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Note

Improved recovery of ovalbumin by reversed-phase high-performance liquid chromatography

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Reversed-phase high-performance liquid chromatography (RP-HPLC) is an unique method of separating proteins and peptides mainly in the order of their hydrophobicities[1–9]. However, it has not been possible to achieve satisfactorily reproducible analyses of labile proteins such as ovalbumin owing to their relatively low recoveries. We wished to improve the RP-HPLC analysis of ovalbumin by increasing its recovery, and have studied the reproducibility and recovery by changing the procedure and conditions using commercially available column-packing materials and apparatus.

We modified the method of Mönch and Dehnen[1] to enable a recovery greater than 80% of ovalbumin: ethylene glycol was used instead of methyl Cellosolve (2-methoxyethanol); proteins injected did not make contact with organic solvent; the loading carrier (solvent A) was aqueous sodium phosphate solution and the column temperature was maintained at 15°C.

MATERIALS AND METHODS

Chemicals and reagents

All the proteins were commercial products: ovalbumin (Calibration Proteins II, Combithek) from Boehringer (Mannheim, G.F.R.); lysozyme chloride (Grade VI), human serum albumin (crystallized and lyophilized) and human transferrin (substantially iron-free) from Sigma (St. Louis, MO, U.S.A.); (Met^5)-enkephalin, (Leu^5)-enkephalin and (Tyr^1)-somatostatin from Protein Research Foundation (Minoh, Osaka, Japan). Ethylene glycol (amino acid analysis grade), isopropanol (HPLC grade) and other analytical grade reagents (orthophosphoric acid, $NaH_2PO_4 \cdot 2H_2O$, Folin-Ciocalteu Reagent, cupric sulphate and sodium tartrate) were purchased from Wako (Osaka, Japan).

Ovalbumin and lysozyme chloride were dissolved in solvent A (0.1 *M* NaH_2PO_4, adjusted to pH 2.0 by adding 6 ml of 85% orthophosphoric acid to 1 l of 0.1 *M* NaH_2PO_4 solution), and other proteins were dissolved in a 1% solution of sodium chloride (saline). Ovalbumin was stored in a deep freezer (at −20°C); it was dissolved in solvent A immediately before use and was used within a day. Distilled and deionized water was used exclusively.

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High-performance liquid chromatography

The liquid chromatograph (Model LC-2; Shimadzu, Kyoto, Japan) was equipped with a gradient programming unit (Model GRE-2, proportioning valve system with one pump), an injector with a 0.25-ml loop (Model SIL-1A) and a variable-wavelength detector (Model SPD-1). The column (50 × 4.0 mm I.D.) was packed by a viscous slurry method with 0.6 g of 10-μm octadecylsilane (ODS)-coated spherical silica gel particles (Nucleosil $10C_{18}$, Charge/batch No. 1061; Macherey, Nagel & Co., Düren, G.F.R.). The number of theoretical plates obtained by using *n*-nonylbenzene was 1200.

The column temperature was maintained at 15°C with a water-bath, and protein analysis was carried out by the use of a linear gradient, from solvent A to solvent B [isopropanol–ethylene glycol–solvent A (3:1:1 v/v); adjusted to pH 2.0 by adding 14 ml of 85% orthophosphoric acid to 500 ml of the mixed solution] at 10 or 5% per minute. The flow-rate was 1.0 ml/min and the pressure was 20–100 kg/cm^2. Five minutes were required for returning to the initial condition. The eluted proteins were detected by their UV absorption at 280 nm.

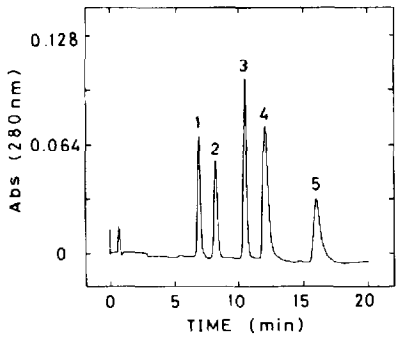
Recovery experiments

Protein recovery experiments were carried out as follows. Ovalbumin was dissolved in solvent A at a concentration of 1.0 mg/ml; 0.20 mg of ovalbumin (200 μl) were injected, and the effluent fraction (1.0 ml) corresponding to the ovalbumin peak was collected. A blank experiment omitting the protein was also carried out.

The amount of protein in the fraction was measured by the method of Lowry *et al.*[10], using ovalbumin as standard protein. The absorption at 750 nm was measured in a double-beam spectrophotometer (Shimadzu, Model UV-190).

RESULTS AND DISCUSSION

A typical chromatogram of the separation of five proteins and peptides



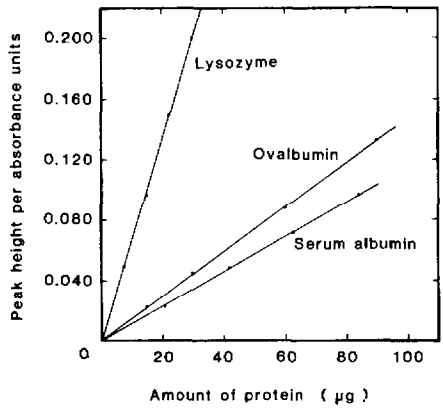


Fig. 1. Separation of typical polypeptides including commercial ovalbumin. Gradient rate: 5% per min. Other conditions as in Materials and methods. Peaks: 1 = (Met^5)-enkephalin (11.2 μg); 2 = (Leu^5)-enkephalin (11.8 μg); 3 = (Tyr^1)-somatostatin (5.2 μg); 4 = human serum albumin (65 μg); 5 = ovalbumin (23 μg).

Fig. 2. Peak-height calibration of three commercial proteins. Gradient rate: 10% per min. Other conditions as in Materials and methods.

TABLE I

RECOVERY OF OVALBUMIN

*Our method**		*Method of Mönch and Dehnen*[1] **	
Ovalbumin found (μg)	*Recovery (%)*	*Ovalbumin found (μg)*	*Recovery (%)*
172	82	228	38
170	81	70	12
164	78	74	12
Mean ± S.D.	80 ± 2.1		21 ± 15

* 210 μg (determined according to Lowry *et al.*[10]) injected.
** 600 μg (determined according to Lowry *et al.*[10]) injected.

[(Met^5)-enkephalin, (Leu^5)-enkephalin, (Tyr^1)-somatostatin, human serum albumin and ovalbumin] using the HPLC method described is shown in Fig. 1. Calibration curves for human serum albumin and ovalbumin were linear over a wide range of protein concentrations (Fig. 2). As a reference, the calibration curve for lysozyme, which is eluted close to human serum albumin, is also included in the figure.

The recovery of ovalbumin was 80 ± 2.1% (n = 3) (Table I), while that of other proteins (lysozyme chloride, human serum albumin, human transferrin and three peptide hormones) was greater than 90% (data not shown). These results compare favourably to those obtained using the method of Mönch and Dehnen[1]. With that method the recovery of ovalbumin was only 21 ± 15% (n = 3) (Table I). Our attempts to apply the method of O'Hare and Nice[6] to the analysis of ovalbumin were unsuccessful.

As a measure of the reproducibility of our method, data on the peak heights of ovalbumin and lysozyme hydrochloride are shown in Table II. The coefficient of

TABLE II

REPRODUCIBILITY

Run number	*Peak height (mm*, 0.32 a.u.f.s.)*	
	Ovalbumin (36 μg)	*Lysozyme chloride (25 μg)*
1	34.6	109
2	32.5	113
3	35.4	107
4	35.3	107
Average	34.5	109
S.D.**	1.35	2.83
C.V. (%)***	3.9	2.6

* Full-scale length of the chart was 215 mm.
** S.D. = Standard deviation (unbiased form).
*** C.V. = Coefficient of variation.

variation for ovalbumin was 3.9% and that for lysozyme hydrochloride was 2.6%.

These results indicate that the method described is applicable to the analysis of ovalbumin as well as to more stable proteins.

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