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Note

Fractionation of peptic glycopeptides from chicken ovalbumin by reversed-phase high-performance liquid chromatography

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We reported previously that Dns-asparaginyl oligosaccharides from chicken ovalbumin can be fractionated by reversed-phase high-performance liquid chromatography (HPLC)[1,2]. However, it was not easy to prepare a glycopeptide containing only one amino acid from glycoprotein because of its incomplete pronase digestion.

We found that the use of pepsin instead of the pronase was more effective in the following respects: (1) it is easy to prepare homogeneous glycopeptides with respect to their peptide portion; (2) the peptic glycopeptides (PGP) could be analysed directly by HPLC without pre-labelling; and (3) the PGP was first separated rapidly (8 min) into several groups due to the differences in their peptide portions and then each group could be further separated into many components depending on their carbohydrate chain differences in a longer time (8 h).

The method elaborated allows us to investigate the microheterogeneity at individual glycosylation sites on each glycoprotein molecule separately.

EXPERIMENTAL

Materials

Con A/Sepharose was obtained from Pharmacia, pepsin (B grade) from Calbiochem and glycopeptidase A from Seikagaku Kogyo. Ovalbumin was prepared from pooled egg white by CM 52-cellulose column chromatography as reported[3]. All solvents for HPLC were filtered through a Toyo Roshi filter (pore size 0.45 μm), and all other reagents were of the highest grade available and were used without further purification.

Preparation of peptic glycopeptides (PGP) from chicken ovalbumin

Glycopeptide was obtained from ovalbumin by pepsin digestion as described previously[4]. Further purification of the PGP desalted by Sephadex G-50 was carried out by paper chromatography using the solvent system 1-butanol–acetic acid–water (3:1:1). The remaining at the origin was eluted with water.

Con A/Sepharose chromatography and glycopeptidase A treatment of PGP

Con A/Sepharose chromatography was carried out as described previously[4].

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The bound fraction was eluted stepwise from the column with 0.2 *M* α-methyl glycoside. Sephadex G-50 chromatography was used to remove salt or/and α-methyl glucoside from the passed PGP fraction and the bound PGP fraction.

Glycopeptidase A treatment was carried out by incubating 50 nmole of PGP in 50 μl of 0.1 *M* citrate–phosphate buffer (pH 5.0) at 37°C for 15 h with 0.04 milliunit of the enzyme.

High-performance liquid chromatography (HPLC)

The HPLC apparatus consisted of a Tri Rotar SR2 (Japan Spectroscopic) solvent delivery system, a Unisil packed ODS column (Gasukuro Kogyo) (5 μm; 25 cm × 4.6 mm I.D.) protected by a Brownlee Labs. MPLC guard column (30 × 4.5 mm I.D.), a Uvidec-100-IV (Japan Spectroscopic) UV spectrophotometer and a Unicorder U-228 recorder (Nippon Denshi Kagaku). HPLC analysis was carried out by applying PGP (10–20 nmole) directly to the column. The column was eluted with a linear gradient from 0 to 4.0% acetonitrile in 25 m*M* sodium borate buffer (pH 7.0) at a flow rate of 1.0 ml/min for 8 h. The effluent was monitored by a UV monitor (absorbance at 213 nm). For isocratic elution, a Radial packed C_{18} cartridge (10 μm; 100 × 8 mm I.D.) equipped with a Z module (Waters Assoc.) was eluted with 6.0% acetonitrile in 25 m*M* borate buffer.

RESULTS AND DISCUSSION

HPLC analysis of peptic glycopeptides from chicken ovalbumin

Ovalbumin glycopeptide (PGP) was separated by isocratic HPLC into two peaks, showing both UV absorption and the presence of hexose (Fig. 1). Hexose in the effluent was monitored by the phenol–sulphuric acid reaction. The two peaks, PGP-A and N, correspond to the peaks PGP-A 1–7 and PGP-N 1–8, respectively, which were separated by linear gradient elution (Fig. 2A). Treatment of the PGP

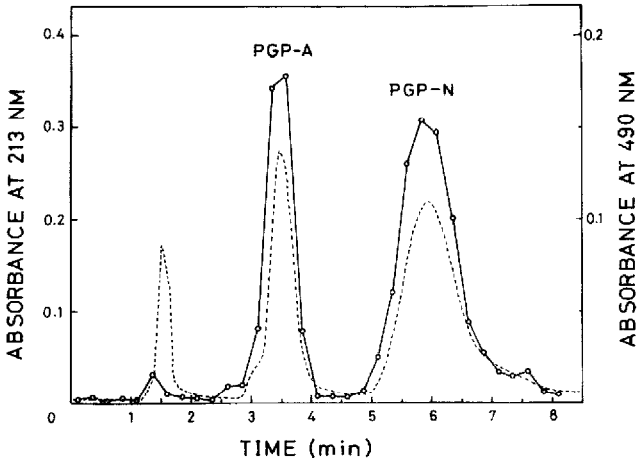

Fig. 1. HPLC analysis of peptic glycopeptides from chicken ovalbumin. About 240 nmole of PGP were fractionated by HPLC under the isocratic elution conditions as described in the text. Broken line, UV absorption (213 nm); solid line, phenol–sulphuric acid reaction (490 nm).

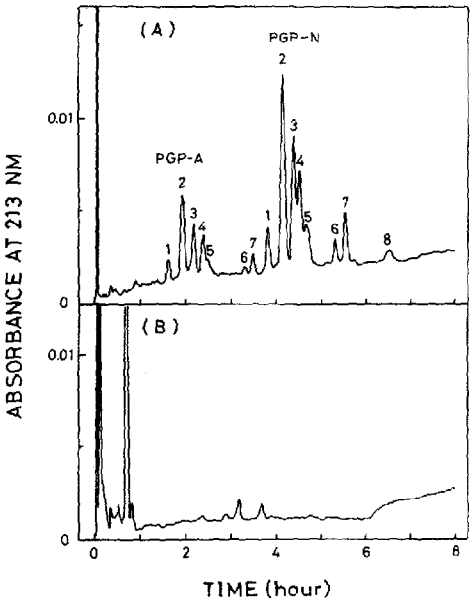

Fig. 2. HPLC analyses of PGP and glycopeptidase A-treated PGP. PGP (20 nmole) was fractionated by HPLC under the gradient elution condition described in the text. (A) PGP; (B) PGP treated with glycopeptidase A.

with glycopeptidase A resulted in the disappearance of all those peaks and the production of the two faster eluted peptide peaks as shown in Fig. 2B. Among above glycopeptide peaks, PGP-A 7,8 and PGP-N 7,8 were recovered as the unbound fraction and the remaining peaks as the fraction bound to Con A/Sepharose (Fig. 3). PGP-A 8 would probably be hidden by the PGP-N 3 and 4 peaks in Fig. 2A.

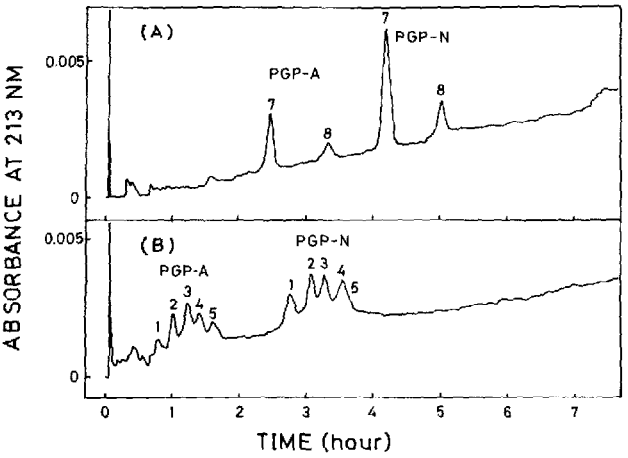

Fig. 3. HPLC analyses of PGP fractions separated by Con A/Sepharose chromatography. (A) The unbound PGP fraction and (B) the PGP fraction bound to the Con A/Sepharose column were analysed by HPLC under the same conditions as in Fig. 2.

These results indicate that the ovalbumin glycopeptide was first separated into two components due to the difference in their peptide portions. These peptides might correspond to the acidic and neutral peptides as reported by Ishihara *et al.*[5].

Each component can be further separated into eight components, depending on their carbohydrate chain difference in a longer analysis (8 h). Correspondence between the elution profiles of PGP-A and PGP-N showed that the microheterogeneity at the two glycosylation sites could not be distinguished.

As the elution condition for PGP was similar to that for Dns-glycopeptide (Dns-GP) and the peptide portion of glycopeptide does not seem to affect the order of their elution from the reversed-phase column as indicated in Fig. 2A, we can speculate that PGP-1, -2, -3, -4, -5, -6, -7 and -8 correspond to the reported components 1, 2, 3, 4, 5, 6 plus 7, 8 plus 9 and 11 plus 12 for Dns-GP, respectively[1]. The fact that Dns-GP 8–12 were derived from the unbound ovalbumin fraction OA[6] supports above suggestion.

As indicated here, this method should be useful for examining the microheterogeneity at individual glycosylation sites in glycoproteins.

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