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Note

Lectin affinity high-performance liquid chromatography columns for the resolution of nucleotide sugars

HIROSHI HARADA* and MASUGU KAMEI
Hohnen Oil Co., Ltd., 1-2-3, Ohtemachi, Chiyoda-ku, Tokyo, 100 (Japan)
and
SEIKO YUI and FUMIHIRO KOYAMA
Sugiyama Chemical and Industrial Laboratory, 11, Kagetori-cho, Totsuka-ku, Yokohama, 245 (Japan)
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Castor beans contain two isolectins called RCA60 and RCA120[1], having molecular weights of approximately 60 000 and 120 000, respectively. They exhibit some differences in hemagglutinating activity, cytotoxicity and carbohydrate binding specificity (or oligosaccharide binding specificity)[1–4]. Nicolson *et al.*[1] reported that hemagglutinating activity of RCA60 and RCA120 is inhibited by sugars containing terminal β-D-galactose-like residues, but RCA60 is additionally inhibited by N-acetyl-D-galactose-like sugars. In general, the separation of RCA60 and RCA120 is carried out by gel filtration or ion-exchange chromatography. We have now applied hydrophobic chromatography to this separation, and obtained good results.

Our previous report[5] demonstrated that RCA120 immobilized on a lectin affinity high-performance liquid chromatography (HPLC) column can efficiently separate UDP-glucose and UDP-galactose, or UDP-N-acetyl-D-glucosamine and UDP-N-acetyl-D-galactosamine. In the present study we immobilized RCA60 on an HPLC gel in order to separate nucleotide sugars. There was a clear difference in the separation pattern of nucleotide sugars between the RCA60 and RCA120 columns. This difference made it possible to separate three kinds of nucleotide sugars.

Here, we demonstrate that hydrophobic chromatography is useful for the separation of RCA60 and RCA120 and that lectin affinity HPLC using RCA60 and RCA120 columns in series is quite useful for the resolution of nucleotide sugars.

MATERIALS AND METHODS

Materials

UDP-glucose, UDP-galactose, UDP-N-acetyl-D-glucosamine and UDP-N-acetyl-D-galactosamine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and dissolved in 0.01 *M* phosphate buffer containing 0.15 *M* sodium chloride (pH 7.4).

Butyl-Toyopearl 650M was obtained from Toyo Soda (Tokyo, Japan). A polyacrylic ester gel(WG003) containing an epoxy residue was obtained from Hitachi (Tokyo, Japan). Superose 6 and 12 columns were obtained from Pharmacia (Tokyo, Japan).

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The fast protein liquid chromatography (FPLC) system was obtained from Pharmacia, and the LC-6A HPLC system from Shimadzu Seisakusho (Kyoto, Japan).

Preparation of RCAs (mixture of RCA60 and RCA120)

RCAs were obtained by the ammonium sulphate fractionation of a castor bean extract according to the method of Ishiguro *et al.*[6], and subsequent affinity chromatography on Lactamyl-Sepharose 6B using the method of Matsumoto *et al.*[7].

Hydrophobic chromatography

A Butyl-Toyopearl 650M column (40 × 2.6 cm) was equilibrated with 20% saturated ammonium sulphate in 0.02 *M* phosphate buffer (pH 7.4). A 10-ml volume of the solution of RCAs (13 mg/ml in starting buffer) was applied to the column. RCA120 was eluted with the starting buffer, and RCA60 with 10% saturated ammonium sulphate in 0.01 *M* phosphate buffer (pH 7.4). All the chromatography was carried out at a flow-rate of 2 ml/min at 4°C and the effluent was monitored at 280 nm.

HPLC

High-performance size-exclusion chromatography was performed by a Pharmacia FPLC system using Superose 6 (300 × 10 mm) and Superose 12 (300 × 10 mm) columns connected in series. The elution buffer was 0.15 *M* sodium chloride in 0.01 *M* phosphate buffer (pH 7.4) containing 0.1 *M* galactose. The flow-rate was 0.8 ml/min. All the chromatographic analyses were carried out at room temperature and the effluent was monitored at 280 nm.

Immobilization of lectins on the epoxy-activated gel

Coupling of lectins to the gel(WG003) was carried out according to the method of Matsumoto *et al.*[8]. The final lectin gels, RCA60-WG003 and RCA120-WG003, contained *ca.* 5.48 mg of RCA60 and 8.87 mg of RCA120 per gram of suction-dried gel, respectively, as determined from the absorbances at 280 nm of the original and filtered lectin solutions.

Lectin affinity HPLC

The lectin-immobilized gels were suspended in 0.01 *M* phosphate buffer containing 0.15 *M* sodium chloride and packed into 250 × 4.6 mm I.D. columns by the slurry packing method at a flow-rate of 1.5 ml/min, using the same buffer. Lectin affinity HPLC was carried out using the Shimadzu LC-6A HPLC system. The elution buffer was 0.01 *M* phosphate buffer (pH 6.2) and the flow-rate was 0.5 ml/min. All chromatographic analyses were conducted at room temperature and the effluent was monitored at 261 nm.

RESULTS AND DISCUSSION

Separation of RCA60 and RCA120

The separation of RCA60 and RCA120 is usually carried out by gel filtration and ion-exchange chromatography. We utilized hydrophobic chromatography with

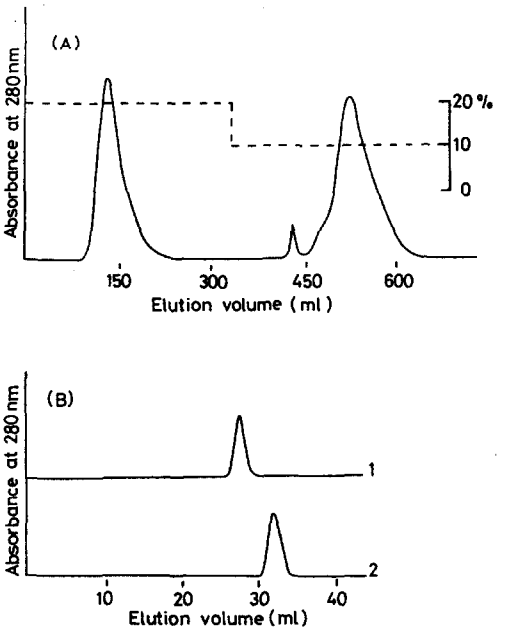

Fig. 1. Separation of RCA60 and RCA120. (A) A mixture of RCA60 and RCA120 (130 mg/10 ml) was applied to the Butyl-Toyopearl 650M column (40 × 2.6 cm). Two step gradients of 20 to 10% saturated ammonium sulphate (dotted line, right-hand scale) in 0.02 *M* phosphate buffer were used and the effluent was monitored at 280 nm. (B) FPLC using columns of Superose 6 and 12 connected in series. The elution buffer was 0.15 *M* sodium chloride in 0.01 *M* phosphate buffer (pH 7.4) containing 0.1 *M* galactose. Curves: (1) the first major fraction from the Butyl-Toyopearl column; (2) the second major fraction from the Butyl-Toyopearl column.

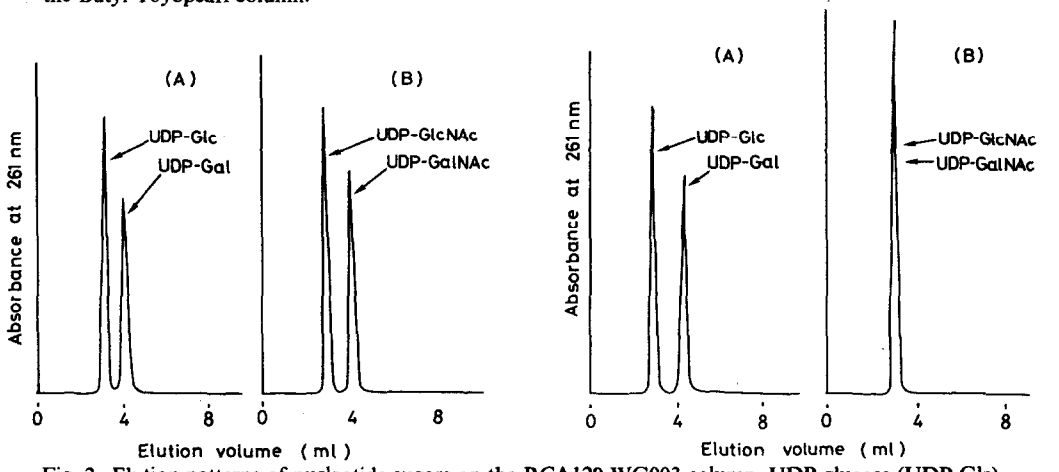

Fig. 2. Elution patterns of nucleotide sugars on the RCA120-WG003 column. UDP-glucose (UDP-Glc) and UDP-galactose (UDP-Gal) (A) and UDP-N-acetyl-D-glucosamine (UDP-GlcNAc) and UDP-N-acetyl-D-galactosamine (UDP-GalNAc) (B) were injected into the RCA120-WG003 column. The concentration of nucleotide sugars was 0.05 mg/ml and the effluent was monitored at 261 nm.

Fig. 3. Elution patterns of nucleotide sugars on the RCA60-WG003 column. UDP-glucose and UDP-galactose (A) and UDP-N-acetyl-D-glucosamine and UDP-N-acetyl-D-galactosamine (B) were injected into the RCA60-WG003 column. Other details as in Fig. 2.

a Butyl-Toyopearl 650M column, and obtained a clear separation of the RCAs into two major fractions using two step gradients from 20 to 10% saturated ammonium sulphate in 0.02 *M* phosphate buffer (Fig. 1A). When the major fractions were examined by high-performance size-exclusion chromatography using the columns of Superose 6 and 12 connected in series, the fraction eluted with 20% saturated ammonium sulphate was found to be RCA120 and that eluted with 10% saturated ammonium sulphate was RCA60 (Fig. 1B). The capacity of Butyl-Toyopearl 650M for RCAs was *ca.* 1 mg/ml gel (data not shown). We thus conclude that hydrophobic chromatography using Butyl-Toyopearl 650M is useful for the large-scale separation of RCA60 and RCA120.

Separation of nucleotide sugars on RCA60 and RCA120 columns

The RCA120-WG003 affinity column could be used for the separation of UDP-glucose and UDP-galactose, UDP-N-acetyl-D-glucosamine and UDP-N-acetyl-D-galactosamine (Fig. 2), and the RCA60-WG003 affinity column for UDP-glucose and UDP-galactose, but not UDP-N-acetyl-D-glucosamine and UDP-N-acetyl-D-galactosamine (Fig. 3). A clear difference in the separation patterns of the nucleotide sugars between the RCA60 and RCA120 columns was noted.

The elution volumes of nucleotide sugars on the RCA60 and RCA120 column are shown in Table I. The elution volume of UDP-galactose was higher than those of the other three kinds of nucleotide sugars on RCA60, and the elution volumes of UDP-galactose and UDP-N-acetyl-D-galactosamine were higher than those of the other two kinds of nucleotide sugars on RCA120. However, the elution volumes of UDP-galactose and UDP-N-acetyl-D-galactosamine on the RCA120 column are almost the same. Thus, the RCA120 column cannot be used to separate these two compounds. Accordingly, we obtained only two peaks when a nucleotide sugar mixture was subjected to HPLC using one lectin affinity column.

Use of two lectin affinity HPLC columns for the resolution of nucleotide sugars

To obtain better resolution, RCA60-WG003 and RCA120-WG003 column were connected in series. Fig. 4 shows that this combination of columns can separate three kinds of nucleotide sugars, UDP-glucose (UDP-N-acetyl-D-glucosamine), UDP-N-acetyl-D-galactosamine and UDP-galactose.

Not only castor bean lectin, but also other plant lectins have different affinities toward nucleotide sugars. For example, concanavalin A (a well known plant lectin)

TABLE I

ELUTION VOLUMES (ml) OF NUCLEOTIDE SUGARS ON THE RCA-60-WG003 AND RCA120-WG003 COLUMNS

Nucleotide sugar	*Lectin affinity column*	
	RCA60	*RCA120*
UDP-Glc	3.09	3.06
UDP-GlcNAc	3.08	3.05
UDP-Gal	4.01	3.95
UDP-GalNAc	3.09	4.23

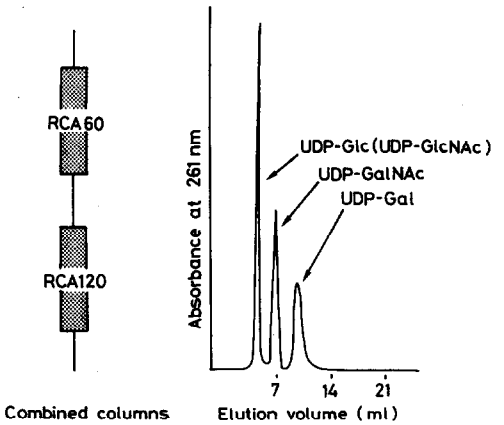

Fig. 4. Resolution of nucleotide sugars on two lectin columns. UDP-glucose (UDP-N-acetyl-D-glucosamine). UDP-N-acetyl-D-glucosamine and UDP-galactose were injected into lectin columns (RCA60 and RCA120) connected in series. Other details as in Fig. 2.

has affinity toward UDP-mannose. When a Con A HPLC column was prepared and a mixture of nucleotide sugars was applied to it. UDP-mannose was clearly separated from the others (data not shown). Using other kinds of lectin affinity HPLC columns, it should be possible to separate many kinds of nucleotide sugars. Moreover, lectin affinity HPLC together with other HPLC methods should provide new means for the resolution of nucleotide sugars.

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