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## Note

# Structure and activity of proteins after reversed-phase high-performance liquid chromatography

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Reversed-phase high-performance liquid chromatography (HPLC) is becoming one of the most versatile techniques for the separation of proteins[1], but reports on recovery and activity after reversed-phase HPLC are scarce[2–5]. Currently available column materials necessitate the use of protein-denaturing solvents. It is necessary to use a low pH to suppress ionization of surface silanols and an organic solvent is needed for elution[1]. Under these conditions, the loss of biological activity is a major concern.

We have studied the effect of these conditions on the recovery and structure of bovine pancreatic ribonuclease (RNase), bovine serum albumin (BSA), horseradish peroxidase (HRP) and ovalbumin, which elute over a wide range of organic solvent concentrations. As a measure of structural changes, the elution profiles on ion-exchange HPLC, the immunological activity and, when appropriate, the enzymatic activity were used.

## EXPERIMENTAL

### *Reagents and materials*

RNase, BSA and HRP were products from Sigma (St. Louis, MO, U.S.A.). Ovalbumin was a product from Millipore (Freehold, NJ, U.S.A.). Absolute ethanol (LiChrosolv), 1-butanol (LiChrosolv) and analytical-reagent grade hydrochloric acid were obtained from E. Merck (Darmstadt, F.R.G.). HRP antiserum was purchased from Nordic (Tilburg, The Netherlands). RNase antiserum was obtained as described by Welling *et al.*[6].

Ovalbumin and BSA antiserum were produced by injecting rabbits at days 1 and 8 subcutaneously with 2 mg of native protein in 0.9% sodium chloride solution mixed with an equal volume of Freund's complete adjuvant.

### *Reversed-phase HPLC*

Chromatography was performed with a system consisting of a Waters M 6000 A pump, an LKB 11300 Ultrograd gradient mixer, a Rheodyne 7125 injector and a Pye Unicam LC-UV detector. The column (40 × 4 mm I.D.) was slurry-packed with Supelcosil LC 318, pore size 30 nm (Supelco, Bellefonte, PA, U.S.A.). Amounts

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of 100–200 μg of protein were chromatographed at a flow-rate of 1 ml/min with a linear gradient from 5 to 50% of solvent B in A during 12 min. Solvent A was 12 m*M* hydrochloric acid in triply distilled water and solvent B was 12 m*M* hydrochloric acid in ethanol–1-butanol (4:1)[7]. The absorbance at 205 nm was monitored. Peak fractions were collected in low protein absorption (Minisorp) tubes (Nunc, Roskilde, Denmark). Pyridine was added to a concentration of 0.1%. Part of the organic solvent was removed by evaporation under a stream of nitrogen and the remaining solution was lyophilized. The recovery was measured by protein determination according to Lowry *et al.*[8].

*Ion-exchange HPLC*

After reversed-phase HPLC, the peak fractions were immediately dialysed for 0.5 h against the starting buffer for ion-exchange HPLC. Chromatography was performed with the same HPLC equipment as used for reversed-phase HPLC. The columns, Mono Q HR 5/5 and Mono S HR 5/5, were obtained from Pharmacia (Uppsala, Sweden). Amounts of 20–200 μg of reversed-phase HPLC-treated proteins or standard proteins were subjected to ion-exchange HPLC. Ovalbumin and BSA were eluted from the anion-exchange column (Mono Q) with a gradient from 0 to 0.5 *M* sodium acetate in 0.01 *M* Tris–HCl (pH 7.5). HRP was eluted from the cation-exchange column (Mono S) with a gradient from 5 to 50 m*M* sodium acetate (pH 4.4). RNase was eluted from the cation-exchange column (Mono S) with a gradient from 0.05 to 0.5 *M* sodium acetate (pH 5.0). All gradients were completed in 12 min at a flow-rate of 1 ml/min. The absorbance was monitored at 280 nm.

*Enzymatic activity*

The enzymatic activity of RNase was determined according to Shapira[9]. The enzymatic activity of HRP was determined as described by the manufacturer with pyrogallol as substrate.

*Immunological assay*

After reversed-phase HPLC, 200 μl of a peak fraction, representing approximately 230–450 μg, were collected and 20 μl of 0.1 *M* Tris were added. After partial evaporation and subsequent lyophilization, 20 μl of demineralized water were added. Double immunodiffusions were performed in a 2% agarose gel in physiological saline. Untreated protein was used at concentrations of 0.2–0.4 mg/ml and the centre well contained undiluted antiserum against the native protein.

## RESULTS AND DISCUSSION

Although trifluoroacetic acid as the ionic modifier has been described more frequently[4,10,11] than hydrochloric acid[12], the latter was used in our reversed-phase HPLC system because it allows detection down to 200 nm[7], proteins elute at lower organic solvent concentrations (data not shown) and proteins were equally well resolved in solvent systems containing either modifier. A disadvantage might be the adverse effect of halide ions on stainless steel[13]. It has been reported, however, that 12 m*M* hydrochloric acid does not have a deleterious effect[12]. We obtained the same results with our chromatographic system, which was flushed with water each day after chromatography.

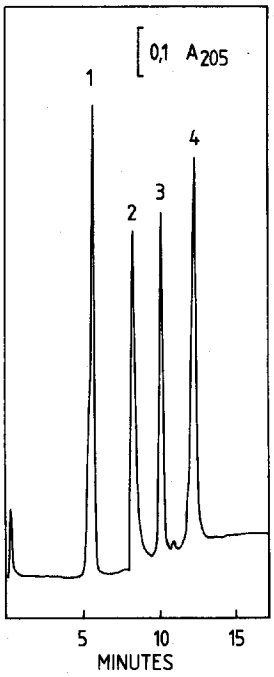


Fig. 1. Elution pattern of (1) RNase, (2) BSA, (3) HRP and (4) ovalbumin from a $C_{18}$ reversed-phase HPLC column. A mixture of the proteins (20 $\mu$g each) was chromatographed as described under Experimental.

For gradients in the reversed-phase HPLC system an ethanol–1-butanol mixture was used instead of the more frequently used propanol[1,4,10,11] because it is less viscous, it is cheaper and proteins are equally well resolved in both systems.

The separation by reversed-phase HPLC of four reference proteins is shown in Fig. 1. RNase, BSA, HRP and ovalbumin eluted at organic solvent concentrations of 17%, 26%, 33% and 41% respectively. Their recoveries, determined after injection of 100 $\mu$g of each protein, were 95%, 83%, 88% and 42%, respectively. This is in agreement with results obtained by others[3,4], who reported similar results after injection of 20–150 $\mu$g of protein.

The chromatographic properties of proteins on an ion-exchange column are largely the results of the charge distribution on the folded polypeptide chain. Any significant change in the folding will affect the charge distribution and, as a consequence, the chromatographic properties. Changes in the elution pattern of native proteins may therefore be taken as an indication that the structure of the native protein was affected. To exclude any effect of a lengthy chromatographic procedure with conventional ion-exchange columns, ion-exchange HPLC, which took only 15–20 min, was used. To determine the extent to which the structure of the four proteins was affected during reversed-phase HPLC, peak fractions were dialysed for 0.5 h against the starting buffer for ion-exchange chromatography and chromatographed.

The results are shown in Fig. 2. The chromatographic properties of RNase were unaffected (Fig. 2a). Two major peaks were seen after chromatography of the

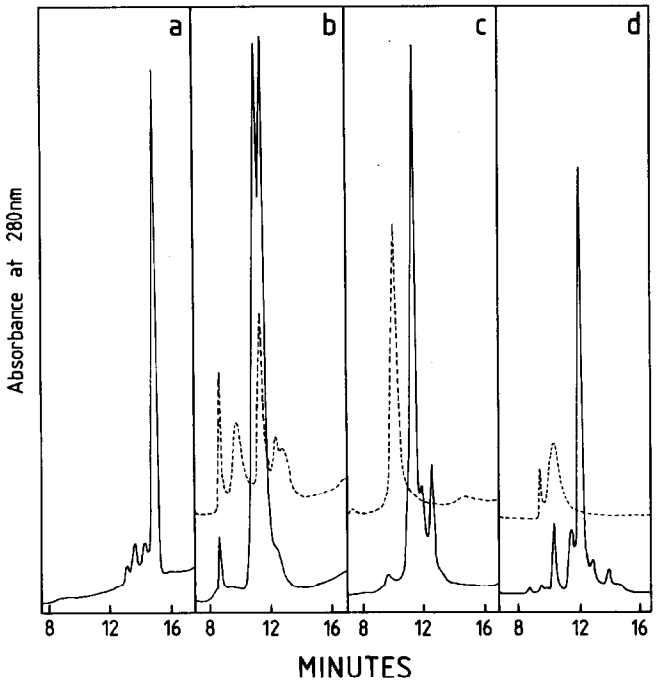


Fig. 2. Elution patterns of (a) RNase, (b) BSA, (c) HRP and (d) ovalbumin from an ion-exchange column after reversed-phase HPLC (------) and without reversed-phase HPLC (——).

control sample of BSA, whereas after reversed-phase HPLC at least five peaks were observed (Fig. 2b). One of the latter peaks eluted at the same position as the control sample. After reversed-phase HPLC of HRP, only one major peak was left, and the elution position had changed (Fig. 2c), indicating that the native structure was affected. The chromatographic properties of ovalbumin changed drastically. Only one broad peak and a smaller peak were observed, both of which eluted earlier than the major peak of the native protein (Fig. 2d).

Another method for determining changes in the structure of a native protein is to measure its immunological reaction. In double immunodiffusion, the immunological activities of RNase and BSA with antisera against the native protein were unchanged after reversed-phase HPLC. With HRP, only weak precipitation lines were observed, and ovalbumin did not react at all with antiserum to the native protein after reversed-phase HPLC.

Two of the four proteins used were enzymes, the enzymatic activities of which were assayed as an indicator of native structure. The enzymatic activity of RNase was not affected, but that of HRP was seriously affected by reversed-phase HPLC. The enzymatic activity of HRP was decreased to 1–5% residual activity.

The results are summarized in Table I. The effect of the reversed-phase HPLC on the native structure of the proteins can be seen to be directly related to the organic solvent concentration. Proteins may be denatured by an organic solvent at low pH but may rapidly renature. A few reports have appeared on the properties of proteins after elution from reversed-phase columns. Prostatic acid phosphatase was still active

TABLE I

EFFECTS OF REVERSED-PHASE HPLC ON THE STRUCTURE AND ACTIVITY OF PROTEINS

| *Parameter* | *RNase* | *BSA* | *HRP* | *Ovalbumin* |
|---|---|---|---|---|
| Elution (% organic solvent) | 16 | 24 | 31 | 38 |
| Yield* | ++ | + | + | ± |
| Immunological activity** | +· | + | ± | − |
| Ion-exchange chromatography*** | + | ± | ± | − |

* ++, 90–100%; +, 60–90%; ±, 30–60%; −, <30%.
** +, Good reaction; ±, weak reaction; −, no reaction.
*** Peak shape and shift of peaks, from no change (+) to large changes (−).

after addition of 45% glycerol to the eluted fractions as a stabilizing agent[2]. HPLC-purified ribisomal proteins were used for reconstitution into 30S ribosomal subunits, resulting in a functional subunit with more than 75% activity[5].

Our results and those of others[2,5] indicate that many proteins subjected to reversed-phase HPLC regain a substantial part of their structural properties.

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