PROTOCOL

Reversed-Phase HPLC Separation of Proteins on Chemically Bonded Silica Gel Columns

Noriyuki Nimura* and Hiroko Itoh

Abstract

Reversed-phase high-performance liquid chromatographic (RP-HPLC) separation of proteins on chemically bonded silica gel columns is described. Efficiency of nonporous alkylsilyl bonded silica gel is compared with that of a macroporous gel that has been widely used for the purpose. A comparative study of the separation under conventional and fast separation conditions is also given. The fast separation technique on the nonporous reversed-phase column has the advantage of improving the recovery of late-eluting hydrophobic and large proteins, such as ovalbumin and apoferritin.

Index Entries: Reversed-phase HPLC; protein; nonporous silica gel; macroporous silica gel; fast protein separation.

1. Introduction

Reversed-phase high-performance liquid chromatography (RP-HPLC) has become one of the most useful techniques for the separation of proteins. In this technique, various chemically bonded silica gel columns are widely used with an acidic mobile phase, such as aqueous trifluoroacetic acid (1). Pore size of the silica gel is one important factor in the separation of proteins. Macroporous silica gels, mean pore diameter of 30–100 nm, have widely been used and give efficient separations. In addition, microparticle nonporous silica gels and organic polymer gels have recently been investigated for use in protein separation (2–4) (see Note 1). Since RP separation of proteins is not seriously influenced by the use of different chemically bonded phases, C4, C8, C18, and phenyl bonded phases are all applicable to this form of separation (3) (see Note 2). Gradient elution is generally used, and an organic modifier, such as acetonitrile or isopropanol, is utilized for this purpose.

The behavior of proteins in RP-HPLC columns is often very different from that of small solutes. This is especially true with regard to protein separation in RP-HPLC, which is based on a characteristic elution mode different from a common reversed-phase-partition mode (5–14). Therefore, general plate theory might not be applicable to this type of separation in its present form. In fact, very short columns have been used for RP-HPLC of proteins (15), the resolution remaining constant as the column length decreased. Furthermore, there have been few effects of particle sizes of column packings on the column efficiency in RP-HPLC separation of proteins (12). Large particle nonporous packing, even up to 20 µm in diameter, yielded the same high efficiency as microparticles 2 or 5 µm in diameter (see Note 3).

A disadvantage of the RP-HPLC technique in protein separation is that the recoveries of large proteins (>100 kDa) or highly hydrophobic proteins tend to be extremely poor. However, it has been found that the recoveries of the late-eluting hydrophobic proteins, such as ovalbumin, increased with increasing flowrate and gradient slope (13), and were also improved by the use of a short column (5,15).
This article describes the RP-HPLC separation of proteins on chemically bonded silica gel columns. Comparative studies of the separations on a nonporous packing material and a macroporous one, under conventional and fast separation conditions, are given. One of the fast separation techniques has the advantage of improving the recovery of late-eluting hydrophobic proteins.

2. Materials

1. HPLC equipment to run a binary solvent gradient with column oven, sampler, UV detector, and integrator. A mixing coil (2 m x 0.8 mm i.d. tube) is fitted between the mixing device and the sample port for the fast separation with a steep gradient. An on-line or helium-purge degasser for degassing the mobile phase should be used. Wavelength of the detector is 215–220 nm (see Note 4).

2. Nonporous C18 silica gels with various particle sizes, 2, 5, and 20 μm (Develosil NP-ODS-2, 5, 20, Nomura Chemical. Co. Ltd., Seto-City, Japan), and macroporous C18 silica gels with particle sizes of 7 and 20 μm (Develosil 300-ODS-7, 20, pore diameter; 30 nm). Column size: 30 × 4.6 mm id.

3. Mobile phase A: 0.1% (v/v) trifluoroacetic acid (TFA) in distilled water. HPLC-grade TFA without any stabilizer should be chosen. Prior to use, filter the mobile phase through a 0.2-μm filter (see Note 5).

4. Mobile phase B: 0.1% (v/v) TFA in 90% aqueous acetonitrile (see Note 6). HPLC-grade acetonitrile should be used, because contaminants often lead to the baseline lability.

5. Protein standards: bovine serum albumin, carbonate anhydrase (bovine erythrocytes), cytochrome c (horse heart), insulin (bovine pancreas), insulin B chain (bovine insulin), α-lactalbumin (bovine milk), lysozyme (chicken egg white), ovalbumin (chicken egg) as a hydrophobic protein, and both apoferritin (horse spleen) and thyroglobulin (bovine) as large proteins. Each sample is dissolved at a concentration of 1 mg/mL in the mobile phase A. This should be stored in a refrigerator (5–10°C).

6. Egg white protein sample: A fresh egg white is diluted 500 times with mobile phase A.

3. Method

1. Conventional separation conditions: flowrate, 2.0 mL/min, column temperature, ambient, linear gradient, from 10 to 100% mobile phase B in 20 min. Fast separation conditions: flowrate, 4.0 mL/min, column temperature, 75°C or ambient, linear gradient, from 22 to 100% mobile phase B in 48 s (on a nonporous column), from 34 to 100% in 48 s (on a macroporous column).

2. Liquid samples of biological materials should be lyophilized and then redissolved in mobile phase A. Should a sample solution contain large amounts of protein, the solution can be directly diluted with the mobile phase A. Solid samples are dissolved directly in the mobile phase A. Protein solutions ranging in concentration from 10 mg to a few mg/1 mL would be acceptable for this HPLC analysis (see Note 7).

3. Prior to the sample injection, the gradient program is run. After the delay time for the gradient (see Note 8), a few milliliters of protein sample are injected onto the HPLC column.

4. The proteins are eluted from the column by a gradient of increasing concentration of buffer B.

4. Notes

1. It is said that hydrophobicity of proteins generally increases with increasing molecular weight. On the other hand, some size-exclusion phenomena are shown on the porous packing material. Therefore, both size-exclusion effects and reversed-phase effects occur during protein separation on a macroporous support (see Fig. 1). However, in practice, differences in retention time between smaller proteins, such as the insulin B chain, and larger ones, such as BSA, become relatively shorter on the macroporous silica column compared to that on the nonporous column (see Fig. 2). Accordingly, a separation based only on hydrophobic interaction is achieved on a nonporous packing material.

2. Retention of proteins is not seriously influenced by changing the alkyl chain length of the bonded phase, because the proteins only interact with the extreme ends of the alkyl chains (14). Accordingly, retention times of proteins do not differ much when different types of chemically bonded phases are used.