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Short communication

Improved liquid chromatographic separation of different proteins by designing functional surfaces of cattle bone-originated apatite

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Abstract

Spherical particles of cattle bone-originated hydroxyapatite (r-HAp) were prepared by dissolution–precipitation, spray-drying using a two fluid-nozzle apparatus, and subsequent heat treatment. The product had effective pore structures for liquid chromatographic separation of albumin, myoglobin, ribonuclease, lysozyme and cytochrome *c*. The activated surfaces of the r-HAp particles were easily prepared with desired proportions of P- and C-sites and appropriate acid–basic strength for selective protein adsorption by optimizing the synthesis conditions. Liquid chromatography columns packed with the particles exhibited high resolution and durability in protein separation, reflecting stable distribution of pore size.

Keywords: Proteins; Hydroxyapatite

1. Introduction

Hydroxyapatite [HAp: $Ca_{10}(PO_4)_6(OH)_2$] has excellent characteristics for adsorbing and desorbing biopolymers such as proteins and nucleic acids without denaturation because of high bioaffinity and biocompatibility, and thereby has been applied as an adsorbent for high-performance liquid chromatography. Even though newer silica-based particles have recently been developed [1], HAp chromatography has effectively been used as a bioactive reagent technique to separate and fractionate the various proteins without organic eluents. One can recognize the responsible reason that each of the proteins obtained through HAp columns can immediately be supplied for the culture of animal cells and other biomaterials experiments without any purification and modification. The adsorption sites on the hexagonal HAp surface are classified as follows: a C-site on the a-face and a P-site on the c-face [2,3]. Acidic proteins and basic proteins are selectively adsorbed on the C- and P-sites on HAp, respectively, and they are easily eluted in phosphate buffer solutions by using a linear molarity gradient method. However, HAp chromatography still has difficulty obtaining high resolution of proteins with durability. To solve these problems, cattle bone-originated apatite (r-HAp) prepared by a dissolution and precipitation is evaluated here instead of synthesized HAp [4]. The r-HAp is characterized by a Ca^{2+}-deficient structure

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containing small amounts of Mg^{2+} and Na^{+} ions [5,6]. The purpose of this study is to fabricate the r-HAp spherical particles with effective surface properties for the separation of proteins in liquid chromatography by using spray-drying techniques and heat treatments, and to design columns with high resolution.

2. Experimental

2.1. Preparation procedure of spherical particles

Cortical bones in cattle femur (Holstein bull, Hokkaido) were calcined and dissolved in a HNO_3 solution (1%, v/v), and reprecipitated by adding a NH_3 aqueous solution at 298 K and aging at pH 10.5 for 24 h. The r-HAp spherical particles with a crystallographic single phase were fabricated by spray-drying techniques (Sakamoto Engineering, DCTRS-3N) accompanied by ultrasonic dispersion using a two fluid-nozzle or a spinning disk at 473 K, and subsequent heat treatments at 673~873 K for 24 h in a stream of water vapor [6]. The two fluid-nozzle (TF) method produced spherical particles 1~10 μm in size without deformation or hollow parts, whereas the spinning disk (SD) method at the rotation rate of 15 000 rpm gave 5~30 μm. The specific surface areas of the particles obtained by the TF method were 10% higher than those by the SD method.

2.2. Chromatography of proteins

The r-HAp spherical particles were easily packed into glass columns (50×8 mm I.D.) by means of a gravitational sedimentation packing method. To investigate the chromatographic separation characteristics of proteins on the columns, bovine serum albumin (BSA), myoglobin from horse skeletal muscle (MOG), ribonuclease A from bovine pancreas (RBN), lysozyme from chicken egg white (LSZ), and cytochrome *c* from horse heart (CTC) were used. After each of the r-HAp columns was attached to an analytical liquid chromatography system (Tosoh, SC-8020), absorbance was measured at 280 nm at a flow-rate of 1.0 ml min^{-1} as chromatograms of the five proteins at 313 K and pH 6.9 by the linear molarity gradient method of a mixed potassium phosphate buffer solution from 10 to 500 m*M*.

3. Results and discussion

Fig. 1 shows typical chromatograms of the proteins attained using the r-HAp particles spray-dried by the TF- and SD-methods and heat-treated at 673 K. One can recognize that each component of the five proteins of 1.0 mg ml^{-1} in the mixed solutions was sufficiently separated on the r-HAp column obtained by the TF method. The characteristic peaks obtained at retention times of about 9.5, 12.4, 13.0, 13.8 and 21.5 min were assigned to BSA, MOG, RBN, LSZ and CTC, respectively. The retention times and the widths for each half of the curves were almost constant, regardless of concentrations of the proteins solutions. For the SD method, on the other hand, MOG, RBN and LSZ cannot clearly be

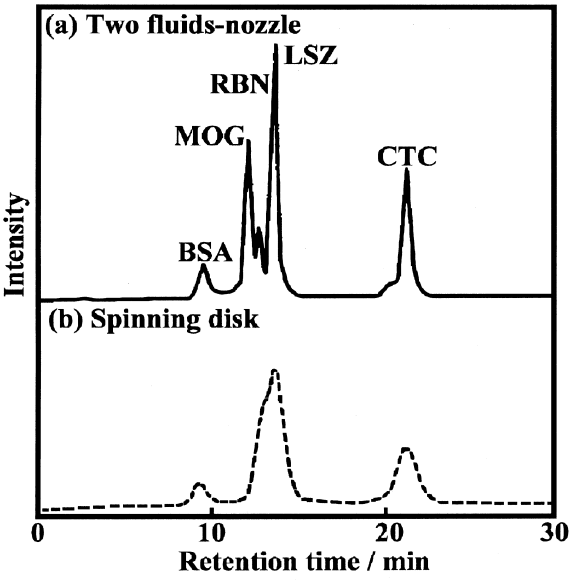

Fig. 1. Chromatograms of the five proteins obtained on the r-HAp columns. Conditions: column size, 50×8 mm I.D.; wavelength of absorbance, 280 nm; 313 K; pH 6.9; flow-rate, 1.0 ml min^{-1}; KH_2PO_4 and K_2HPO_4 buffer solution; linear molarity gradient method, 10~500 m*M*, 30 min; five proteins of 1.0 mg ml^{-1} in the mixed solutions as bovine serum albumin (BSA), myoglobin from horse skeletal muscle (MOG), ribonuclease A from bovine pancreas (RBN), lysozyme from chicken egg white (LSZ), cytochrome *c* from horse heart (CTC).

separated on the column obtained, even when the column length was 2~3-times longer and the particles were largely loaded into columns, or the particles were sieved in the size of 5~10 μm as for the TF method. These results strongly exhibit that the lower resolution of the SD column is not resulted from the particle size distribution.

Fig. 2 shows curves of pore size distribution measured by the N_2-adsorption method for the r-HAp particles. The pore volumes in the pore diameters of 14~30 nm for the particles obtained by the TF method were much larger than those by the SD method. This result suggests that the TF method flowing up the r-HAp slurry with a stream of N_2 gas is more effective procedure to design particles with available micropores than the SD method flowing away the r-HAp slurry from a spinning disk. Concerning the particle-quality difference between the TF and SD methods, our speculation is as follows. In the TF method, the liquid droplets sprayed from small hole of nozzle form the particles with the small size and large pore volumes in an instantaneous evaporation process of water, whereas in the SD method, the liquid droplets sprayed from hole of the spinning disk under a high rotation rate produce the particles with the larger size and much smaller pore volume, resulting from a slow dehydration rate. This pore diameter range is possibly available for diffusion of the proteins molecules, because the molecular sizes of BSA, MOG, RBN, LSZ and CTC are 4×4×14, 5.7ϕ×0.9, 3.8×2.8×2.2, 3.0×3.0×4.5, and 2.5×2.5×3.7 nm, respectively. Based on these results, it was found that the r-HAp spherical particles which were spray-dried by the TF-method and heat-treated subsequently at 673~873 K had effective pore structures for liquid chromatographic separation of the five different proteins.

Fig. 3 shows a drastic difference in the chromatographic separation of the same proteins between the TF column and a commercial HAp (Tosoh, HA-1000) after the sample injection of 300 times. At the first injection, the plate numbers for the TF column and the commercial column in the separation condition of open column chromatography were kept in almost equivalent. The plate numbers for BSA, MOG, RBN, LSZ and CTC were 2000~10 000. Although the chromatograms on the HA-1000 initially gave clear separation of the five proteins, the resolution of the proteins significantly decreased, and the reason for the decay in resolution was found to

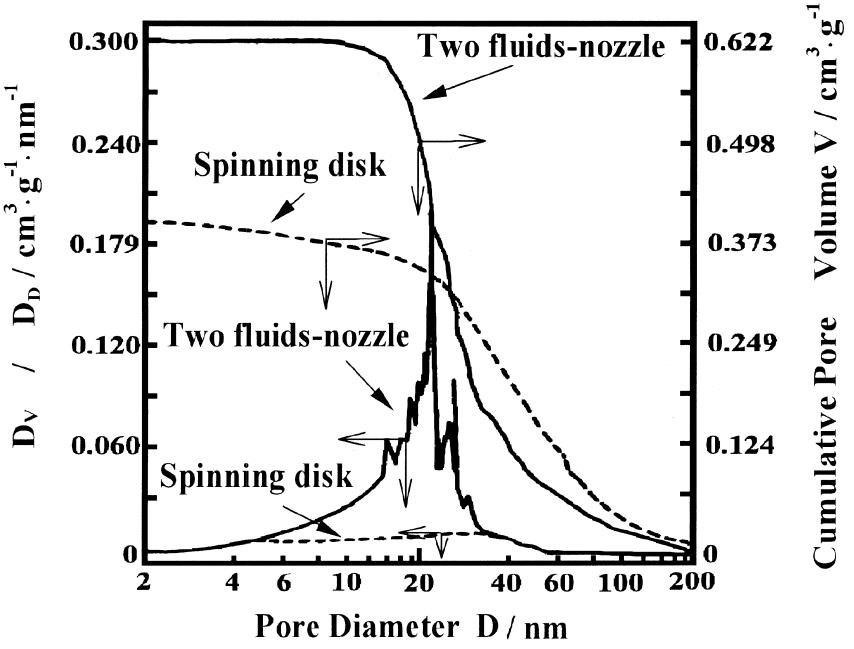

Fig. 2. Curves of pore size distribution measured by the N_2-adsorption method for the r-HAp particles.

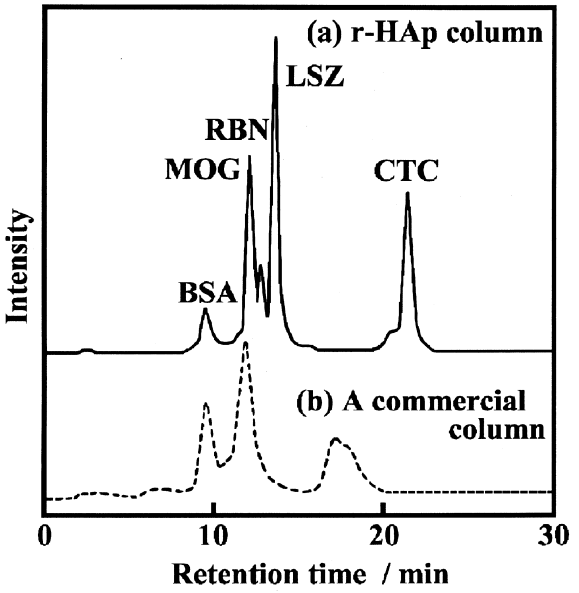

Fig. 3. Chromatograms of the five proteins after the sample injection of 300 times on the different columns. (a) r-HAp columns; (b) a commercial column. Conditions: column size, 50×8 mm I.D., 313 K; pH 6.9; flow-rate, 1.0 ml min^{-1}; KH_2PO_4 and K_2HPO_4 buffer solution; linear molarity gradient method, 10~500 m*M*, 30 min; the five proteins of 1.0 mg ml^{-1} in the mixed solutions. The symbols of the proteins are the same as those in Fig. 1.

be due to drastic variation in the pore size distribution. However, the chromatograms on the r-HAp column obtained by the TF method always exhibited reproducible patterns even at the sample injection of 300 times, reflecting a little change of the total pore volume from 0.411 to 0.400 cm^3 g^{-1} in the r-HAp particles before and after use.

The separation qualities of the columns used are strongly influenced by not only pore structure of the r-HAp particles but also the total number, proportion and chemical nature of the surface P- and C-sites. Detailed adsorption characteristics for each of the five proteins solutions on the r-HAp surfaces were investigated at pH 6.9~7.1 and 277~295 K. All the adsorption isotherms for the proteins obeyed a Langmuir equation, indicating that the proteins can be adsorbed in a homogeneous monolayer on the r-HAp surfaces. The adsorption characteristics of the five proteins were classified into two groups relating to isoelectric points (E_0) of the proteins and saturated amounts of the adsorbed proteins. The amounts of RBN, LSZ and CTC [designated as A_{SB} (basic group)] with $E_0>7.0$ were 2~10 times larger than those of BSA and MOG [designated as A_{SA} (acidic group)] with $E_0<7.0$. The basic and acidic groups can selectively be adsorbed on P- and C-sites of the r-HAp surfaces, respectively, suggesting that the numbers of P-sites would be more than the numbers of C-sites. Heats of adsorption for RBN, LSZ and CTC, which were calculated from the adsorption isotherms by the Clausius–Clapeyron equation, were smaller than those for BSA and MOG. Moreover, as the heat-treatment temperature of the r-HAp powders was elevated from 673 to 873 K, the A_{SB} and A_{SA} values gradually increased from 0.014~0.108 to 0.022~0.149 μmol m^{-2}. The ratios of A_{SB} to A_{SA}, which mean surface proportions of P- and C-sites, decreased from 1.5~7.8 to 1.3~6.9, and heats of adsorption for BSA and MOG decreased from 48~55 to 30 kJ mol^{-1}, whereas those for RBN, LSZ and CTC increased from 11~26 to 30 kJ mol^{-1}.

Based on the results obtained above, one can conclude that the surfaces of r-HAp spherical particles with effective pore structure are easily modified into desired proportions of P- and C-sites and acid–basic strengths which are suitable for adsorption of various proteins, by choosing appropriate spray-drying conditions using the TF-method and heat-treatment temperatures. This surface modification technique can be used to design material with high resolution for proteins and stable columns.

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