatogram of the Pa and Pb mixture.

The slower cleavage of c and d (Fig. 6) than that of a and b showed the former two to be ϵ-aminodansyl GGH and the latter, α-aminodansyl GGH since this enzyme acts preferentially on dansyl GGH whose ϵ-amino group is not blocked. Under the present conditions, the products from a and b could be separated from each other, but not those from c and d (Figs. 5 and 6). Thus, the complex chromatogram in Fig. 1 obtained from GP-I treated with the crude enzyme is due to the close elution of c and d with that of the products from a and b.

Prolonged treatment of component c resulted in the appearance of two fluorescent products, monodansyl galactosyl hydroxylysine (GH) and monodansyl hydroxylysine produced by the successive action of β-galactosidase on dansyl galactosyl hydroxylysine (Fig. 7). Monodansyl galactosyl hydroxylysine produced from a and b could not be cleaved further by the enzyme.

It is significant that the known α-glucosidase inhibitor, mannosamine, at a concentration of 1.0 mM, also completely inhibited α-glucosidase activity in the crude enzyme preparation using monodansyl derivatives in the HPLC analysis.

Moro et al. [8] separated the dansyl derivatives of urinary GGH and GH by HPLC under conditions similar to ours. They reported monodansyl GGH, didansyl GGH, monodansyl GH and didansyl GH to be eluted in this order from the reversed-phase column. In our experiment, the monodansyl GH eluted before the didansyl GGH. This discrepancy may possibly arise from differences in the solvent system used.

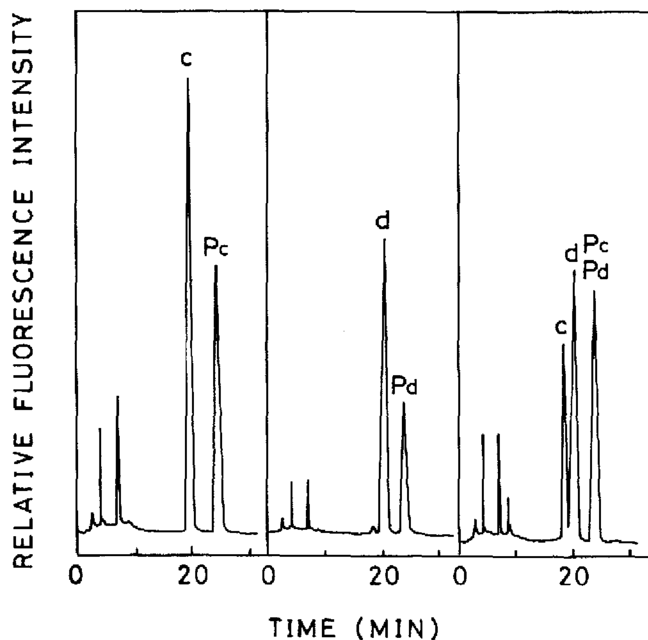

Fig. 6. Treatment of ϵ-aminodansyl GGH (c and d) with chicken liver enzyme. Monodansyl GGH (components c and d) was treated with chicken liver enzyme and analysed by HPLC as described in Fig. 5. Pc and Pd indicate the deglucosylated products, monodansyl GH as in Fig. 5. The right-hand panel shows the chromatogram of the c and d mixture treated with the crude enzyme.

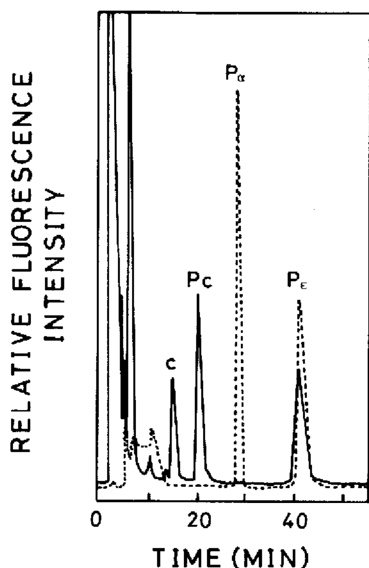

Fig. 7. Prolonged treatment of ε-aminodansyl GGH with chicken liver enzyme. Component c was treated with chicken liver enzyme for two days and analysed by HPLC under the same conditions as in Fig. 5. The broken line indicates the elution profile of α-aminodansyl hydroxylysine (P_α) and ε-aminodansyl hydroxylysine (P_ϵ) prepared from GP-I by hydrolysis with 6 *M* hydrochloric acid at 100°C for 7.5 h.

In the present study, the glycopeptide-mapping method was found applicable to the fractionation of dansyl derivatives from collagen glycopeptides. This method should also facilitate detection of α-glucosidase, which acts specifically on GGH even in a crude enzyme preparation such as chicken liver homogenate.

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