Journal of Chromatography, 481 (1989) 391–396
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 750

Note

Recovery of proteins and peptides with nanogram loads on non-porous packings

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(First received May 17th, 1989; revised manuscript received June 23rd, 1989)

Non-porous packings were introduced by Horvath *et al.*[1] in 1967 and the operational variables were studied in detail for packings with fairly large particle sizes (*ca.* 50 μm in diameter)[2,3]. A major advantageous property of non-porous packings, *i.e.*, the absence of sample diffusion in pores that occur in porous packings, leads to rapid separation with high efficiency and resolution. In the last decade, the technology of packings for high-performance liquid chromatography (HPLC) has greatly advanced so that non-porous microparticles (1.5–7 μm in diameter) have been developed enabling the rapid separation of proteins and peptides[4–18] and nucleic acids[19,20] within 10 min.

Recently, it has become important to separate submicrogram amounts, *i.e.*, picomole or nanomole levels of proteins and peptides since quality control or on-line monitoring is required for invaluable biomolecules like recombinant products[21]. For this reason, the porous packings were examined as regards the quantitative recovery of nanogram amounts of proteins by reversed-phase chromatography (RPC)[22–25] and size-exclusion chromatography (SEC)[23,25] on microbore columns since the recovery is higher due to a smaller (packingweight)/(sample weight) ratio. Those columns, however, gave longer analysis times than conventional columns and, moreover, a sophisticated HPLC system equipped with a pump and a gradient mixer is necessary. In contrast to porous packings, another advantageous property of non-porous packings is the small surface area (<5 m^2/g), which indicates the high recovery of sample with small loads. Hence, Burke *et al.*[6] reported the quantitative recovery of protein from nanogram loads on non-porous cation exchangers of 7 μm in diameter.

More recently, non-porous spherical resins of 2.5 μm in diameter have become commercially available as packed columns of TSKgel DEAE-NPR and SP-NPR for ion-exchange chromatography (IEC) and Octadecyl-NPR for RPC. Kato *et al.*[11,18] reported the quantitative recovery of proteins and peptides from microgram loads on these columns. In this paper, the quantitative recovery of proteins and peptides, and micropreparative separation of enzyme from nanogram loads, on these columns are described.

EXPERIMENTAL

All chromatographic separations were performed at 25°C with a high-performance liquid chromatograph consisting of a CCPM pump (Tosoh, Tokyo, Japan),

0021-9673/89/$03.50

a Model 7125 sample injector (Rheodyne, Cotati, CA, U.S.A.) with a 50-μl sample loop, an UV-8000 detector (Tosoh) operated at 215 or 220 nm (0.04–0.32 a.u.f.s.) for RPC, or an FS-8000 fluorescence detector (Tosoh) operated at 280 nm for excitation and 340 nm for emission (attenuation 3–16) for IEC, and an FBR-2 recorder (Tosoh). A line-filter with an 0.45-μm membrane was installed between the pump outlet and the sample injector. The dead volumes between the dynamic gradient mixer (volume 1.6 ml) and the injector and those of the tubings were kept to a minimum.

Proteins between 25 and 500 ng (10 ng/μl) were separated by a 10-min linear gradient from 0 to 0.5 *M* NaCl in 20 m*M* Tris–HCl (pH 8.0) by IEC on TSKgel DEAE-NPR (Tosoh), or a 10-min linear gradient from 0 to 0.5 *M* sodium sulphate in 20 m*M* acetate buffer (pH 5.0) by IEC on SP-NPR (Tosoh). Proteins and peptides between 12.5 and 500 ng were separated by a 10-min linear gradient from 0 to 80% acetonitrile in 5 or 100 m*M* $HClO_4$, or the same gradient from 15 to 80% in 5 m*M* $HClO_4$. All separations were performed by an high-pressure gradient system at a flow-rate of 1.5 ml/min. All columns were 35 mm × 4.6 mm I.D. The mass recovery of samples was determined by measuring the peak area of the eluate since the UV absorption and fluorescence intensity are proportional to the sample concentration in the eluent. Hexokinase activity was determined according to the standard procedures[26].

Cytochrome *c* was digested with N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)–trypsin according to the standard procedures[27]. The protein (1 mg/ml) and TPCK–trypsin (5 mg/ml) were incubated at a substrate to enzyme ratio of 100:1 in 10 m*M* Tris–HCl (pH 8.3) containing 0.1 *M* sodium acetate and 0.1 m*M* calcium chloride at 37°C for 2 h. A 500-ng amount of the digest in 0.5 μl was directly injected into the column for RPC without terminating the reaction.

Ovalbumin (chicken egg) was obtained from Seikagaku (Tokyo, Japan) and mouse monoclonal antibody for human proline hydroxygenase (IgG_1) was from CosmoBio (Tokyo, Japan). Hexokinase was obtained from Oriental Yeast (Osaka, Japan) and all other proteins were from Sigma (St. Louis, MO, U.S.A.). All peptides were obtained from the Peptide Institute (Osaka, Japan).

RESULTS AND DISCUSSION

Table I summarizes the recovery of proteins and peptides with 400-ng loads obtained by IEC on TSKgel DEAE-NPR and SP-NPR, and by RPC on TSKgel Octadecyl-NPR. All proteins and peptides were recovered in high yields from submicrogram loads. The results obtained by IEC with 400-ng loads reveal are in good agreement with those obtained with 5-μg loads reported by Kato *et al.*[11], while IgG_1 showed slightly lower recovery.

Figs. 1 and 2 show the relationship between the sample loads and peak area of the eluate in the separation of proteins and peptides with less than 400-ng loads by IEC on TSKgel DEAE-NPR and SP-NPR, and by RPC on TSKgel Octadecyl-NPR. The sample loads showed good linearity with the peak area. As indicated in Table I, the recovery of the proteins from 400-ng loads were almost quantitative so that the proteins would also be recovered in high yields with more than 25-ng loads by IEC and with more than 12.5 ng by RPC, which suggests that the small surface area of non-porous resins contributes to the high recovery of the sample even when present in very small amounts.

TABLE I

RECOVERY OF PROTEINS AND PEPTIDES FROM 400-ng LOADS BY ION-EXCHANGE CHROMATOGRAPHY ON TSKgel DEAE-NPR AND SP-NPR AND BY REVERSED-PHASE CHROMATOGRAPHY ON TSKgel Octadecyl-NPR

Column	*Protein or peptide*	*Recovery (%)*
DEAE-NPR	Ovalbumin	103
	Soybean trypsin inhibitor	98
	IgG_1	89
SP-NPR	Lysozyme	94
Octadecyl-NPR	Ribonuclease A	95
	Insulin	94
	Cytochrome *c*	102
	Myoglobin	99
	Somatostatin	99
	Bradykinin	101
	Angiotensin I	106

As demonstrated above, it is evident that proteins and peptides can be recovered in high mass yields even with submicrogram loads. We also evaluated the recovery of enzymatic activity with submicrogram loads. Fig. 3 shows the micropreparative separation of crude hexokinase by IEC on TSKgel DEAE-NPR. The separation was

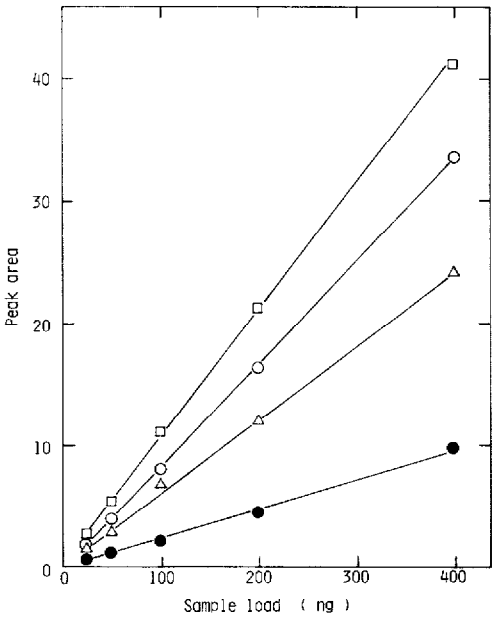

Fig. 1. Relationship between sample load and peak area of the eluate in the separation of proteins by IEC on TSKgel DEAE-NPR and SP-NPR. Proteins of ovalbumin (□), STI (○) and IgG_1 (△) were separated on TSKgel DEAE-NPR and lysozyme (●) was separated on TSKgel SP-NPR. Conditions as described in Experimental.

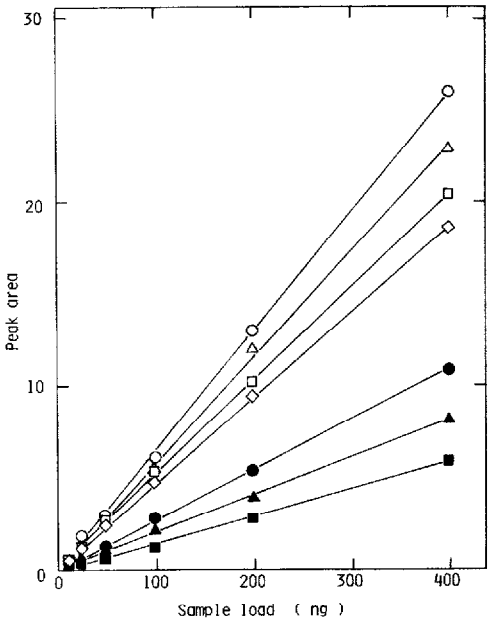

Fig. 2. Relationship between sample load and peak area of the eluate in the separation of proteins and peptides by reversed-phase chromatography on TSKgel Octadecyl-NPR. Proteins of ribonuclease A (○), cytochrome *c* (□), myoglobin (◇) and peptides of insulin (△), somatostatin (●), bradykinin (▲) and angiotensin I (■) separated on TSKgel Octadecyl-NPR. The conditions of chromatography were described in Experimental.

completed within 5 min and hexokinase was eluted at 4.1 min. The hexokinase fraction as indicated in the figure was collected with 80% recovery of hexokinase activity. The recovery of activity from the sample from 500-ng loads was fairly high, although that from 25 μg was slightly higher[11].

Fig. 4 shows the separation of proteins with 50-ng loads by RPC on TSKgel Octadecyl-NPR. The proteins were separated completely with high resolution within 6 min. Each peak was also sharp due to the small particle size (2.5 μm). Perchloric acid

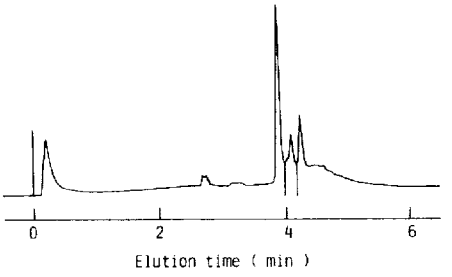

Fig. 3. Separation of hexokinase from nanogram loads by IEC on TSKgel DEAE-NPR. A 500-ng amount of crude hexokinase was separated by a 10-min linear gradient from 0 to 0.5 *M* NaCl in 20 m*M* Tris–HCl (pH 8.0) at a flow-rate of 1.5 ml/min. Recovery of activity was 80%.

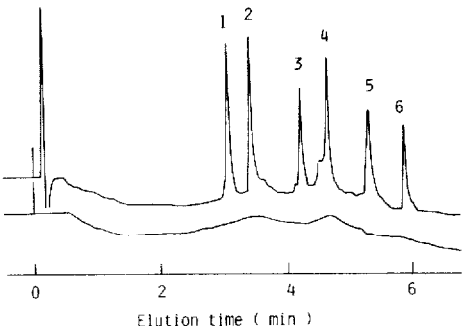

Fig. 4. Separation of a protein mixture from nanogram loads by RPC on TSKgel Octadecyl-NPR. A mixture containing 50 ng of each protein (1 = ribonuclease A; 2 = insulin; 3 = cytochrome *c*; 4 = lysozyme; 5 = transferrin and 6 = myoglobin) was separated by a 10-min linear gradient from 15 to 80% acetonitrile in 5 m*M* perchloric acid at a flow-rate of 1.5 ml/min and was detected by UV absorbance at 220 nm (0.04 a.u.f.s.). The trace of the blank gradient is also shown.

as a solvent seemed to give a better background compared with trifluoroacetic acid (TFA) when the submicrogram samples were separated by RPC[18].

Fig. 5 shows the monitoring of cytochrome *c* digested by TPCK–trypsin by RPC on TSKgel Octadecyl-NPR. The reaction mixture containing 500 ng proteins was directly injected into the column without termination of the reaction. Separation of the digest was completed within 7 min and many peptides derived from cytochrome *c* increased in peak height with time. This separation indicates rapid monitoring of enzymatic reaction and also suggests the application to peptide mapping of protein with submicrogram loads. Although Kalghatgi and Horvath[12] reported the same procedures previously with 10-μg sample loads, one-twentieth of the sample loads, *i.e.*, 500 ng was found to be enough for separation.

In conclusion, non-porous resins with 2.5-μm spherical particles yielded rapid separations of proteins and peptides with sharp peaks by IEC and RPC even though the sample loads were of the order of nanograms. Non-porous resin-packed columns

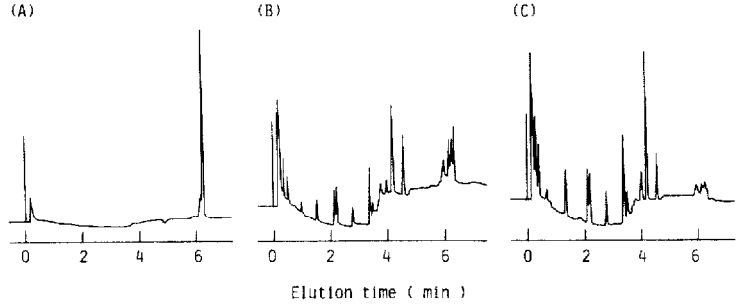

Fig. 5. Separation of tryptic digests of cytochrome *c* from nanogram loads by RPC on TSKgel Octadecyl-NPR. Cytochrome *c* was digested by TPCK–trypsin for 1 h (B) and 2 h (C). A 500 ng amount of cytochrome *c* (A) or the digests was separated by a 10-min linear gradient from 0 to 80% acetonitrile in 100 m*M* perchloric acid at a flow-rate of 1.5 ml/min and detected by UV absorbance at 215 nm on 0.32 a.u.f.s. in (A) and 0.08 a.u.f.s. in (B) and (C).

are applicable to conventional HPLC systems although microbore columns require sophisticated systems. Moreover, non-porous resins are chemically stalbe so that the separation by IEC at high pH, and column cleaning with sodium hydroxide, are possible although silica-based packings are unstable under such conditions. Non-porous resins, therefore are very useful not only for quality control, on-line monitoring, purity check of biomolecules like peptide mapping of recombinant products but also for micropreparative separation of active biomolecules.

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