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Note

High-performance ion-exchange chromatography of peptides on a pellicular ion exchanger

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Totally porous supports have commonly been employed in high-performance liquid chromatography (HPLC). On the other hand, pellicular supports, which were once explored in the early stages of HPLC but did not come into general use, have been drawing attention during the last few years particularly in the separation of biopolymers. After Unger and co-workers had shown that pellicular supports of small particle diameter (1.5 μm) are very useful for rapid separations of proteins[1–3], various types of pellicular supports were prepared and some of them have become commercially available. TSKgel DEAE-NPR and TSKgel SP-NPR are such supports for ion-exchange chromatography[4]. They were prepared by introducing diethylaminoethyl or sulphopropyl groups into non-porous spherical hydrophilic resins of 2.5 μm in diameter. We recently demonstrated that proteins[4], oligonucleotides[5] and DNA restriction fragments[6] could be separated rapidly with high resolution on these supports. We have now investigated the separation of peptides on SP-NPR and the results are presented here.

EXPERIMENTAL

Chromatographic measurements were performed at 25°C on a 35 mm × 4.6 mm I.D. stainless-steel column with a system consisting of a Model CCPM double-plunger pump and a Model UV-8000 variable-wavelength UV detector operated at 215 nm (TOSOH, Tokyo, Japan). All peptides were purchased from the Peptide Institute (Osaka, Japan), except insulin, which was obtained from Sigma (St. Louis, MO, U.S.A.).

RESULTS AND DISCUSSION

Fig. 1 shows the separation of a peptide mixture. As exemplified here, peptides of relatively high molecular weights could be separated rapidly with high resolution. However, small peptides of molecular weight below about 1000 were usually eluted as broad tailing peaks (see Table I). The reason is probably that SP-NPR is resin-based

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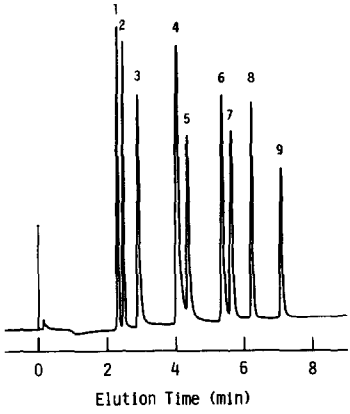

Fig. 1. Separation of a peptide mixture on TSKgel SP-NPR. A mixture of γ-endorphin (1), bombesin (2), luteinizing hormone-releasing hormone (3), somatostatin (4), α-melanophore-stimulating hormone (5), substance P (6), glucagon (7), insulin (8) and β-endorphin (9) was separated with a 30-min linear gradient of sodium sulphate from 0 to 0.25 *M* in a mixture of 20 m*M* acetate buffer (pH 3.5) and acetonitrile (60:40, v/v) at a flow-rate of 1.5 ml/min. Sample loads were 0.25 μg for components 2, 3 and 5 and 0.5 μg for others. The 20 m*M* acetate buffer (pH 3.5) was prepared by adding 20 m*M* sodium acetate to 20 m*M* acetic acid until the pH became 3.5.

TABLE I

PEAK WIDTHS OF PEPTIDES OBSERVED ON SP-NPR

Conditions as in Fig. 1. Peak widths were measured at half-height and multiplied by 1.70.

Peptide	*Molecular weight*	*Peak width (ml)*	*Elution volume (ml)*
TRH[a]	369	0.22	2.13
Leu-enkephalin	556	0.82	1.79
Met-enkephalin	574	0.84	1.49
Angiotensin III	931	0.75	8.75
Oxytocin	1007	0.15	1.07
Angiotensin II	1046	0.43	6.00
Bradykinin	1060	0.42	7.91
LH-RH[b]	1182	0.17	4.48
Angiotensin I	1297	0.47	8.70
Substance P	1348	0.19	8.23
Mastoparan	1480	0.41	11.85
Bombesin	1620	0.13	3.80
Somatostatin	1638	0.24	6.27
α-MSH[c]	1665	0.19	6.63
α-Endorphin	1746	0.15	3.70
γ-Endorphin	1859	0.15	3.49
Motilin	2700	0.18	9.60
VIP[d]	3326	0.27	16.73
β-Endorphin	3465	0.19	11.15
Glucagon	3483	0.16	8.66
Insulin	5808	0.17	9.53

[a] Thyrotropin-releasing hormone.
[b] Luteinizing hormone-releasing hormone.
[c] α-Melanophore-stimulating hormone.
[d] Vasoactive intestinal polypeptide.

and is assumed to have very small pores, although it is said to be non-porous. If small molecules enter such small pores, the diffusion rate there should be slow, which must result in broad and tailing peaks. Some peptides with higher molecular weights were also eluted as slightly broad peaks.

Elution conditions for obtaining good peptide separations were examined. When peptides were separated with eluents that did not contain organic solvents, some were eluted late as broad peaks, probably owing to hydrophobic interactions between the peptides and the support. We then examined the effect of addition of acetonitrile to the eluent. Peptides were separated with 30-min linear gradients of sodium sulphate from 0 to 0.25 *M* in mixtures of 20 m*M* acetate buffer (pH 3.5) and acetonitrile (100:0 to 50:50, v/v) at a flow-rate of 1.5 ml/min. All peptides tested were eluted without significant retardation on addition of more than 20% of acetonitrile to the buffer. The addition of less than 20% of acetonitrile was not sufficient to eliminate the abnormal elution behaviour of some peptides. At acetonitrile concentrations above 20% the peak widths were almost uanchanged, although the selectivity changed to some extent with the content of acetonitrile.

The effect of salt concentration gradient was examined by separating a mixture of insulin and β-endorphin with linear gradients of sodium sulphate from 0 to 0.25 *M* in a mixture of 20 m*M* acetate buffer (pH 3.5) and acetonitrile (60:40, v/v) at a flow-rate of 1.5 ml/min. The gradient time was varied between 5 and 60 min. The resolution for the separation of insulin and β-endorphin is plotted against gradient time in Fig. 2. The resolution increased with increasing gradient time up to 30 min, then became constant. If we consider the separation time and dilution of the sample, which increase as the gradient time becomes longer, gradient times of *ca.* 30 min corresponding to a gradient of about 8 m*M*/min sodium sulphate appear to be a good compromise. Sodium sulphate was employed because it is not corrosive to stainless steel even at low pH and its UV absorption at low wavelengths, *e.g.*, 215 nm, is low. These properties of the salt are important in the separation of peptides on cation exchangers because peptides are usually separated at low pH and detected at low

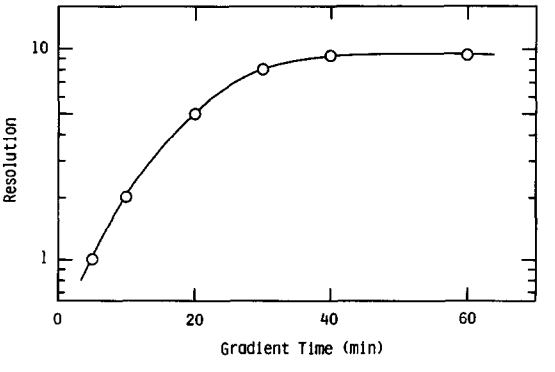

Fig. 2. Dependence of resolution on gradient time in the separation of peptides on TSKgel SP-NPR. A mixture of insulin and β-endorphin was separated under the conditions in Fig. 1 except that the gradient time was varied between 5 and 60 min. The resolution was calculated from the peak widths and elution volumes of the two peptides.

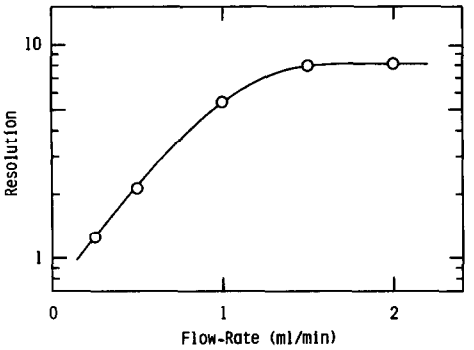

Fig. 3. Dependence of resolution on flow-rate in the separation of peptides on TSKgel SP-NPR. A mixture of insulin and β-endorphin was separated under the conditions in Fig. 1 except that the flow-rate was varied between 0.25 and 2.0 ml/min. Resolutions calculated as in Fig. 2.

wavelength. Some other salts such as sodium perchlorate may be used also in place of sodium sulphate.

The effect of flow-rate was examined by separating a mixture of insulin and β-endorphin with a 30-min linear gradient of sodium sulphate from 0 to 0.25 *M* in a mixture of 20 m*M* acetate buffer (pH 3.5) and acetonitrile (60:40, v/v) at flow-rates of 0.25–2.0 ml/min. The resolution for the separation of insulin and β-endorphin is plotted against flow-rate in Fig. 3. The resolution increased with increasing flow-rate up to 1.5 ml/min, then became constant at higher flow-rates. Although the separation time becomes slightly shorter as the flow-rate increases, the samples are more diluted and the pressure drop becomes higher almost proportionally with the increase in flow-rate. Therefore, flow-rates of *ca.* 1.5 ml/min appear to be a good compromise.

The loading capacity was evaluated by separating some peptides individually with various sample loads. The peak widths remained constant at sample loads up to about 2 μg, then increased with further increase in sample load. Accordingly, the maximum sample load in order to obtain the highest resolution is only about 2 μg for pure samples, although it is expected to be much larger for crude samples as in the separation of proteins[4]. This low loading capacity must be due to the small surface area of SP-NPR and is the greatest disadvantage of pellicular supports.

As demonstrated above, peptides with molecular weights above about 1000 can be separated rapidly with high resolution on the pellicular cation exchanger SP-NPR, whereas small peptides with molecular weights below about 1000 are eluted as broad tailing peaks. The elution with a 30-min linear gradient of sodium sulphate from 0 to 0.25 *M* in a mixture of 20 m*M* acetate buffer (pH 3.5) and acetonitrile (60:40, v/v) at a flow-rate of 1.5 ml/min is generally a good compromise with respect to resolution, separation time and dilution of sample, although shorter gradient times, *e.g.*, 10 min, are recommended when very rapid separations are required.

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