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Note

High-performance liquid chromatographic analysis of changes of asparagine-linked oligosaccharides in regenerating rat liver

IKUKO ISHII

Faculty of Pharmaceutical Sciences, Nagoya City University, Nagoya 467 (Japan)

NORIKO TAKAHASHI*

Department of Biochemistry, Nagoya City University Medical School, Mizuho-ku, Nagoya 467 (Japan)

SHIGEMI KATO and NOBU AKAMATSU

Department of Biochemistry, St. Marianna University School of Medicine, Miyamae-ku, Kawasaki 213 (Japan)

and

YUTAKA KAWAZOE

Faculty of Pharmaceutical Sciences, Nagoya City University, Nagoya 467 (Japan)

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In our previous report [1], time dependency of the alterations in oligosaccharides of tissue glycoproteins of regenerating liver was examined in detail from 6 h to 10 days after partial hepatectomy. We used a [^{3}H]mannose incorporation method. The results indicated that a higher proportion of complex type oligosaccharides was observed in regenerating liver 24 h after partial hepatectomy than in the control. (The proportion was based on the susceptibility difference between complex-type and high-mannose-type oligosaccharides to endo-β-N-acetyl-glucosaminidase H digestion.) This tendency increased gradually with time and was most pronounced at 6 days. The ratio of complex-type to high-mannose-type oligosaccharides decreased gradually to the control level by 10 days, when the deficit of liver had been restored.

In this paper, on the basis of our metabolic results using isotopes [1, 2], we

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examined at critical times as demonstrated in previous papers, i.e. 1, 6 and 10 days after partial hepatectomy.

The separation and identification of pyridylamino derivatives of oligosaccharides on extremely small scales were carried out using high-performance liquid chromatography (HPLC) with a reversed-phase C_{18} column [3, 4]. The procedure has not yet been used to examine dynamic aspects of glycoprotein processing.

In the present work, we successfully employed the HPLC technique to determine how asparagine-linked oligosaccharides were processed during regeneration of rat liver. We showed the detailed differences in the oligosaccharide chains between the regenerating liver and the controls, without any isotope-labelling procedure.

EXPERIMENTAL

Enzymes and standard oligosaccharides

Pepsin was purchased from Sigma (St. Louis, MO, U.S.A.). N-Oligosaccharide glycopeptidase (almond) EC 3.5.1.52), by which all kinds of desialylated asparagine-linked oligosaccharide chains were released quantitatively from glycopeptides [5—7], was the product of Seikagaku Kogyo (Japan). All standard oligosaccharides described below were prepared by the method of Ishihara et al. [8]: the high-mannose-type (Man_{5-9} $GlcNAc_2$) and bi-antennary oligosaccharides with fucose were prepared from porcine thyroglobulin (a kind gift from Dr. O. Tarutani, Gunma University); bi-antennary without fucose was from fibrinogen; tri- and tetra-antennary with fucose were from porcine pancreatic lipase; tri- and tetra-antennary without fucose were from α_1-acid glycoprotein. The oligosaccharides obtained were reductively aminated with a fluorescent reagent, 2-aminopyridine, by the use of sodium cyanoborohydride. The pyridylamino derivatives of the oligosaccharides thus prepared were purified by gel filtration with Sephadex G-15 by the method of Hase et al. [4]

Other chemicals

Bio-Gel P-4 (200—400 mesh) was purchased from Japan Bio-Rad Labs. (Japan); Sephadex G-15 and Con A-Sepharose 4B were from Pharmacia Fine Chemicals (Sweden); sodium cyanoborohydride was from Aldrich (U.S.A.); 2-aminopyridine from Wako (Japan) was recrystallized from 1-hexane.

Preparation of liver powder

Male Wistar rats were partially hepatectomized by the method of Higgins and Anderson [9]. Sham-operated rats were used as controls. Of each liver 2 g (wet weight) were used to prepare the liver powder for analysis as described previously [1].

Preparation of N-linked oligosaccharides from liver glycoproteins by glycopeptidase digestion

The oligosaccharide preparations to be analysed were obtained from 30 mg of each of the liver powders from the regenerating livers and the controls. The procedure of N-oligosaccharide glycopeptidase digestion of desialylated and

pepsin-digested liver glycopeptides was described previously [7]. The digestion product was fractionated on a column of Bio-Gel P-4. Further purification of the oligosaccharides by ion-exchange resins has been described previously [8]. The oligosaccharides were coupled with 2-aminopyridine, and the pyridylamino derivatives thus obtained were purified by Sephadex G-15 column chromatography by the method of Hase et al. [4].

Separation of oligosaccharides by Con A-Sepharose

In order to separate bi-antennary and multi-antennary oligosaccharides, the pyridylamino derivatives of oligosaccharides were further chromatographed on an immobilized Con A-Sepharose column (0.9 ml), equilibrated in 5 m*M* sodium acetate buffer (pH 6.0) containing 0.1 *M* sodium chloride and magnesium chloride, manganese chloride, calcium chloride (1 m*M* each).

After the fraction (350 μl) containing oligosaccharides was applied to the Con A column, elution was carried out first with 9 ml of the above buffer and then with 10 ml of the buffer containing 100 m*M* methyl α-D-glucoside. Non-retained (multi-antennary oligosaccharides) and retained (bi-antennary oligosaccharides) fractions were diluted with 200 and 400 ml of water, respectively, and were each adsorbed to 5 ml on a Dowex 50 (H^+) column. The columns were washed with 25 ml of water and the pyridylamino derivatives adsorbed on the resin were eluted with 40 ml of 0.1 *M* aqueous ammonia. The eluate was evaporated to dryness, dissolved in water and further purified by Sephadex G-15 gel chromatography.

Analysis of oligosaccharides by HPLC

The separation and identification of pyridylamino derivatives were carried out by HPLC using a Shimadzu LC-3A with a column (250 × 4 mm) packed with TSK-Gel LS 410 (5 μm, C_{18}, Toyo-Soda). Elution was performed at 50°C with 0.1 *M* phosphate buffer (pH 3.8) containing 0.1—1.0% 1-butanol at a flow-rate of 1 ml/min, and fluorescence was detected using excitation and emission wavelengths of 320 and 400 nm, respectively.

Analysis of component monosaccharides

Each oligosaccharide fraction separated by HPLC was hydrolyzed by 2 *M* hydrochloric acid containing 2 *M* trifluoroacetic acid at 100°C for 6 h [10]. The resulting monosaccharides were analysed by HPLC using a Shimadzu Model LC-3A with a column of ISA-07/S2504 (250 × 4 mm) [11].

RESULTS AND DISCUSSION

The mixture of pyridylamino derivatives of standard oligosaccharides was analysed directly by HPLC without pretreatment by Con A-Sepharose. Fig. 1 shows a typical chromatogram for this case. The shaded areas indicate the region of high-mannose-type oligosaccharides. Effective separation of the oligosaccharides ($Man_{5-9}GlcNAc_2$) was attained within 30 min. Complex-type oligosaccharides (bi-, tri- and tetra-antennary species with and without fucose) were clearly separated from the high-mannose-type. This pattern is effective enough to evaluate the ratio of complex-type to high-mannose-type oligosaccharides

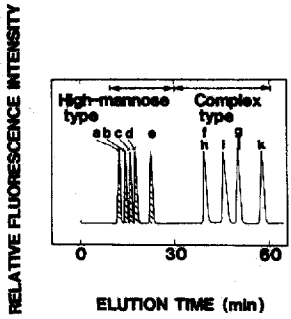

Fig. 1. HPLC profiles of the pyridylamino derivatives of standard oligosaccharides. Shaded areas indicate the regions of high-mannose-type oligosaccharides. After the high-mannose-type, the complex-type oligosaccharides were eluted. Peaks: a = M_8GN_2; b = M_9GN_2; c = M_7GN_2; d = M_6GN_2; e = M_5GN_2;

f = G-GN-M(G-GN-M)M-GN-GN;

g = G-GN-M(G-GN-M)M-GN-GN; F

h = G-GN(G-GN)M(G-GN)M(G-GN)M-GN-GN;

i = G-GN(G-GN)M(G-GN-M)M-GN-GN;

j = G-GN(G-GN)M(G-GN)M(G-GN)M-GN-GN; F

k = G-GN(G-GN)M(G-GN-M)M-GN-GN. F

The symbols used for the structures are: G = galactose; M = mannose; F = fucose; GN = N-acetylglucosamine.

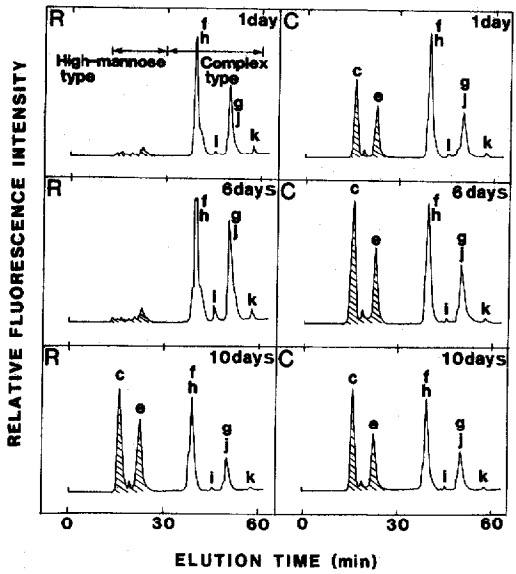

Fig. 2. Comparison of oligosaccharide profiles of rat liver glycoproteins by HPLC. Oligosaccharide fractions obtained by N-oligosaccharide glycopeptidase digestion of glycopeptides from six kinds of livers (1, 6 and 10 days after partial hepatectomy and their controls) were aminated and applied on HPLC as described in the text. (R) Regenerating liver; (C) control. Peaks c, e, f, g, h, i, j and k correspond to those of Fig. 1.

(cf. Fig. 2). The recovery of oligosaccharide in each case was 90—100% of that applied to the column. The chromatographic behaviour of each oligosaccharide in the mixture is essentially identical to that of the homogeneous species [3, 4].

Fig. 2 shows the patterns of the pyridylamino derivatives from regenerating livers and controls 1, 6 and 10 days after the operation, respectively. The main components in the high-mannose region were $Man_7GlcNAc_2$ and $Man_5GlcNAc_2$. The ratio of complex-type to high-mannose-type oligosaccharides changed appreciably during liver regeneration. Until 6 days after partial hepatectomy, the oligosaccharides were mostly complex-type, and high-mannose-type oligosaccharides were markedly decreased in the regenerating liver. The initial sharp decrease in carbohydrate content in the regenerating rat liver was observed by Van Dijk et al. [12]. Taken together, it is suggested that most of the high-mannose-type oligosaccharides (the intermediates in glycoprotein biosynthesis) are processed rapidly in the regenerating rat liver. The ratio of complex-type to high-mannose-type recovered to the control level by 10 days after partial hepatectomy. A significant decrease in high-mannose-type oligosaccharides, i.e. an increased processing of glycoproteins during a period of rapid increase in cell number, suggests that complex-type oligosaccharides may be synthesized in preference to the high-mannose-type.

The complex-type oligosaccharides in the mixture of pyridylamino derivatives of asparagine-linked oligosaccharides were further separated into two portions by Con A-Sepharose column chromatography, since Con A-Sepharose is known to bind bi-antennary oligosaccharides but neither tri- nor tetra-antennary oligosaccharides [13—15]. They were designated in Fig. 3 as "retained" and "non-retained" complex-type, respectively. High-mannose-type oligosaccharides were not eluted from Con A-Sepharose under the elution conditions employed. As shown in Fig. 3, the amount of multi-antennary oligosaccharides with and without fucose (non-retained) increased markedly in the regenerating liver 6 days after partial hepatectomy. Quantitatively, complex-type oligosaccharides per mg of liver powder reached a maximum value

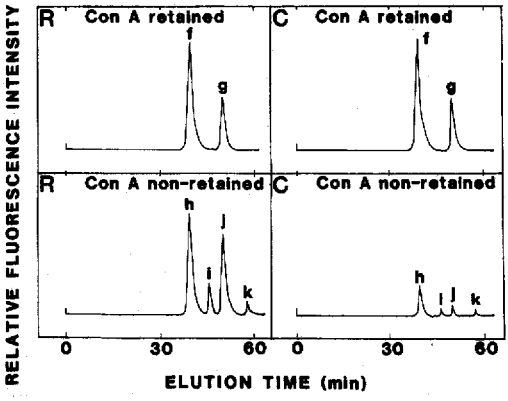

Fig. 3. HPLC analysis of complex-type oligosaccharides in regenerating liver 6 days after partial hepatectomy and the control. Retained (bi-antennary) and non-retained (multi-antennary) oligosaccharides were separated by Con A-Sepharose. (R) Regenerating liver; (C) control. Peaks f, g, h, i, j, and k correspond to those of Fig. 1.

(11.8 μg) on the 6th day after partial hepatectomy (control value was 6.6). It is of interest that the amount of multi-antennary oligosaccharides increased temporarily in the regenerating process. The chromatogram of the regeneration liver 10 days after partial hepatectomy settled down at the control level shown in Fig. 3 (data not shown).

The identity of each oligosaccharide fraction isolated by the HPLC technique was further confirmed by determination of the component monosaccharides [10, 11]. In this study, we primarily showed that the separation method of asparagine-linked oligosaccharides by HPLC after N-oligosaccharide glycopeptidase digestion is valuable, especially for demonstrating the distribution of high-mannose-type and complex-type oligosaccharides.

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