SYNTHESIS OF HYDROXYAPATITE PARTICLES INTENDED FOR THE
SELECTIVE ADSORPTION OF BASIC PROTEINS

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Abstract: In this study, we report a simple method for synthesizing HAp particles intended for the selective adsorption of basic proteins. HAp particles for the adsorption of basic proteins were prepared from a mixture of calcium acetate and diammonium hydrogen phosphate solutions, both adjusted at pH 10 (HAp-pH10) and a non-adjusted pH (HAp-none). For HAp-none, the amount of bovine serum albumin (BSA, an acidic protein) adsorbed per unit specific surface area was larger than the amount of lysozyme (LSZ, a basic protein) adsorbed. The ratio of the amounts of protein adsorption, i.e., LSZ/BSA, was 0.32 for HAp-none. The amount of LSZ adsorbed on HAp-pH10 was larger than those for BSA. The ratio of LSZ/BSA was 2.38 on HAp-pH10 aged for 1 h. An increase in the aging time (72 h) caused an increase in LSZ/BSA to 3.17.

INTRODUCTION

Hydroxyapatite (Ca10(PO4)6(OH)2, HAp) has attracted interest in the study of protein adsorption behavior. Synthesized HAp crystals are important for the columns of high-performance liquid chromatography. HAp has been reported to exhibit selective adsorption with crystal structures. HAp crystalized in the P63/m space group has unit-cell parameters of a = b = 0.943 nm and c = 0.688 nm. HAp has two crystal planes, a-plane and c-plane. They possess different charges capable of adsorbing different proteins1−3. The a-plane has Ca2+ ions, whose positive charge possesses the ability to adsorb the acidic groups of proteins. PO43− groups are arranged hexagonally on the c-plane. Therefore, positively charged proteins tend to adsorb on the c-plane. For this reason, morphological control of HAp crystals has been studied widely with the aim of selective adsorption between acidic proteins and basic proteins.

Regarding the control of the crystal morphology of HAp crystals, numerous studies have reported the synthesis of HAp particles elongated along the c-axis. Yoshimura and Neira et al. reported the synthesis of HAp whiskers with a sharp-faced hexagonal morphology using hydrothermal treatment of HAp with nitric acid, urea, and cetyltrimethylammonium bromide (CTAB). They also reported the preparation of HAp particles with hexagonal-, prism-, and needle-like morphologies using hydrothermal synthesis4,5. Aizawa et al. reported the synthesis of calcium-deficient apatite fibers using a homogeneous precipitation method. The fibers had long axes of 60−100 μm, and were elongated along the c-axis6−7. Ohtsuki et al. reported needle-shaped HAp through hydrothermal processing of a mixture composed of dicalcium phosphate dihydrate (DCPD; CaHPO4·2H2O) and β-tricalcium phosphate (β-TCP; Ca3(PO4)2)8. Most reports on the morphological control of HAp showed rod- or needle-like crystals elongated along the c-axis, such that acidic proteins such as bovine serum albumin (BSA) were advantageously adsorbed9,10. This is because rod or needle-like crystals elongated along the c-axis have a large a-plane surface area compared with the c-plane surface area. In contrast, Nagata et al. reported the synthesis of HAp plate-like particles using a hydrothermal treatment with alcohol or ethylamine11,12. However, in these plate-like HAp crystals, the wide crystal planes were the a-plane. Aizawa et al. reported the synthesis of plate-shaped HAp particles with a preferred orientation along the c-plane using a homogeneous precipitation method via an enzyme reaction of urea with urease13. However, their protein adsorption behavior has not been reported.

For these reports, HAp particles tended to elongate along the c-axis. It is difficult to readily obtain HAp crystals with a wide c-plane for the selective adsorption of basic proteins. The synthesis of HAp particles for the adsorption of basic proteins
has not yet been established. This difficulty is caused by the α-plane of synthesized HAp particles. Acidic protein adsorption is influenced by the growth of the α-plane stemming from elongation along the c-axis.

This study aims to obtain HAp particles with high adsorption selectivity for basic proteins. In this study, we report a simple method to synthesize HAp particles intended for the selective adsorption of basic proteins.

**EXPERIMENTAL**

**Materials**

Calcium acetate solution [(CH₃COOH)₂Ca·H₂O] (99.0%), diammonium hydrogen phosphate [(NH₄)₂HPO₄] (99.0%), and phosphate buffer (11 mM, pH 7.4) were procured from Wako Pure Chemical Industries, Japan. BSA (isoelectric point [pI] = 4.7, an acidic protein) and lysozyme (LSZ, pI = 11.1, a basic protein) were procured from Sigma-Aldrich. All materials were of analytical grade and were used without further purification.

**Preparation of HAp**

HAp was synthesized from a mixture of a 50 mM solution of (CH₃COOH)₂Ca·H₂O and a 30 mM solution of (NH₄)₂HPO₄, both solutions being adjusted to a pH of 10. Two-hundred milliliters of the 30 mM (NH₄)₂HPO₄ solution was added to 200 mL of the 50 mM (CH₃COOH)₂Ca·H₂O solution. The resulting mixture was subsequently stirred for 10 min at 20 °C. The temperature of the mixture was increased at 1 °C/min using a cool stirrer from NISSIN (SWC-9000). The mixture was further stirred at 60 °C for 72 h. The time at which the mixture was maintained at 60 °C is hereafter referred to as the aging time. The products were recovered by filtration, washed repeatedly with distilled water, and freeze-dried (HAp-pH10). For comparison, additional samples of both solutions were not adjusted to a pH of 10, with the rest of the synthesis method except for the reaction temperature being the same as previously described (HAp-none). In addition, HAp was synthesized using various aging times. The synthesis method was the same as for HAp-pH10, with the exception of the reaction times. The mixture was further stirred at 60 °C for 3, 5, 24, 48, and 72 h.

**Characterization of HAp**

The morphology of the samples was studied using field-emission scanning electron microscopy with a Hitachi S-4300 electron microscope (Japan) operated at 20 kV. The products were characterized by powder X-ray diffraction (XRD) using a Rigaku RINT2000/PC equipped with a Cu–Kα radiation source operated at 30 mA and 40 kV. Data were collected in the 2θ range from 3.0 to 60.0° at a scan speed of 2.000°/min.

All the samples were characterized by nitrogen gas adsorption/desorption isotherms using a Shimadzu TriStar3000 system (Japan).

**Protein adsorption on HAp**

The protein adsorption behavior was measured using BSA and LSZ. Five milligrams of HAp particles were mixed in 1 mL of a 0.5 mg/mL protein solution dissolved in a 11-mM phosphate buffer solution. The mixture was stirred overnight at 4 °C. The mixture was then centrifuged at 12000 rpm for 12 min. The amount of unadsorbed protein in the supernatant was determined using a Bradford protein assay. The amount of protein adsorbed on HAp was calculated on the basis of the amount of protein in the supernatant.

The equation used to calculate the protein adsorption per unit specific surface area is

\[ q = \frac{(C_o - C_a)}{(A_{HA} \cdot M)}, \]  

where \( q \) (mg·m⁻²) is the amount of protein adsorbed per unit specific surface area, \( C_p \) (mg·mL⁻¹) is the concentration of the initial protein solution, \( C_s \) (mg·mL⁻¹) is the concentration of protein in the supernatant, \( A_{HA} \) (m²·g⁻¹) is the specific surface area of the sample, and \( M \) (mg) is the mass of the sample used in the adsorption experiments.

**RESULTS AND DISCUSSION**

**Influence of the pH values on the amount of basic protein adsorption**

The protein adsorption properties of the HAp particles were studied using two types of proteins: BSA, an acidic protein and LSZ, a basic protein. The results for protein adsorption on HAp-pH10 and HAp-none are shown in Figure 1. For HAp-none, the amount of BSA adsorbed per unit specific surface area was larger than those of LSZ. The ratio of the amount of protein adsorption, i.e., LSZ/BSA, was 0.32 for HAp-none. In contrast, the amount of LSZ adsorbed on HAp-pH10 was larger than that for BSA.

The value of LSZ/BSA was 3.17. The results of protein adsorption indicate that the amount of protein adsorption was significantly affected by the pH value of the solutions used for HAp preparation.
SEM images of synthesized HAp for different pH values are shown in Figure 2. The SEM images show that the HAp-pH10 particles have a grain-like morphology, although the morphology of HAp-none particles are rod-like. The pH value of the solutions used for HAp preparation changes the morphology of products. Figure 3 shows the XRD patterns of the products synthesized using different pH solutions. The peaks associated with HAp (JCPDS: 90432) are observed in the pattern for the products. The patterns confirmed that the samples were single-phase HAp.

**Influence of the aging time on the amount of basic protein adsorption**

HAp particles were synthesized with various aging times with the intention of increasing the amount of basic protein adsorption and to improve the selective protein adsorption of basic protein.

Products aged for various times were observed using SEM [Fig. 4(a)–(f)]. The morphology of these products was grain-like. The products aged for 1–72 h contained grain-like crystals with both widths and lengths of 20 ± 5 nm. The sizes of the products aged for 1–72 h did not vary remarkably. Figure 5 shows the XRD patterns of the products synthesized using different aging times. In the patterns of the products synthesized at 60 °C at aging times of 1, 3, 5, 24, 48, and 72 h, all the peaks were assigned to the HAp. The patterns confirm that the samples were single-phase HAp [Fig. 5(a)–(g)]. The sharpness of the XRD peaks among the patterns for HAp aged for 1–72 h were similar; thus, HAp samples aged for 1–72 h exhibited similar crystallinities.

The ratios of LSZ/BSA are shown in Table 1. These results show that the ratio of LSZ/BSA was 2.38 on HAp-pH10 aged for 1 h. An increase in the aging time (72 h) caused an increase in the ratio of LSZ/BSA to 3.17. A longer aging time improved the selectivity of basic protein adsorption.

The values of the ζ-potential of HAp particles are shown in Table 1. Compared with the results of protein adsorption, the ratio of LSZ/BSA was not significantly affected by the values of the ζ-potential. The values of the ζ-potential for all HAp particles obtained in this study were negative, and so the amount of protein adsorbed would not be remarkably influenced. From these results, in this study, the inhibition of crystal growth along the c-axis of HAp synthesized at pH 10 resulted in an increase in the amount of LSZ adsorbed and a decrease in the amount of BSA adsorbed.
CONCLUSIONS

HAp particles for the selective adsorption of basic proteins were successfully obtained using a simple method. The synthesized morphology and protein adsorption behavior were significantly changed by adjusting the pH values of the preparation solutions. The grain-like HAp particles synthesized at a pH of 10 significantly increased the adsorption amount of LSZ. The value of LSZ/BSA was 3.17 for HAp-pH10 aged for 72 h.

REFERENCES