A Newly Designed Adsorbent Prepared from Hydroxyapatite Originating from Cattle-Bones for Chromatographic Separation of Albumin and Lysozyme

Toshiyuki Akazawa,* Masayoshi Kobayashi,† and Kohei Kodaira††

Hokkaido Industrial Research Institute,
Nishi 11, Kita 19, Kita-ku, Sapporo 060

†Department of Chemical System Engineering, Kitami Institute of Technology, 165 Koencho, Kitami 090

††Division of Materials Science and Engineering, Graduate School of Engineering, Hokkaido University, Nishi 8, Kita 13, Kita-ku, Sapporo 060

(Received March 18, 1997)

Functional spherical particles 5—30 μm in size were prepared by a spray-drying method with ultrasonic dispersion from hydroxyapatite (r-HAp) which was derived from bones of cattle. The liquid chromatographic characteristics for the separation of bovine serum albumin (BSA) and lysozyme from egg white (LSZ) on the r-HAp particles were investigated in comparison with those of stoichiometric hydroxyapatite (s-HAp) derived from reagents. The specific surface area (S_{AD}), the crystallite size (C_S), and the microstructure of the HAp's particles were strongly influenced by the heat-treatment at 273—1073 K (designated by HAp(273—1073)) in a stream of water vapor. In liquid chromatography (LC) analysis with columns packed with the HAp powders, for r-HAp, mixed solutions of 0.5—2.0 mg cm^{-3} BSA and LSZ were sufficiently eluted using a linear molarity gradient of sodium phosphate buffers at 313 K. The resolutions (R_S) of the proteins and the widths for each half of the chromatographic curves (W_{HB} for BSA or W_{HL} for LSZ) were sensitively varied in connection with S_{AD} and C_S. The optimal values of the LC operating parameters for all the HAp's were evaluated as R_S of r-HAp(673) and W_{HB} and W_{HL} of r-HAp(873).

Hydroxyapatite (HAp: $Ca_{10}(PO_4)_6(OH)_2$) has been applied as a column packing material of liquid chromatography (LC) to separation of biopolymers such as proteins, nucleic acids, and viruses,[1—4] since Tiselius[5] discovered it as an adsorbent with excellent chromatographic separation characteristics of the biopolymers on HAp powders in 1965. HAp chromatography has merely been carried out only in an experiential manner, because deactivation or denaturation of adsorbate molecules on a HAp surface seldom occurs, in contrast with SiO_2 chromatography. However it cannot always be regarded as the best means for analyzing the samples, because functional HAp particles with suitable resolution and durability life for LC column have not been developed yet by using a simple procedure. Okuyama et al.[6] demonstrated that porous and spherical HAp particles (which were granulated to be 5—20 μm in particle size and 10—100 nm in pore size) were available for the column packing material for high performance liquid chromatography. Honda et al.[7] showed that the HAp coated-polyethylene composite particles could be prepared by the dry impact-blending preparation method. The particles obtained were applied for the separation of mixed proteins (for example, albumin, lysozyme, and cytochrome).

In spite of a number of studies on the HAp adsorbents for LC which were attempted by many researchers,[8—12] quantitative correlations between the column ability for separation of biopolymers and the preparation conditions of HAp have not been clarified enough yet. The surface characteristics of HAp for LC packing materials seem affected by an appropriate combination of two important parameters: 1) homogeneity or size of spherical particles granulated and 2) morphology or size of crystallites consisting of the surfaces. In our recent papers,[13—15] it has been emphasized that Ca^{2+}-deficient HAp (r-HAp) containing 0.6% Mg^{2+} and 0.04% Na^+ ions (which was prepared by the dissolution–precipitation of cattle bones) has structure-sensitive surfaces. The morphology and size of crystallites which appear on the surface can easily be changed depending on the heat-treatment temperature. If the r-HAp particles are fabricated by a comparatively simple procedure as the spray-drying technique, and the distribution of the given adsorption sites (C- and P-sites) on the surface or the adsorption heat for various proteins on both the sites is easily designed, the r-HAp will become a newly effective adsorbent for LC.

Bovine serum albumin (BSA)[16] and lysozyme from egg white (LSZ)[17] which are representative acidic and basic proteins, are selectively adsorbed on the active C- and P-sites on HAp surface, respectively.[18,19] Kawasaki et al.[18] and Okuyama et al.[6] verified that BSA and LSZ adsorbed on the HAp surface were reversibly eluted from both the sites

by carrying out the linear molarity gradient of potassium or sodium phosphate buffer solutions.

The first purpose of this study is to establish a production process of desired spherical HAp particles. The surface characteristics of the particles which are available for LC of BSA and LSZ can easily be designed by a spray-drying method and the sequence of heat-treatment. The second one is to elucidate important factors controlling the chromatographic characteristics of the two proteins.

Experimental

Preparation and Characterization of Spherical HAp Particles. For the preparation of hydroxyapatite from cattle-bones, cortical bones in cattle femur (Holstein bull, Hokkaido) were calcined at 1373 K and dissolved to HNO_3 solution as previously described.[20] The sample solution was adjusted to about pH 10.5 by adding an aqueous NH_3 solution and aged at 298 K for 24 h to reprecipitate HAp (r-HAp). After filtration and washing of the precipitate with distilled water, HAp aqueous slurries of 5—20% were prepared as samples for spray-drying. On the other hand, s-HAp was synthesized from guaranteed grade $Ca(NO_3)_2 \cdot 4\,H_2O$ and $(NH_4)_2HPO_4$ reagents by the wet method as reference samples.[20] The two HAp's slurries were granulated in a homogeneous form of sphere using a spray dryer (Sakamoto Engineering Co., Ltd., DC-TRS-3N) together with ultrasonic dispersion at 423—523 K under an atomizer rotation of 12000—18000 rpm. The HAp's powders thus prepared were heated at 273—1073 K (designated by HAp-(273—1073)) in a stream of water vapor controlled with N_2 gas.

For evaluating the degree of dispersion of the HAp slurries, the viscosity of the slurries was measured by a rotation viscometer (BM type, rotator No. 2). For the characterization of the HAp's powders obtained, the crystallite size (C_S) of HAp single phase was evaluated by using either a (200) plane or a (002) plane of X-ray diffraction (XRD) patterns.[21] Photographs of scanning electron microscope (SEM: JEOL LTD, JSM-5800LV) were taken to observe both the morphology and size of the particles or crystallites. The ratio of Ca^{2+} to P^{5-} ion (Ca/P) composition was determined by electron probe microanalysis (EPMA). The BET specific surface area (S_{AD}) of the particles was measured by N_2 adsorption at 77 K.[20]

Evaluation of Chromatographic Separation Characteristics of BSA and LSZ on the HAp's. Comparing separation quality of protein's chromatogram between the two HAp's columns, BSA and LSZ were used as an acidic protein selectively adsorbed on the C-sites and a basic protein preferentially adsorbed on the P-sites, respectively.

The two types of spherical HAp's particles prepared were easily packed into glass columns (8 mmϕ × 50 mm) by means of the gravitational sedimentation packing method. After each of the columns was attached to an analytical system of liquid chromatography (Tosoh Co., Ltd., SC-8020), a sodium phosphate buffer solution of 10 mmol dm^{-3} (an equimolar mixture of Na_2HPO_4 and NaH_2PO_4) was flowed through it under a rate of 1.0 cm^3 min^{-1} at 313 K and pH 6.9. In an equilibrium state of the system, 0.5—2.0 mg cm^{-3} BSA and LSZ sample solutions were injected and the absorbancies at 280 nm were continuously monitored with time. The chromatographic separation for BSA and LSZ was carried out for 30 min by a linear molarity gradient of the buffer solution from 10 to 500 mmol dm^{-3} at pH 6.9.

Results and Discussion

Spherical Particles Design of the HAp's with Homogeneous Surfaces. Preparation Characteristics Due to the Spray-Drying Method. For formation of spherical HAp particles with desired homogeneous surfaces, one must find an optimal combination of several spray-drying conditions: drying temperature, atomizer rotation, and slurry concentration. First of all, for the available drying temperature, higher temperatures will be needed to keep the first shape of fine liquid droplets sprayed from the atomizer, because the water content in the droplets must instantaneously be evaporated before the sprayed droplets reach the inner wall of the drying chamber. For the atomizer rotation, the particle size distribution of HAp tended to shift to the small particle size with increasing the rotation number from 12000 to 18000 rpm. Consequently, as the best spray-drying condition, an atomizer rotation of 15000 rpm at 523 K was chosen to obtain spherical particles larger than 5 μm in size without deformation or hollow parts, which can easily be packed into the LC glass columns.

As is well known, the slurry concentration is one of the important parameters to make up the dense grains-associated particles with homogeneous surfaces. Figure 1 shows surface textures of the r-HAp particles prepared by the spray-drying method under the different conditions. These powders always gave a single phase of HAp and the molar ratio of Ca/P became 1.65, which is close to 1.67 of stoichiometric HAp and almost agrees with the values obtained by the static drying method at 323 K shown in our previous paper.[14,15,20] These results suggest that chemical composition of HAp is not influenced by the abrupt spray-drying at 523 K. In the 20% HAp aqueous slurry, the particles obtained consist of some heterogeneous aggregates derived from higher slurry concentration, whereas in the 5% HAp aqueous slurry, they are constituted by comparatively small grains. The viscosities of the HAp slurries were 0.040 Pa s for the 5% slurry and 0.180 Pa s for the 20% slurry, indicating that the former slurry containing HAp gel was finely dispersed better than the latter one. Moreover, in the 5% aqueous slurry prepared with ultrasonic dispersion, although the viscosity was not changeable, the r-HAp gel locally aggregated in the slurry was finely broken down and dispersed well by the strong physical energy of the spray-drying, so that the surface of spherical particles formed would be more homogeneous. Also, for s-HAp, spherical particles were similarly obtained by the same spray-drying conditions.

Accordingly, spherical particles of the HAp's with homogeneous small grains can be fabricated continuously by the specified spray-drying method accompanied with ultrasonic dispersion under an atomizer rotation of 15000 rpm at 523 K.

Surface Characteristics of Spherical HAp Particles Influenced by the Heat-Treatment. The specific surface areas of the preheated HAp powder formed by the spray-drying were 144 m^2 g^{-1} for r-HAp(273) and 69 m^2g^{-1} for s-HAp(273); such values were higher than those (95 m^2 g^{-1} for r-HAp(273) and 60 m^2g^{-1} for s-HAp(273)) obtained in the static drying method.[20] These results suggest that the spray-drying process developed in this study appreciably de-

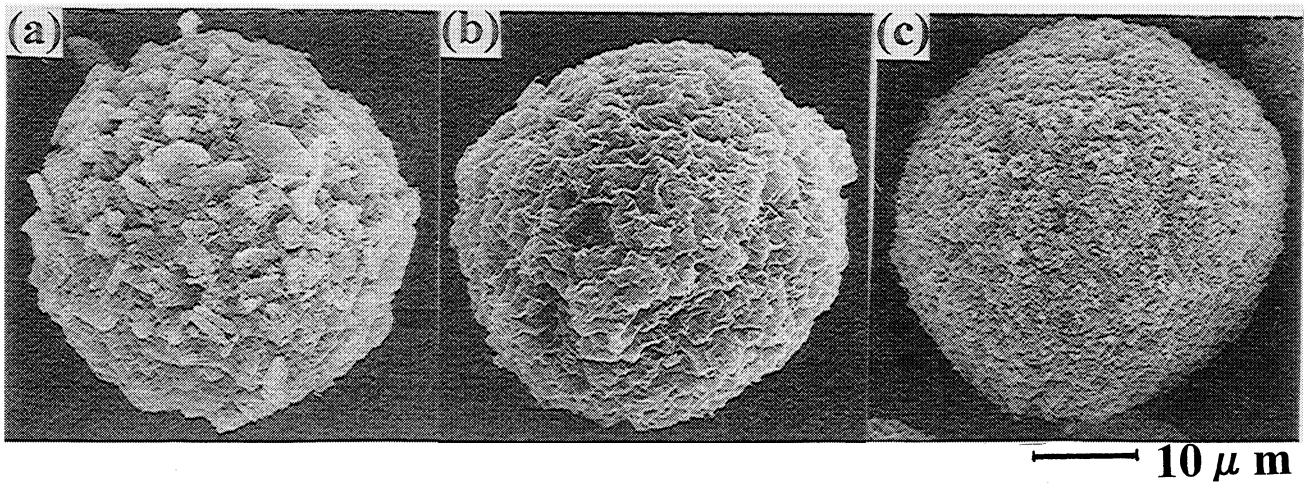

Fig. 1. SEM photographs of the spherical r-HAp particles prepared by the spray-drying method accompanied with an ultrasonic dispersion, which was carried out in the various HAp slurry concentrations under an atomizer rotation of 15000 rpm at 523 K: (a) 20% slurry without the dispersion, (b) 5% slurry without the dispersion, (c) 5% slurry with the dispersion.

presses coalescence of the HAp gels and that the spherical particles formed possess an advantage over larger pore volumes, which is caused by the abrupt evaporation of water content in the liquid droplets. In the heat-treatment of the HAp powders, however, heating temperature dependencies on S_{AD} of the particles prepared by the spray-drying showed a very similar tendency to those of the static drying.[20] Figure 2 shows the BET specific surface areas of the two HAp powders prepared by the spray-drying method as a function of the heating temperatures. S_{AD} monotonously decreases as the heating temperature of HAp rises. At the temperatures of 273—673 K, S_{AD} of r-HAp gave 74—144 $m^2 g^{-1}$ values, which were 44—210% larger than that of s-HAp, whereas at the temperatures of 873—1073 K, S_{AD} of the two HAp's fell in the range of 10—33 $m^2 g^{-1}$. The typical examples of surface textures for the heat-treated HAp powders are shown

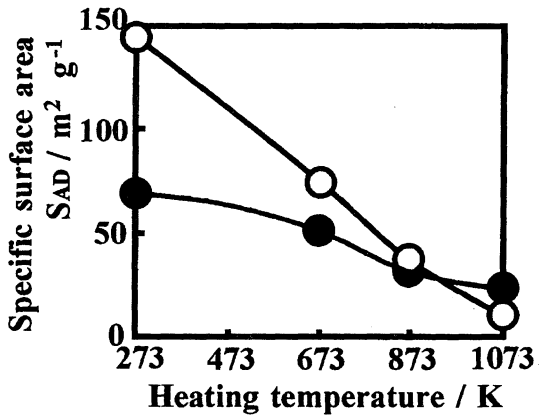

Fig. 2. Influences of heat treatment on the BET specific surface areas of the HAp powders prepared by the spray-drying method: ○ r-HAp, ● s-HAp.

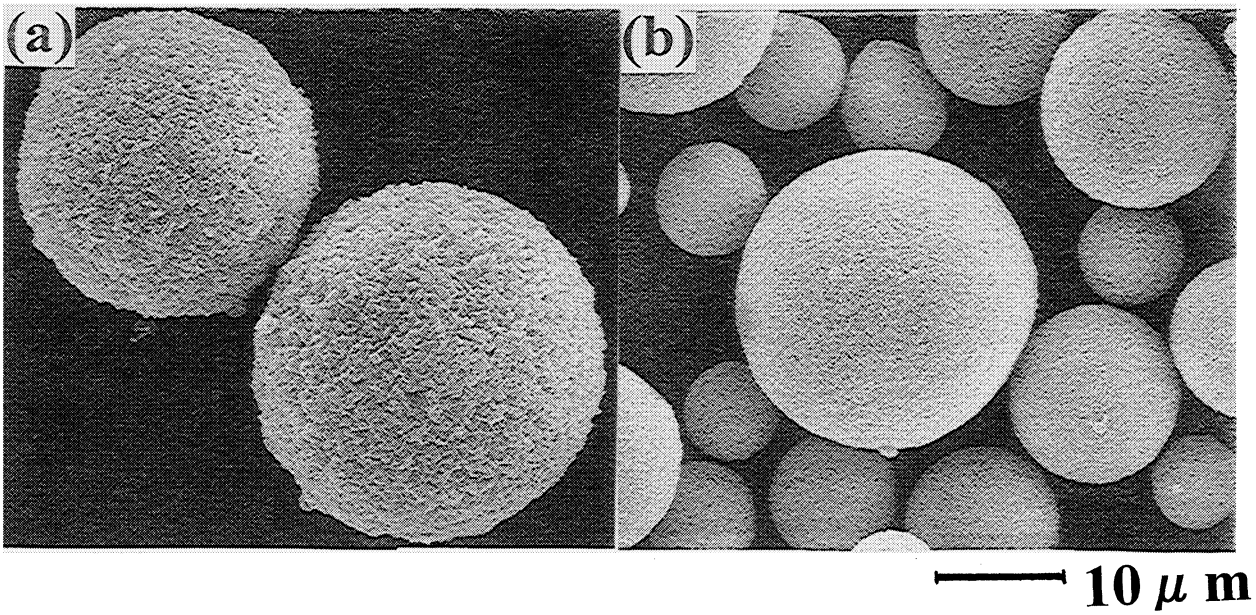

Fig. 3. SEM photographs of the HAp particles heated at 873 K in a stream of water vapor: (a) r-HAp, (b) s-HAp.

in Fig. 3. One can recognize that the two HAp particles seem to keep the spherical shape and the grain homogeneity even after the heat-treatment at 873 K. r-HAp particles show porous cocoon like-surfaces, whereas s-HAp particles give dense surfaces associated with small grains, in spite of the close value of S_{AD} for the two HAp's (see Fig. 2).

Considering anisotropic growth of hexagonal crystal the crystal size was separately expressed as C_{SA} using a (200) plane and C_{SC} using a (002) plane of HAp structure. Both the C_{SA} and C_{SC} for r-HAp grew little at the heating temperatures of 273—673 K, but at the temperatures of 673—1073 K, they gradually increased from 19 to 39 nm. Hence, for s-HAp, C_{SA} were almost constant at 273—873 K and increased from 24 to 34 nm at 873—1073 K, but C_{SC} fell in the narrow range of 35—40 nm at 273—1073 K. Figure 4 shows microstructures of the two HAp's particles heated at 673 and 1073 K. A significant difference in the microstructures of spherical particles between the HAp's prepared is clearly observed, depending on the heating temperatures. At 673 K, the microstructure of r-HAp shows a cluster of microcrystals with random growth, whereas s-HAp consists of many prismatic crystals grown anisotropically, as can be seen in the comparison of typical surface textures of HAp between Fig. 4 (a), (c) and (b), (d). As sintering of the grains in the HAp particles occurred at 1073 K, the microstructures separately transformed to spherical or ellipsoidal grains 50—200 nm in size for r-HAp and to prismatic grains with 3—5 aspect ratios for s-HAp. From these results, one can presume that the crystal of r-HAp isotropically grows at 673—1073 K and that of s-HAp grows on the a-face along the *c*-axis at 873—1073 K. This extraordinary difference between the two HAp's in the C_S and the microstructures may be attributed to the morphology discrimination of the gels which originates from the wet preparation procedure used.

Figure 5 shows surface textures of two HAp gels freeze-dried in an electronic microscope. One can recognize different network structures for the two gels: that is, flocks of microcrystals for r-HAp and needle-like crystals with high aspect ratios for s-HAp. Yamashita et al.[22] demonstrated that crystal growth on various ceramics treated by electric poling in a simulated body fluid produced the bone-like crystals of partially carbonated HAp containing Mg^{2+}. On the calcined cattle bones we have supplied here, crystal growth was accelerated and characteristic surface textures, as seen

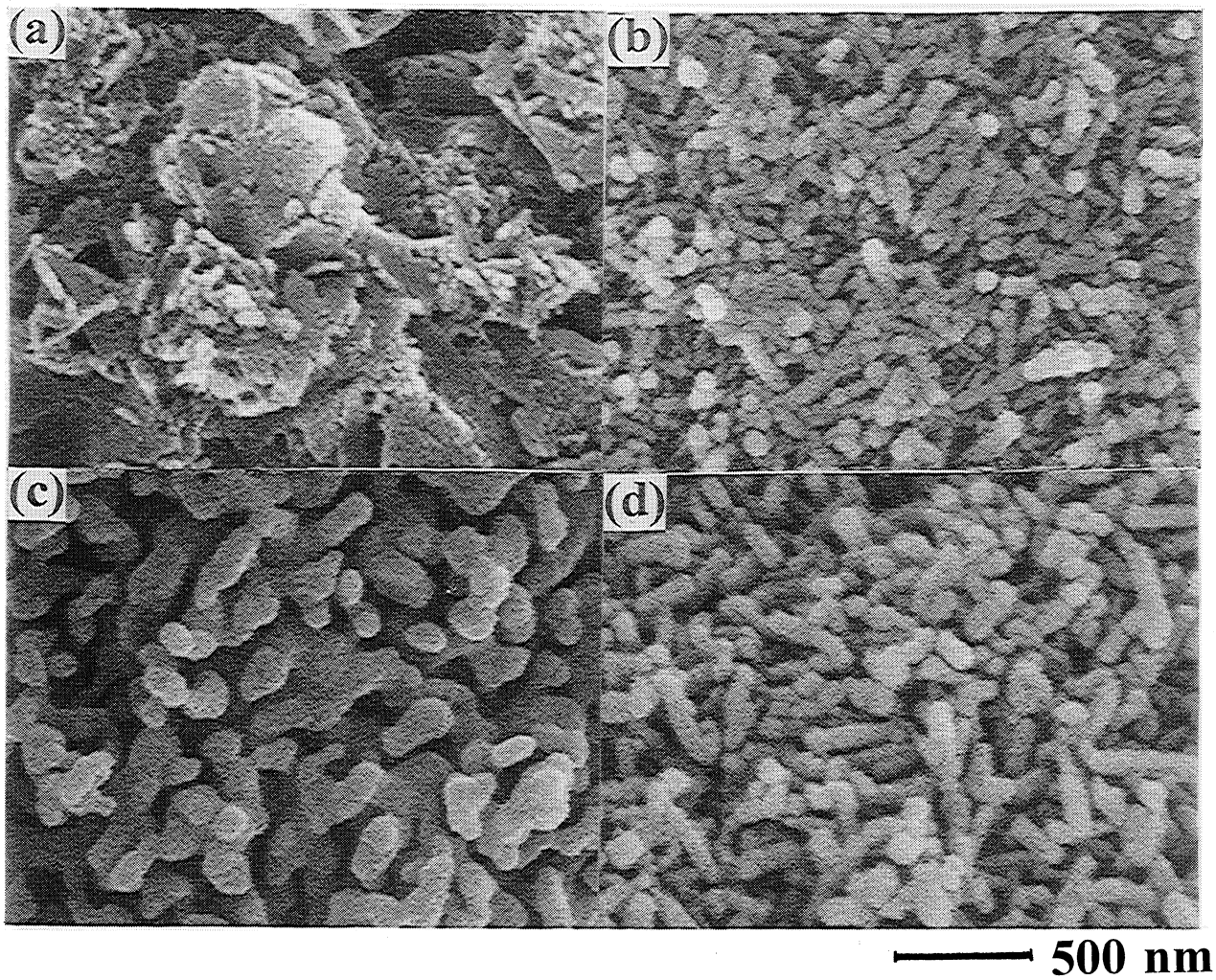

Fig. 4. SEM photographs of the HAp particles heated at 673 and 1073 K in a stream of water vapor: (a) r-HAp(673), (b) s-HAp(673), (c) r-HAp(1073), (d) s-HAp(1073).

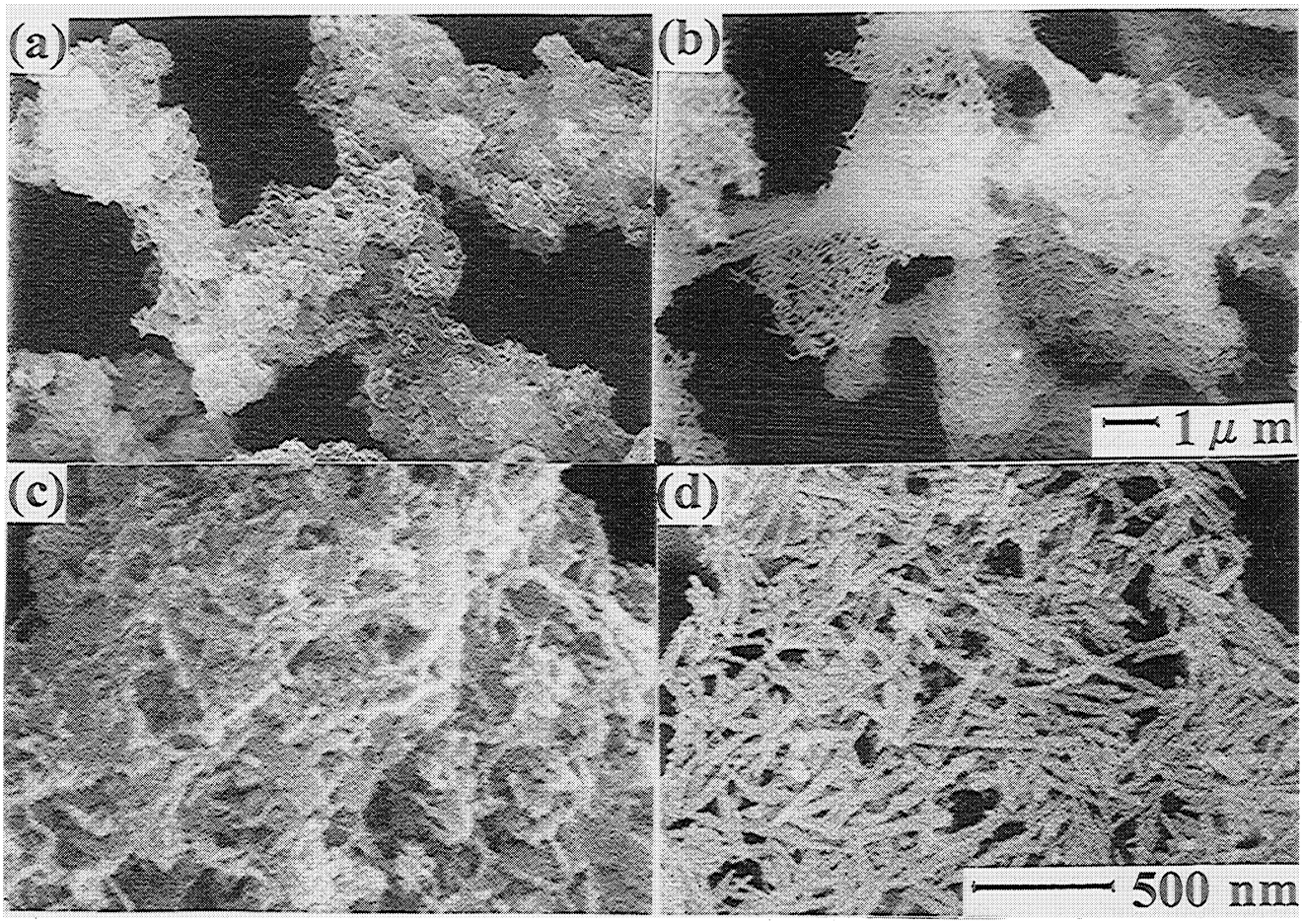

Fig. 5. SEM photographs of the HAp gels freeze-dried in an electronic microscope: (a) r-HAp (×10000), (b) s-HAp (×10000), (c) r-HAp (×50000), (d) s-HAp (×50000).

in Fig. 5 (c), were observed. Thus, in r-HAp, the Ca^{2+}-deficient HAp structure containing small amounts of impurities would be responsible for the characteristic behavior of crystal growth.

Based on the results obtained above, it is found that desired spherical particles of the two HAp's can be prepared by the spray-drying method, and that, from the viewpoint of response to the desire, they can be designed in widely different surface characteristics by choosing the heating temperature or the starting materials.

Chromatographic Characteristics for the Separation of BSA and LSZ through the Two HAp Columns. Chromatograms of the Proteins on the HAp's. A variety of chromatographic behavior for the separation of BSA and LSZ was evaluated using the columns packed with the HAp powders prepared under various conditions. Figure 6 illustrates the typical chromatograms of BSA and LSZ through the two HAp's columns, whose packing powders were heat-treated at 873 K. The chromatograms show that BSA of 1.0 mg cm^{-3} and LSZ of 1.0 mg cm^{-3} in the mixed solutions are sufficiently separated by these columns. For r-HAp, the characteristic peaks at the retention times of about 8.5 and 12.2 min can be assigned to BSA and LSZ, respectively. The retention times (t_{WB} for BSA, t_{WL} for LSZ) and the widths for each half of the curves (W_{HB} for BSA, W_{HL} for LSZ) were

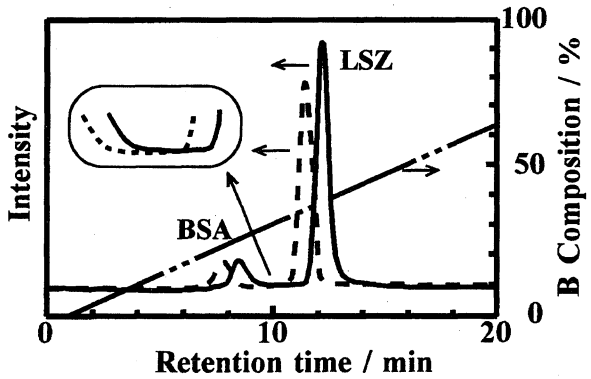

Fig. 6. Chromatograms of BSA and LSZ through the two HAp columns, whose packing powders were heated at 873 K: — r-HAp, - - - - - - s-HAp, –··– Gradient line, Injected samples; 1.0 mg cm^{-3} BSA and 1.0 mg cm^{-3} LSZ (0.020 cm^3), Column size (8 mmϕ×50 mm), Flow rate; 1.0 cm^3 min^{-1}, Linear molarity gradient conditions (A liquid; 10 mmol dm^{-3} → B liquid; 500 mmol dm^{-3} Na_2HPO_4 and NaH_2PO_4 buffer solutions, 30 min).

almost constant (t_{WB} = 8.48±0.28 min, t_{WL} = 12.27±0.14 min, W_{HB} = 46.0±5.3 s, W_{HL} = 34.8±1.1 s), regardless of the concentrations of BSA and LSZ solutions. These chromatograms obtained thus always gave reproducible patterns,

even at the sample injection numbers more than 300. This indicates that r-HAp has more excellent durability life for chromatography than a commercial HAp (Tosoh, Co., Ltd., HAP-1000). On the commercial column of HAP-1000, it was separately reconfirmed that the separation for the two proteins became unclear at the sample injection numbers around 250 in the same chromatographic conditions.

For s-HAp, the retention times were shorter (t_{WB} = 7.79±0.16 min, t_{WL} = 11.56±0.21 min) and the widths for each half of chromatographic curves were a little larger (W_{HB} = 49.40±7.30 s, W_{HL} = 39.3±0.35 s) than those of r-HAp. These results are probably due to the difference in the pore structure of the two HAp's particles formed.

Based on the above obtained results, r-HAp would have a more effective surface for LC to separate BSA and LSZ than would s-HAp.

Resolutions of the Proteins and the Widths of Each Half of the Chromatographic Curves. Figure 7 illustrates influences of the heating temperature of HAp on the chromatographic separation behavior of BSA and LSZ through the r-HAp columns. The t_{WB} and t_{WL} evaluated gradually decreased and the geometry of the peaks drastically varied as the heating temperature of r-HAp rose. These phenomena may be due to two reasons: 1) reduction of pore volume for BSA or LSZ molecular diffusion due to the accelerated sintering of pore structure with elevating the heating temperature[20,21] and 2) variations in the total number, surface proportion and chemical nature of the P- and C-sites on the two HAp's surfaces. For the s-HAp powders prepared, BSA and LSZ were similarly separated through the columns within about 14.5 min retention time.

The column ability for chromatography can be represented as the resolution of sample and the width of half of the chromatographic curve. In the separation of BSA and LSZ using the HAp columns, resolutions (R_S)[23] are expressed by the following equation:

$$R_S = 1.18\,(t_{WL} - t_{WB})/(W_{HL} + W_{HB}). \quad (1)$$

If one evaluates quantitatively the effects of the heating temperature on the chromatographic characteristics of BSA and LSZ, R_S was calculated from the t_{WB}, t_{WL}, W_{HB}, and W_{HL} in all the chromatograms obtained; the results are visualized in Fig. 8 as a function of the heating temperature of the two HAp's. One can recognize that R_S, W_{HB}, and W_{HL} vary as the heating temperature increases and that R_S of r-HAp is considerably higher than that of s-HAp, whereas W_{HB} and W_{HL} of r-HAp are a little bit smaller, except for W_{HL} of HAp's (1073). This clear difference in the R_S, W_{HB}, and W_{HL} between the two Hap's may be caused by variation of the surface proportion and chemical nature of the C-site and P-site distributed in the characteristic pore structures of the particles. At the temperatures of 673—1073 K (designated by Region II, see Refs. 13, 14, 15, and 20), the acid strength of the P-sites and basic strength of the C-sites on r-HAp significantly change, together with the variation in total number or surface proportion of the two sites. As the P-sites on r-HAp are partially substituted by impurities such as 0.6% Mg^{2+} and 0.04% Na^+ ions, in the Region II, the P-sites having the strong acid strength for LSZ adsorption can be formed. The surface nature of the C- and P-sites and the pore structure of HAp's particles may thus considerably affect R_S, W_{HB}, and W_{HL} of the proteins.

As a result, R_S of r-HAp (673), W_{HB} of r-HAp (873), and W_{HL} of r-HAp (873) were respectively evaluated to be about 3.53, 46 s and 35 s as optimal values in all the HAp's. For the HAp's (1073), the R_S was steeply reduced but the W_{HB} and W_{HL} clearly increased. This variation is attributed to the decrease in the total number of the P- and C-sites, which

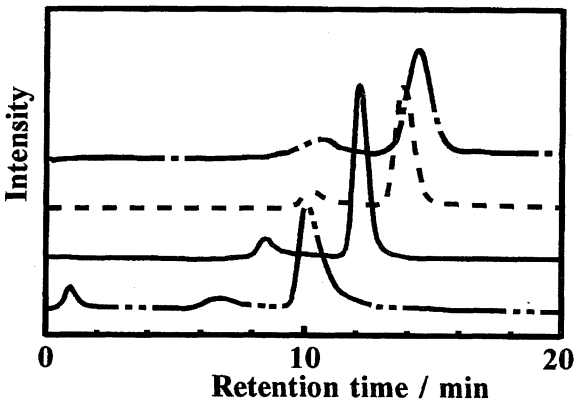

Fig. 7. Effects of heating temperature on the chromatographic separation characteristics of BSA and LSZ through the r-HAp columns, whose packing powders were heated at 273—1073 K: –·– r-HAp(273), --- r-HAp(673), —— r-HAp(873), –··– r-HAp(1073), Injected samples; 1.0 mg cm^{-3} BSA and 1.0 mg cm^{-3} LSZ (0.020 cm^3), Column size (8 mmϕ×50 mm), Flow rate; 1.0 cm^3 min^{-1}, Linear molarity gradient conditions (A liquid; 10 mmol dm^{-3} → B liquid; 500 mmol dm^{-3} Na_2HPO_4 and NaH_2PO_4 buffer solutions, 30 min).

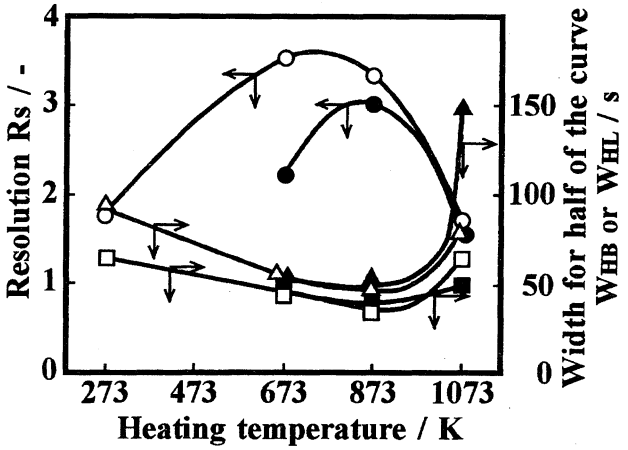

Fig. 8. Resolutions (R_S) of the proteins and widths of each half of the curves (W_{HB} for BSA or W_{HL} for LSZ) as a function of the heating temperature of the two HAp's: Injected samples; 0.5—2.0 mg cm^{-3} BSA and LSZ solutions (0.020 cm^3), ○ R_S on r-HAp, ● R_S on s-HAp, □ W_{HL} of r-HAp, ■ W_{HL} of s-HAp, △ W_{HB} of r-HAp, ▲ W_{HB} of s-HAp.

implies rearrangement of the adsorption sites.

One can conclude that the parameters controlling the chromatographic characteristics of the two proteins are the heat-treatment and starting material of HAp, and that r-HAp is used as one of the most useful adsorbents for HPLC, because of the advantages that desired particle sizes and various acid or basic strength for the optimal chromatographic separation of BSA and LSZ can easily be designed by regulating the atomizer rotation number of the spray-drying and the heat-treatment temperature of HAp.

Conclusions. Spherical particles of HAp single phase derived from cattle bones (r-HAp) or reagents (s-HAp) were obtained by spray-drying the aqueous slurries containing HAp gels at 523 K; the characteristic network structures in each of the HAp gels were clearly discriminated from the viewpoint of crystal growth direction. The specific surface area (S_{AD}) of the particles obtained monotonously decreased as the heat-treatment temperature rose in a stream of water vapor. At the temperatures of 273—673 K, the S_{AD} of r-HAp was 74—144 $m^2 g^{-1}$, which were 44—210% larger than that of s-HAp, whereas at the temperatures of 873—1073 K, S_{AD} of the two HAp's fell in the range of 10—33 $m^2 g^{-1}$. Although the crystallite size (C_S) and the microstructure for the HAp's were not changeable between 273—673 K, at the 873—1073 K range, the C_S gradually increased and the microstructures transformed from microcrystals to spherical or ellipsoidal grains for r-HAp and to prismatic grains for s-HAp. For r-HAp, the acid strength of P-sites or the basic strength of C-sites can sensitively be varied together with the morphology and size of the crystallites by changing the heating temperature. Because of the advantages of the surface nature and pore structure contributing to the chromatographic characteristics of BSA and LSZ on the HAp columns, r-HAp would exhibit higher resolutions of the proteins and smaller widths of each half of the chromatographic curves than s-HAp.

r-HAp produced by the spray-drying and heat treatment processes can effectively be applied as a new adsorbent with high resolution to LC for the separation of various proteins by choosing the heating temperature of HAp.

The authors thank Mr. H. Minoshima and Mr. T. Uchiyama of Hokkaido Industrial Research Institute, for their assistance in the fabrication of spherical HAp particles using the spray dryer.

References

1) G. Bernardi, *Methods Enzymol.*, **21**, 95 (1971).
2) G. Bernardi, *Methods Enzymol.*, **22**, 325 (1971).
3) G. Bernardi, *Methods Enzymol.*, **27**, 471 (1973).
4) S. Tsuru., N. Shinomia, Y. Katsura, Y. Uwabe, M. Noritake, and M. Rokutanda, *Bio-Med. Mater. Eng.*, **1**, 1 (1991).
5) A. Tiselius, S. Hjerten, and O. Levin, *Arch. Biochem. Biophys.*, **65**, 132 (1965).
6) T. Okuyama, T. Ogawa, and M. Ebihara, *Gypsum Lime*, **210**, 323 (1987).
7) F. Honda, H. Honda, and M. Koishi, *J. Chromatogr., A*, **696**, 19 (1995).
8) G. Bernardi and T. Kawasaki, *Biochem. Biophys. Acta*, **160**, 301 (1968).
9) T. Kawasaki, W. Kobayashi, K. Ikeda, S. Takahashi, and H. Monma, *Eur. J. Biochem.*, **157**, 291 (1986).
10) S. Inoue and A. Ono, *Yogyo-Kyokai-Shi*, **95**, 759 (1987).
11) K. Itatani, M. Aizawa, F. S. Howell, A. Kishioka, and M. Kinoshita, *Phosphorus Res. Bull.*, **1**, 35 (1991).
12) K. Itatani, T. Nishioka, S. Seike, M. Aizawa, F. S. Howell, A. Kishioka, and M. Kinoshita, *J. Am. Ceram. Soc.*, **77**, 801 (1994).
13) T. Akazawa, M. Kobayashi, T. Kanno, and K. Kodaira, *Chem. Lett.*, **1996**, 779.
14) T. Akazawa, M. Kobayashi, and K. Kodaira, "The 2nd International Symposium on Inorganic Phosphate Materials '96 Nagoya," Sept. 4—6, 1996, Abstr., p. 9.
15) T. Akazawa, M. Kobayashi, and K. Kodaira, "The Annual Meeting of the Tohoku-Hokkaido Branch in J. Ceram. Soc. Jpn.," 1996, Abstr., p. 84.
16) K. Aoki, T. Takagi, and H. Terada, "Kesseiarubumin Seitai Niokeru Sono Yakuwari," Kodansha, Tokyo (1984), pp. 1 and 13.
17) K. Imabori and H. Yamakawa, "Seikagakujiten," Tokyokagakudojin, Tokyo (1989), pp. 1333 and 1334.
18) T. Kawasaki, M. Niikura, and Y. Kobayashi, *J. Chromatogr.*, **515**, 91 (1990).
19) A. C. Juriaanse, J. Arends, and J. J. Ten Bosch, *Colloid Interface Sci.*, **76**, 220 (1980).
20) T. Akazawa and M. Kobayashi, *J. Ceram. Soc. Jpn.*, **104**, 284 (1996).
21) T. Akazawa, M. Kobayashi, and K. Kodaira, *J. Ceram. Soc. Jpn.*, **104**, 1030 (1996).
22) K. Yamashita, N. Oikawa, and T. Umegaki, *Chem. Mater.*, **8**, 2697 (1996).
23) L. R. Snyder, J. L. Glajch, and J. J. Kirkland, "Practical HPLC Method Development," ed by A. Takahashi and S. Araki, Tokyokagakudojin, Tokyo (1992), pp. 17 and 26.