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High-performance affinity chromatography of concanavalin A

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ABSTRACT

D-Glucose was immobilized as a form of maltamyl group on Asahipak GS-520, a synthetic polymer-type gel, and employed as an affinity adsorbent for high-performance affinity chromatography. The method proved useful for the rapid fractionation of tetravalent and divalent molecular species contained in the usual preparations of concanavalin A. It also afforded an adequate means for the efficient purification of a monovalent derivative of concanavalin A produced by a photochemical reaction.

INTRODUCTION

Even in a conventional form, affinity chromatography shows high efficiency for the rapid purification of substances that can interact with a certain biospecific affinity adsorbent from non-interacting impurities. However, when the purpose is to fractionate a series of substances with similar but slightly different affinities toward the same adsorbent, the use of high-performance affinity chromatography (HPAC) has many merits. This paper deals with the application of HPAC to the fractionation of tetravalent and divalent molecular species of concanavalin A (Con A), a lectin with specific binding ability toward the α-D-manno- or α-D-glucopyranoside residues of polysaccharides. The utility of HPAC is further illustrated in the isolation of a monovalent Con A derivative from a complex mixture produced from native Con A by photochemical reaction.

EXPERIMENTAL

Affinity adsorbent for HPAC

The matrix employed for the preparation of the adsorbent was Asahipak GS-520 (particle size 9 ± 0.5 μm), a hard gel of a synthetic polymer type developed for high-performance gel permeation chromatography by Asahi Chemical (Tokyo, Japan). D-Glucose was immobilized on the matrix mainly by the method of Matsumoto *et al.*[1], as follows. Asahipak GS-520 (2 g of dry powder) was added to a mixture of epichlorohydrin (1.1 ml), 2 *M* sodium hydroxide solution (4.7 ml) and

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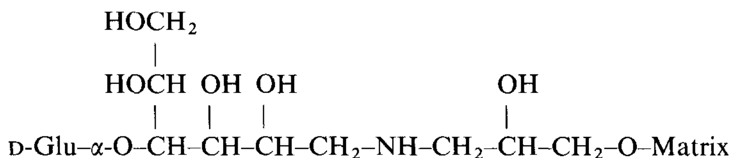
dimethyl sulphoxide (10.9 ml) and shaken for 15 h at 30°C. The activated gel was washed with water. Amino groups were introduced to the gel by shaking in 28% ammonia solution (10 ml) for 1.5 h at 40°C. Amino-Asahipak was then treated with maltose (189 mg) and $NaBH_3CN$ (92.4 mg) in 0.2 M K_2HPO_4 (5.44 ml) for 33 days at room temperature. Maltose was immobilized on the gel as a form of maltamyl group with the following structure:



Remaining amino groups on the gel were blocked by acetylation with acetic anhydride. Maltamyl-Asahipak GS-520 thus prepared was found to contain 35–40 μmol of immobilized glucose per gram of dry gel, when assayed by the Park and Johnson method[2] after hydrolysis in 0.5 M sulphuric acid at 100°C for 4 h. It was later found that maltamyl-Asahipak with almost the same glucose content could be obtained in about 20 h if amino-Asahipak was allowed to react at 80°C with maltose and $NaBH_3CN$.

Maltamyl-Asahipak was suspended in 0.01 M Tris–HCl buffer (pH 7.4) containing 0.1 M NaCl, 2 mM $MnCl_2$ and 2 mM $CaCl_2$ and packed in a stainless-steel column, which was then connected to a Tosoh HLC-803D high-performance liquid chromatograph equipped with a GE-4 gradient unit and a UV-8 Model II spectrophotometer.

Concanavalin A

Con A was extracted from finely ground powder of jack bean seeds (Sigma, St. Louis, MO, U.S.A.) and purified according to the method of Agrawal and Goldstein[3]. This preparation is called whole Con A. Tetravalent and divalent molecular species (α-Con A and β-Con A, respectively) were separated from whole Con A by affinity chromatography on Sephadex G-100 as described previously[4] or on maltamyl-Asahipak as described below.

Photochemical reaction to produce monovalent Con A

The reaction was carried out as reported previously[5]. Briefly, α-Con A (100 mg), chloroacetamide (468 mg) and methyl-α-D-mannopyranoside (180 mg) were dissolved in 100 ml of 0.01 M Tris–HCl buffer (pH 7.4) containing 1.0 M NaCl, 10 mM $MnCl_2$ and 10 mM $CaCl_2$. The solution was irradiated with a 200-W high-pressure mercury lamp through a filter of 1.0% $CuSO_4$ (1.2 cm pass) for a defined period under continuous bubbling with argon.

Amino acid composition analysis

Proteins were hydrolysed with 4 M methanesulphonic acid containing 0.2% 3-(2-aminoethyl)indole at 115°C for 24 h in evacuated tubes and subjected to composition analysis on a Hitachi 835 amino acid analyser.

RESULTS AND DISCUSSION

Con A, a typical lection, has been widely used as a probe for cell surface analyses and as a modulator of cell functions. Most of the cell biological studies were carried out with commercial Con A specimens. All the specimens, including that prepared by the method of Agrawal and Goldstein[3], are mixtures of at least two molecular species with different sugar-binding valencies[6,7] and the mixing ratio of the two is variable from one preparation to the other. The first Con A species is a tetravalent tetramer composed of homologous "intact subunit", with a molecular weight of 102 000 at physiological pH[8]; we call it α-Con A. The second species, β-Con A, consists of two pairs of "split subunits". These subunits look as if they have been formed by a single cleavage at a central portion of the polypeptide chain of the intact subunit. The molecular weight and the sugar-binding valency of β-Con A are half those of α-Con A. Many studies have suggested that the effect of lectins on various cell functions alters depending on the binding valency of lectin molecules toward cell surface receptors[9–13]. Therefore, the use of homogeneous Con A preparations with the defined sugar-binding valency may be strongly recommended for future studies in cell biology and other fields.

Separation of α-Con A and β-Con A

Fractionation of α- and β-Con A in a whole Con A preparation has usually been carried out by selective elution with a concentration gradient of D-glucose from a column of Sephadex G-100, which has D-glucose residues at the non-reducing termini, as a specific bioaffinity adsorbent[4]. However, this affinity chromatography is very time consuming because Sephadex is a soft gel and does not tolerate high pressure to allow rapid elution.

HPAC on maltamyl-Asahipak was therefore tried in order to achieve an effective fractionation of the Con A species. A whole Con A preparation was applied to a column (7.5 × 0.75 cm I.D.) of this adsorbent and eluted at a flow-rate of 1 ml/min by a linear gradient of D-glucose concentration from 0 to 0.1 *M* under the conditions described in the legend of Fig. 1. As shown in Fig. 1, the divalent and tetravalent Con A were well separated from each other within only 14 min. The efficiency of the column is in marked contrast to that of a conventional Sephadex column, which requires more than 24 h to separate the two species with the same resolution as in Fig. 1. The sample size used in this experiment was only 50 μg, but even 10 mg of an α- and β-Con A mixture were found to be similarly well separated with the same maltamyl-Asahipak column.

It was thought at first that high-performance gel permeation chromatography should be utilizable for the separation of α-Con A from β-Con A, because there is a large molecular-weight difference between them. However, the two Con A species were eluted closely together, either from an Asahipak GS-510 column (Asahi Chemical) (50 × 0.76 cm I.D.) or from a TSKgel G-3000SW column (Tosoh) (60 × 0.75 cm I.D.).

The ability of these columns to act as high-performance molecular sieves seems to have been adversely affected by some interaction of the column matrices with Con A. Thus HPAC by maltamyl-Asahipak appears to be the best means of separating Con A species at least for analytical purposes.

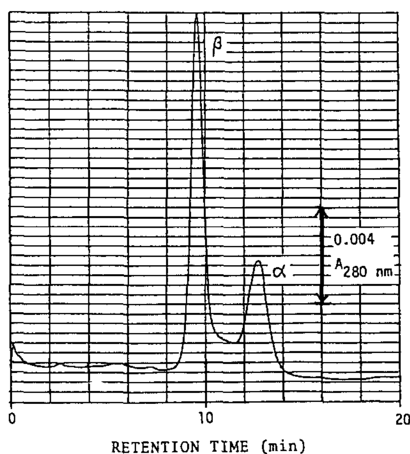


Fig. 1. Separation of α-Con A and β-Con A by affinity chromatography on maltamyl-Asahipak. The sample dissolved in 1 ml of 0.01 *M* Tris–HCl buffer (pH 7.4) containing 0.1 *M* NaCl, 2 m*M* $MnCl_2$ and 2 m*M* $CaCl_2$ was loaded on a column (7.5 × 0.75 cm I.D.) of the adsorbent equilibrated with the same buffer and eluted at a flow-rate of 1 ml/min by increasing the concentration of D-glucose in the same buffer linearly from 0 to 0.1 *M* in 15 min. The column operation was carried out at room temperature.

HPAC separation of monovalent Con A

The utility of the maltamyl-Asahipak column is further demonstrated by the following example of a more complicated system. We have previously reported that α-Con A can be converted into a monomeric monovalent form (m-Con A) by a light-induced alkylation reaction with chloroacetamide[5]. The reaction affords a complex mixture involving unfavourable products. In the previous work, m-Con A present in the reaction mixture was isolated by affinity chromatography on a column of Sephadex G-100 from various side-reaction products by a glucose-concentration gradient elution for 3 days (see Fig. 1 in ref. 5). The m-Con A thus purified has been widely used in cell biological studies as a valuable tool which can bind to a cell surface receptor but does not make a cross-link between them[9–13].

HPAC on maltamyl-Asahipak was successfully applied to the rapid isolation of m-Con A. Fig. 2 indicates the results of this experiment, in which two columns of the adsorbent connected in tandem were used. Elution was carried out by raising the glucose concentration from 0 to 0.1 *M* within 30 min at a flow-rate of 0.5 ml/min. Fig. 2A shows the chromatogram for the reaction product obtained by mercury-lamp irradiation of α-Con A in the presence of chloroacetamide for 20 min, and Fig. 2B shows that for the product obtained by 80-min irradiation. In both instances the reaction mixtures were separated into five peaks within 40 min. The material in peak I was identical with the desired Con A derivative, which was confirmed to have monovalent binding features and monomeric molecular weight. The molecular weight was determined by the combined use of high-performance gel permeation chromatography (on a column of Asahipak GS-510) and the low-angle laser light-scattering technique according to Takagi[14]. Peaks 0, II, III and IV seem to consist of inactivated, divalent, trivalent and tetravalent materials, respectively. The presence of the trivalent material was not observed in our previous work with chromatography on a Sephadex G-100 column[5], indicating the superior resolution of the maltamyl-Asahipak column.

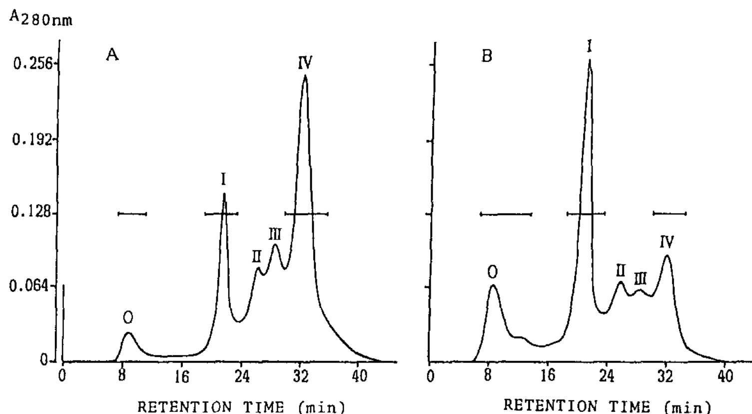


Fig. 2. Separation of photoalkylation products from α-Con A,by affinity chromatography on maltamyl-Asahipak. α-Con A (0.8 mg in 1 ml) after irradiation with chloroacetamide under the conditions described under Experimental for (A) 20 min or (B) 80 min was loaded on the adsorbent, which was packed in two columns (each 7.5 × 0.75 cm I.D.) connected tandem. Elution was carried out by raising the glucose concentration linearly from 0 to 0.1 *M* in 30 min at a flow-rate of 0.5 ml/min. Other chromatographic conditions as in Fig. 1. Peaks 0, I, II, III and IV consist of inactivated, monovalent, divalent, trivalent and tetravalent materials, respectively, derived from α-Con A.

The HPAC method was also useful for a quick survey of the reaction conditions most suitable for the formation of m-Con A and for the characterization of this derivative. The yields of m-Con A and other products were calculated from the chromatogram and plotted against the reaction times (Fig. 3). The results suggest that a 60-min reaction is sufficient for the formation of m-Con A. The amino acid compositions of m-Con A and a tetravalent material isolated from the reaction mixtures obtained after various irradiation times were compared with that of native α-Con A. The differences in the compositions, which are regarded as the contents of modified amino acid residues in the products, are plotted in Fig. 4. Comparison of these contents clearly indicates that excessive modification of the polypeptide chain at

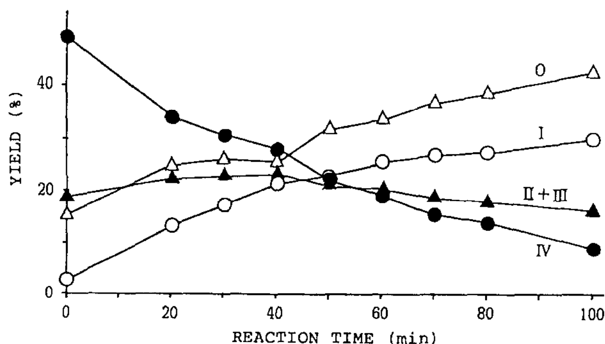


Fig. 3. Yields of tetravalent, di- + trivalent, monovalent and inactivated materials separated by affinity chromatography on maltamyl-Asahipak, as shown in Fig. 2, from α-Con A after photoreaction with chloroacetamide for various times.

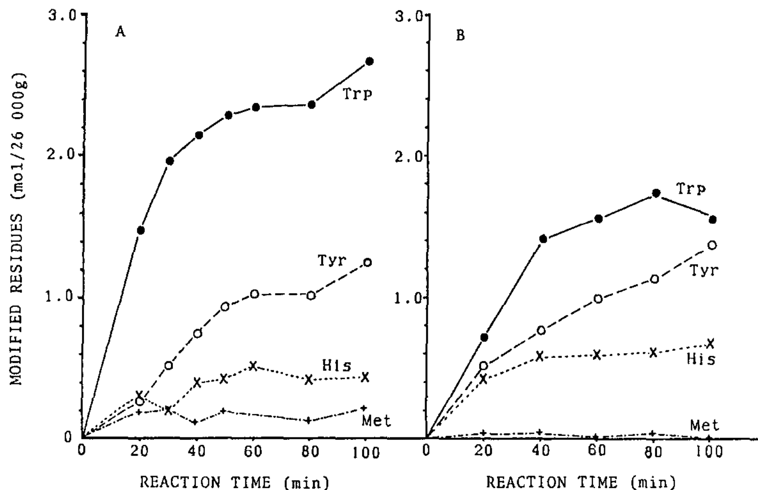


Fig. 4. Amounts of modified amino acids determined in (A) a monovalent material (= m-Con A) and (B) a tetravalent material isolated as shown in Fig. 2 from α-Con A after photoreaction for various times.

one tryptophan residue may be responsible for the conversion of α-Con A into the monomeric form. This result is consistent with our previous proposal[12] on the importance of a tryptophan residue for monomer–monomer interactions in Con A.

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REFERENCES

1 I. Matsumoto, H. Kitagaki, Y. Akai, Y. Ito and N. Seno, *Anal. Biochem.*, 116 (1981) 103.
2 J. T. Park and M. J. Johnson, *J. Biol. Chem.*, 181 (1949) 149.
3 B. B. L. Agrawal and I. J. Goldstein, *Biochim. Biophys. Acta*, 147 (1967) 262.
4 Y. Oda, K. Kasai and S. Ishii, *J. Biochem.*, 89 (1981) 285.
5 I. Tanaka, Y. Abe, T. Hamada, O. Yonemitsu and S. Ishii, *J. Biochem.*, 89 (1981) 1643.
6 Y. Abe, M. Iwabuchi and S. Ishii, *Biochem. Biophys. Res. Commun.*, 45 (1971) 1271.
7 J. L. Wang, B. A. Cunningham and G. M. Edelman, *Proc. Natl. Acad. Sci. U.S.A.*, 68 (1971) 1130.
8 G. N. Reeke, Jr., J. W. Becker and G. M. Edelman, *J. Biol. Chem.*, 250 (1975) 1525.
9 K. Itaya, M. Itoh, I. Tanaka and S. Ishii, *Biomed. Res.*, 2 (1981) 501.
10 H. Suya, Y. Abe, I. Tanaka, S. Ishii and K. Itaya, *J. Biochem.*, 92 (1982) 1251.
11 M. Saito, F. Takaku, M. Hayashi, I. Tanaka, Y. Abe, Y. Nagai and S. Ishii, *J. Biol. Chem.*, 258 (1983) 7499.
12 S. Ishii, Y. Abe, I. Tanaka and M. Saito, *J. Protein Chem.*, 3 (1984) 63.
13 M. Kuroki, N. Kamo, Y. Kobatake, Y. Abe and S. Ishii, *Cell Struct. Funct.*, 11 (1986) 199.
14 T. Takagi, *J. Biochem.*, 89 (1981) 363.