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Short communication

Rapid separation of peptides and proteins on 2-μm porous microspherical reversed-phase silica material

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Abstract

The fast LC of peptides and proteins on a reversed-phase column packed with 2-μm porous silica microspherical silica gel (TSKgel Super-ODS, 50 mm × 4.6 mm I.D.) was investigated. This short column packed with small particles showed high resolutions for peptides and proteins with high yields (over 80%). The relationship between the sample amount and peak area of a basic peptide indicated good linearity over a wide range of sample amount applied (1–500 ng). In comparison of the chromatograms of a tryptic digest obtained on porous and non-porous columns, many sharp peaks were recognized on the Super-ODS column owing to its high hydrophobicity. Using a short gradient time and high flow-rate, a peptide mixture could be separated successfully within 1 min.

Keywords: Stationary phases, LC; Peptides; Proteins

1. Introduction

High-performance reversed-phase liquid chromatography (RPLC) has been widely used for analysing peptides and proteins on columns packed with 5-μm porous packing materials. On the other hand, many researchers have also demonstrated the usefulness of non-porous packing materials based on resin and silica gels for separating macromolecules such as peptides, proteins and polynucleic acids in various separation modes, e.g., ion-exchange (IEC) [1–5], reversed-phase (RPC) [6–13] and hydrophobic interaction chromatography (HIC) [14,15].

The advantages of such non-porous columns are that they enable one (1) to check the peak band expansion in pores and (2) to utilize microspheres with high mechanical durability. However, these non-porous columns are not appropriate for analysing hydrophilic compounds because of their low adsorption abilities in comparison with conventional porous columns.

Fast LC on porous microspherical packings (particle size ca. 3 μm) has been reported for separating compounds such as amino acid derivatives [16], peptides and proteins [17]. These small porous packings showed excellent resolution. Danielson and Kirkland [18] reported the merits of the use of a smaller RPC gel, based on a porous silica gel (particle diameter 1.5 μm) for the separation of macromolecules.

Recently, a new reversed-phase column, TSKgel Super-ODS, based on 2-μm porous silica gel became commercially available [19]. We report here fast peptide and protein separations with this column compared with non-porous and porous conventional RPC columns.

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2. Experimental

2.1. Instrumentation

Chromatography was carried out on a Model SC-8020 HPLC system controller, equipped with a CCPM-II computer-controlled dual pump, an MX-8010 dynamic mixer (inner volume 100 μl), an AS-8020 autosampler and a UV-8020 variable-wavelength UV detector with a 2-μl microflow cell (time constant $\nu = 50$ ms) (all from TOSOH, Tokyo, Japan). The sampling time for data processing was 50 ms.

2.2. Chemicals and materials

The HPLC columns employed were TSKgel Super-ODS (50 mm × 4.6 mm I.D.), based on 2.3-μm porous silica gel having a 12-nm pore diameter, TSKgel Octadecyl-NPR (35 mm × 4.6 mm I.D.), based on 2.5-μm non-porous hydrophilic resin, TSKgel Octadecyl-2PW (150 mm × 4.6 mm I.D.), based on 5-μm porous hydrophilic resin having a 20-nm pore diameter), and TSKgel ODS-80Ts (150 mm × 4.6 mm I.D.), based on 5-μm porous silica having an 8-nm pore diameter (all from TOSOH).

Acetonitrile was of HPLC grade from Kanto Chemical (Tokyo, Japan). Deionized water was prepared with a Milli-Q water-purification system (Millipore, Bedford, MA, USA) and perchloric acid was purchased from Wako (Osaka, Japan). All proteins and peptides were obtained from Sigma (St. Louis, MO. USA).

The sample recoveries were calculated from the ratios of the peak area of the blank injection without the column and those with the column.

A tryptic digest of β-lactoglobulin was prepared by mixing a protein solution [50 mg of the protein in a 25-ml portion of 50 m*M* phosphate buffer (pH 7.0)] and 1 mg of trypsin at 37°C for 4 h with shaking and then adding 0.5 ml of 0.1 *M* HCl. A 2-μl portion of the solution was directly applied to the ODS columns.

3. Results and discussion

3.1. Recovery

The use of a fully end-capped RPC gel based on purified silica gels and rapid separation lead to high yields as ionic interactions between the solutes and the packing surface and denaturation of protein samples are avoided. The concentration of ligands on the Super-ODS gel was 3.58 μmol/g gel and the metal content was below 1 ppm for Fe, Al and Cu. Table 1 gives the recoveries of peptides and proteins when 500-ng of each sample were loaded on the TSKgel Super-ODS column. The peptides and proteins were recovered with high yields from the Super-ODS column and also from non-porous and

Table 1
Recoveries of peptides and proteins on TSKgel Super-ODS

Peptide/Protein	Molecular mass	Recovery (%)	
		Super-ODS	Octadecyl-NPR
Bradykinin	(1060.2)	99	89
Angiotensin I	(1296.5)	104	103
Leu-Enkephalin	(555.6)	102	99
Cytochrom c	(12327)	89	95
Ribonuclease A	(13700)	90	94

Column: TSKgel Super-ODS (50 × 4.6 mm I.D.); eluent: 13 m*M* $HClO_4/CH_3CN$; 10-min linear gradient of CH_3CN from 10% to 80% was employed at a flow-rate of 1.5 ml/min; sample, 0.5 mg of each sample were injected; detection, UV 215 nm. The data on TSKgel Octadecyl-NPR were cited from Ref. [12] with permission.

porous columns [3,20]. Good linearity between the sample amount (x, ng) and peak area (y, arbitrary units), for bradykinin ($y = -0.72 + 0.37x$, $r^2 = 0.9997$) was obtained over a wide range of the loaded amounts (1–500 ng).

3.2. Sample loading capacity

Maximum sample capacity is in proportion to the column length [21] (equal to the gel volume) or effective pore volume for a given protein size. This is the main reason why packings having a larger pore size (more than 30 nm) are recommended for analysing large molecules such as proteins. Fig. 1 illustrates the chromatograms of crude hexokinase (10 and 800 μg applied). The resolution between the two main peaks decreased from 33.28 (10 μg) to 16.56 (800 μg), and to 7.19 with a 1.5-mg loading.

3.3. Comparison of elution profiles on various RPC packings

Fig. 2 shows a comparison of the elution profiles of a mixture of proteins on various RPC columns. Table 2 summarizes the resolution and

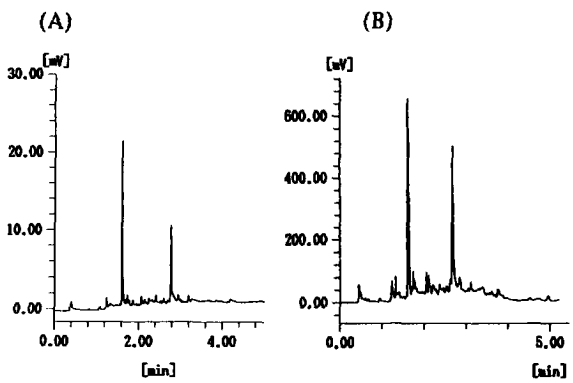

Fig. 1. Chromatograms of crude hexokinase obtained on a TSKgel Super-ODS column. Conditions: column, 50 mm × 4.6 mm I.D., eluent, 13 mM $HClO_4$–CH_3CN with a 10-min linear gradient of CH_3CN from 20% to 80%; sample, crude hexokinase (yeast), (A) 10 and (B) 800 μg; flow-rate, 1.5 ml/min; detection, UV at 220 nm.

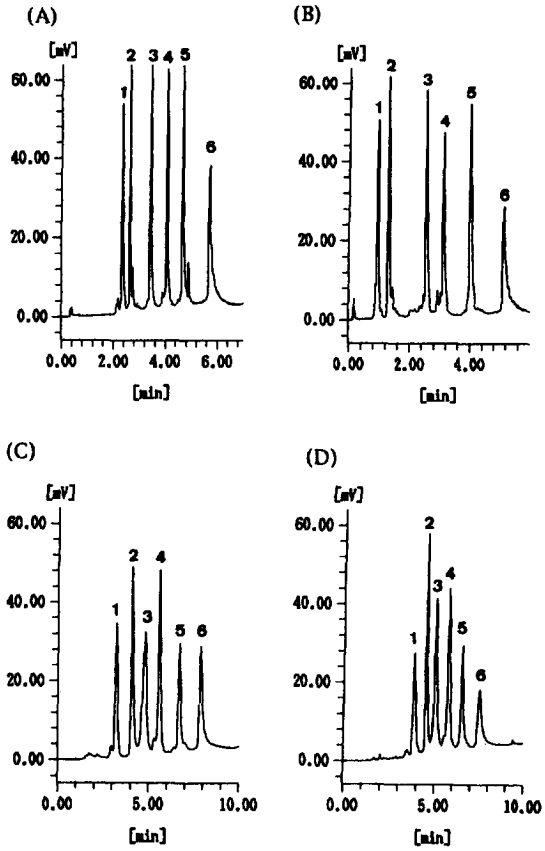

Fig. 2. Comparison of the elution profiles of the protein mixture on various RPC columns: (A) TSKgel Super-ODS (50 mm × 4.6 mm I.D.); (B) TSKgel Octadecyl-NPR (35 mm × 4.6 mm I.D.); (C) TSKgel Octadecyl-2PW (150 mm × 4.6 mm I.D.); (D) TSKgel ODS-80Ts (150 mm × 4.6 mm I.D.). Eluent: 13 mM $HClO_4$–CH_3CN: (A) and (B) 10-min linear gradients of CH_3CN from 20% to 68%; (C) and (D) 15-min linear gradients of CH_3CN. Sample: 1 = ribonuclease A; 2 = insulin; 3 = cytochrome c; 4 = lysozyme; 5 = β-lactoalbumin; 6 = myoglobin. A 0.5-μg amount of each protein was applied. Flow-rate, (A) and (B) 1.5; (C) and (D) 1.0 ml/min; detection, UV at 220 nm.

separation factors between the solutes obtained from Fig. 2. The chromatograms were obtained under the optimized conditions which gave the best results on each RPC column. Higher resolutions were obtained on the Super-ODS and Octadecyl-NPR columns than on the Octadecyl-2PW and ODS-80Ts columns. The smaller pore size (8 nm) of ODS-80Ts and the larger particle size of Octadecyl-2PW seem to be the main

Table 2
Comparison of resolution and separation factors of peptides and proteins on various RPC packings

Sample No.	TSKgel Super-ODS		TSKgel Octadecyl-NPR		TSKgel Octadecyl-2PW		TSKgel ODS-80Ts	
	R_s	α	R_s	α	R_s	α	R_s	α
1/2	3.32	1.15	2.97	1.46	3.87	1.51	3.08	1.28
2/3	9.56	1.37	10.58	2.12	2.36	1.28	2.46	1.17
3/4	6.14	1.21	4.81	1.25	3.15	1.24	3.03	1.21
4/5	6.71	1.17	6.48	1.30	4.46	1.27	3.28	1.18
5/6	8.92	1.25	7.48	1.30	4.20	1.23	3.17	1.19

Chromatographic conditions as described in Fig. 2.

reasons for their poorer results. On the other hand, Super-ODS and Octadecyl-NPR columns gave higher resolutions owing to the smaller particle sizes (2.3 and 2.5 μm, respectively).

3.4. Fast separation of peptides and proteins

Fig. 3 shows the chromatogram of the tryptic digest of β-lactoglobulin on the Super-ODS and Octadecyl-NPR columns. Although hydrophilic fragments on Super-ODS were sufficiently retained and effectively separated, they were eluted at the void volume from the Octadecyl-NPR column without retention. Higher hydrophobicity is another advantage of porous RPC packings.

Fig. 4 demonstrates the fast separation of a mixture of standard peptides on the short Super-ODS column within 1 min.

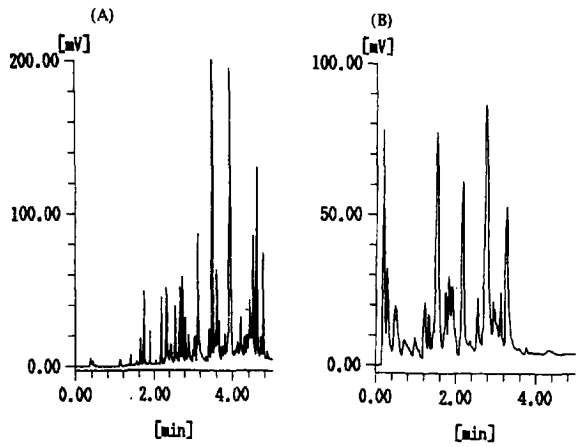

Fig. 3. Chromatograms of the tryptic digest of β-lactoglobulin on TSKgel Super-ODS and Octadecyl-NPR columns: (A) TSKgel Super-ODS (50 mm × 4.6 mm I.D.); (B) TSKgel Octadecyl-NPR (35 mm × 4.6 mm I.D.). Eluent: 13 m*M* $HClO_4$–CH_3CN with 10-min linear gradients of CH_3CN from 0 to 80%. Sample, 2-μl-paortions of the tryptic digest of β-lactoglobulin were applied; flow-rate, 1.5 ml/min; detection, UV at 220 nm.

4. Conclusion

Peptide and protein samples can be effectively and rapidly separated with high yields on a porous microparticle RPC column which possesses a high surface coverage and small particle

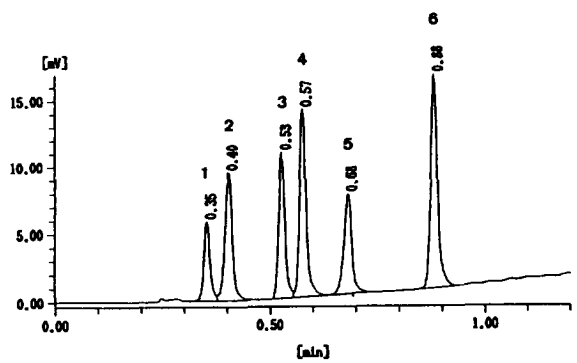

Fig. 4. Fast separation of the peptide mixture on a TSKgel Super-ODS column. Conditions: column, 50 mm × 4.6 mm I.D.; eluent, 13 m*M* $HClO_4$–CH_3CN, with a 2-min linear gradient of CH_3CN from 23% to 56%. Sample: 1 = oxytocin; 2 = α-endorphin; 3 = bombesin; 4 = leu-enkephalin; 5 = γ-endorphin; 6 = somatostatin. Flow-rate, 2 ml/min; detection, UV at 220 nm.

size. The advantages of the use of the small porous particles and a short column are the possibilities of separating hydrophilic peptide fragments and of applying large amounts of samples in comparison with non-porous RPC columns.

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