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Note

Fractionation of high-molecular-weight proteins with mixed Toyopearl packings

JOHN GERMERSHAUSEN*, RICHARD BOSTEDOR, RICHARD LIOU and JOHN D. KARKAS
Department of Biophysics, Merck Sharp & Dohme Research Laboratories, P.O. Box 2000, Rahway, NJ 07065 (U.S.A.)
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Gel-permeation chromatography of proteins has advanced rapidly in the past few years with the introduction of semi-rigid packing materials[1–3]. Excellent resolution of protein mixtures can now be achieved routinely with the TSK-SW type columns (Toyo Soda, Japan). These columns are suitable for analytical or semi-preparative scale protein separations. However, they have limited use for the resolution of high-molecular-weuight proteins (over 10^6 daltons) and, being silica-based, cannot be used at alkaline pH. Also, they are sold prepacked and the cost of preparative-scale columns can be prohibitive. Recently[4] we reported on a similar packing material, Toyopearl HW55F, which is now sold in bulk quantities at considerably lower prices and, consequently, can be easily adapted to preparative- or even industrial-scale separations[4,5]. This material, a hydrophilic polymer of vinyl-type monomers, is available in various grades having different particles sizes and porosities for the fractionation of different ranges of molecular weights[5]. We report here on two of these grades, HW55F and HW65F, which separately have unique properties that, when combined, allow for the resolution of high-molecular-weight ($>10^6$ daltons) proteins.

EXPERIMENTAL

Materials

Toyopearl HW55F and HW65F were generously supplied by Toyo Soda, Japan, as aqueous suspensions containing 0.02% NaN_3. The material is now sold by numerous suppliers under the name Fractogel TSK-HW55F and HW65F. Calf thymus DNA was from Calbiochem. Cytochrome *c*, chymotrypsinogen A, ovalbumin, bovine serum albumin, aldose, catalase, ferritin and thyroglobulin were purchased as kits from Pharmacia. Human low-density lipoprotein (LDL) was prepared according to Kroon[6]. The molecular weights of the standards are shown in the legend to Fig. 1.

Chromatography

The columns (60 × 1 cm, stainless-steel) were fitted with glass extensions and rapidly packed at 6 ml/min as previously described[4]. The mixed-resin column was

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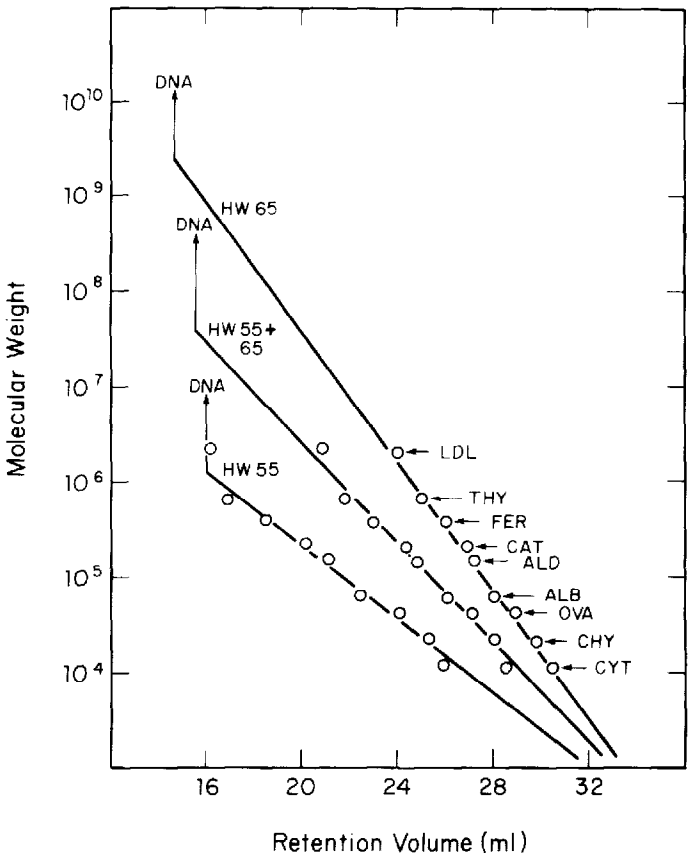

Fig. 1. Standard curves. Individual protein standards were chromatographed on each of the three (60 × 1 cm) columns as described in *Chromatography*. The plots of log MW *vs.* retention volume are shown for the HW55F, HW65F, and HW55F–HW65F (4:5, w/w) packings as indicated. Protein standards were LDL, MW $2.3 \cdot 10^6$; THY, thyroglobin, $6.7 \cdot 10^5$; FER, ferritin, $4.4 \cdot 10^5$; CAT, catalase, $2.32 \cdot 10^5$; ALD, aldolase, $1.58 \cdot 10^5$; ALB, bovine serum albumin, $6.9 \cdot 10^4$; OVA, ovalbumin, $4.5 \cdot 10^4$; CHY, chymotrypsinogen A, $2.5 \cdot 10^4$; and CYT, cytochrome *c*, $1.25 \cdot 10^4$ daltons. The excluded volume was determined with calf thymus DNA, $> 10^9$ daltons.

packed similarly with HW55F–HW65F (4:5, w/w), stirred with a magnetic stirrer just before packing. All operations were performed at room temperature with a Varian 5000 liquid chromatography system. The eluting solvent contained 0.1 *M* potassium chloride in 0.05 *M* potassium phosphate buffer (pH 6.5). Each column was standardized by individual injections of 0.1 ml of a 10 mg/ml solution of each protein standard and a 1 mg/ml solution of calf thyumus DNA dissolved in the eluting buffer. Flow-rate was 0.5 ml/min in all cases. Detection was by UV (254 nm). Recoveries were greater than 90% in all cases.

RESULTS

Individual protein standards were chromatographed on each of three 60 × 1 cm columns packed with Toyopearl HW55F, HW65F or HW55F–HW65F (4:5,

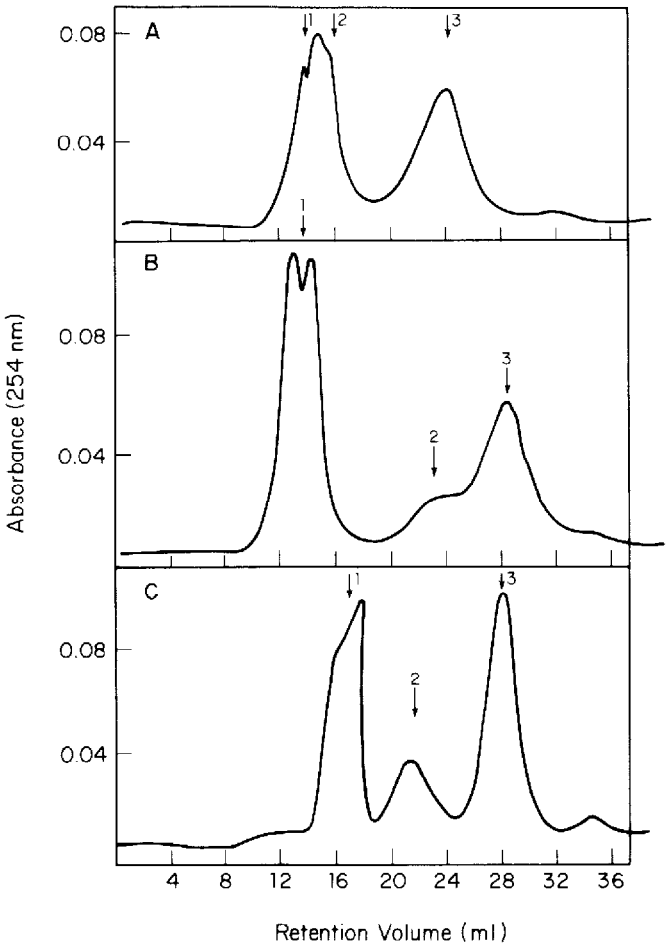

Fig. 2. Separation of LDL from DNA and chymotrypsinogen A. A mixture containing 10 μg calf thymus DNA (1), 25 μg low-density lipoprotein (2) and 25 μg chymotrypsinogen A (3) in a final volume of 50 μl was injected into each of three (60 × 1 cm) columns: HW55F (A), HW65F (B) and HW55F–HW65F (4:5, w/w) (C). The mixtures were chromatographed at 0.5 ml/min as described in *Chromatography*.

w/w). A plot of log molecular weight (MW) *versus* retention volume for each column is shown in Fig. 1. The exclusion volume was determined with DNA since no protein standards are available of sufficiently high molecular weight to be excluded from all three columns. The exclusion limits were approximately 10^6, $5 \cdot 10^7$ and 10^9 daltons for the HW55F, HW55F–HW65F, and HW65F columns, respectively (Fig. 1). The log plots were linear in each case, with the mixed-resin column having a slope intermediate between the two individual packings (Fig. 1).

The mixed-bed column was used to separate LDL (MW $2.3 \cdot 10^6$) from DNA (MW $> 10^9$) and chymotrypsinogen A (MW $2.5 \cdot 10^4$) as shown in Fig. 2. The individual packings, while having unique properties of their own (Fig. 2A and B), were limited by either exclusion limit (HW55F, Fig. 2A) or resolving power (HW65F, Fig. 2B). The mixture provided adequate resolving power while retaining the exclu-

sion properties necessary for the separation of this high-molecular-weight protein (Fig. 2C).

DISCUSSION

The fractionation of high-molecular-weight ($> 10^6$ daltons) proteins or protein complexes by gel filtration chromatography has historically created problems for the chromatographer. Numerous packing materials (for example Sephadex and TSK-SW resins) are available which will separate high-molecular-weight proteins from typical, intermediate-molecular-weight (10^4–10^5 daltons) proteins. However, these materials exclude the high-molecular-weight proteins along with even larger macromolecules, or aggregates, which seem to appear in almost every protein mixture. The Sepharose and agarose packings have partially overcome this problem by providing much higher exclusion limits. However, these packings, like the Sephadex-type materials, suffer from a relative lack of rigidity which limits flow-rate and also from the fact that they are composed of carbohydrates which are substrates for microbial growth. The Toyopearl HW materials, described here and in a previous communication[4], are semi-rigid polymers of vinyl monomers and consequently can accommodate extremely fast flow-rates without significant back pressure. For instance, a flow-rate of 6 ml/min through a 60 × 1 cm column produces only 4 atm pressure. Also, these materials do not support bacterial growth and therefore have extended column life. The HW55F and HW65F columns used in this study were packed more than two years ago and were stored at room temperature.

The use of mixed-bed Toyopearl resins has been reported previously by Barker *et al.*[7] for the separation of dextran from glucose. The authors used various combinations of HW55S and HW65S packings. However, the "superfine" grade of Toyopearl which they used, while providing somewhat better resolution than the "fine" grade used in this study, develops considerably higher back pressures and consequently increases analysis time. The HW55F and HW65F packings are better suited to preparative and even industrial-scale gel filtration for which fast flow-rates are required. In addition, the Toyopearl resins can be used at virtually any pH, ionic strength and temperature as well as with a large array of organic solvents and protein denaturants[5]. Furthermore, as illustrated above, mixtures of resins can be used with the ratio varied to suit the individual needs of the chromatographer.

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