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Protein release properties on carbonate ion-containing apatite in a flow system

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Release properties of bovine serum albumin (BSA) and egg white lysozyme (LSZ) on hydroxyapatite containing carbonate ion (CHAp) of various contents were examined with our new flow experimental system. The desorption ratio: the r_{de}, defined as the ratio of desorbed amount to initially-adsorbed one, was employed as an index of binding strength of these proteins to the surface. Dependency of the content of the carbonate ion on the r_{de} showed reverse tendency for BSA and LSZ; the r_{de} of BSA increased as an increase of content of the carbonate ion, while that of LSZ decreased as the increase of the content. This result clarified a difference of primary adsorption sites between BSA and LSZ. As incorporation of the carbonate ion into apatite retarded the growth of the crystal along the *c axis*, the exposed area of the *c face*, i.e. the primary adsorption site of LSZ, the *p site* increased and that of the *a (b) face*, i.e. the primary site of BSA, the *c site* decreased.

Key-words : Hydroxyapatie, Carbonate ion, Protein, Adsorption and release, Flow system

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1. Introduction

Hydorxyapatie (HAp) has been a typical biomaterial used as bone-substitute due to its chemically analogue to bone[1)] and biocompatibility. Recently, HAp has been paid attention as a protein-drug carrier with the controlled-release ability.[2),3)] An advantage of HAp as the career is that it does not cause unfolding, aggregation, and chemical degradation of protein during resorption of HAp and drug-releasing in the body as polymers sometimes do.[4)]

In vitro investigations on release properties of protein on HAp have been frequently carried out prior to in vivo ones. However, most of them have been done with a batch or a semi-batch system in which readsorption of protein plausibly occurs or eluent is changed regularly to avoid the readsorption and to keep a constant composition as much as possible. Under such cases, intrinsic behavior tends to become vague. Actually, in our batch system, desorbed amounts of proteins from HAp showed maxima being followed by gradual decrease at a elapsed time, indicating their readsorption and making the release properties unclear.[5)] Furthermore, a drug-loaded carrier is exposed to flow of body fluid, e.g. blood flow. For simulating a fluid-flow condition in the body, we developed a new flow system and successively monitored adsorption and desorption behavior on carbonate-containing apatite (CHAp) with the system.[6)] The ratio of the desorbed amount of protein to the initially adsorbed one; the r_{de} was larger in this flow system than in the previous batch system. Because the r_{de} was considered to an index of binding strength between protein and the surface of apatite, this result showed a large difference between the binding strength under the dynamic condition and that under the static one and feasibility of this flow system as a system to evaluate the release properties of proteins under a body fluid-flow environment.

In the present paper, we made an attempt to clarify the difference of binding strength of two proteins to the HAp surface with our newly-developed flow system. For this purpose, four kinds of HAp of various contents of the carbonate ion (CHAp) were prepared, where the carbonate ion in HAp structure has known to change the crystallographic properties of HAp. Adsorption and desorption behavior and the r_{de} were compared for the four CHAp's and two kinds of proteins with different isoelectric points and thus different primary adsorption sites were employed.

2. Materials and methods

2.1 Preparation of the samples

Preparation of the carbonate-containing apatite (CHAp) was done as in the previous paper.[7)] A mixture of 0.06 M $(NH_4)_2HPO_4$ and 0.06 M NH_4HCO_3 aq. soln. was flown into 0.1 M $Ca(NO_3)_2 \cdot 4H_2O$ aq. soln. at $4.5 \pm 0.5\,cm^3/min$ with a peristaltic pump under nitorgen gas bubbling. CHAp was produced and the crystal was grown under agitation with a magnetic stirrer at 323 ± 5 K for 24 h at constant pH's of 6.0, 7.0, 8.5, or 10.5 with a pH controller. The content of the carbonate ion of each sample was 1.1, 2.7, 5.8, or 8.1 mass %, and the specific surface area was 75, 91, 151, or 162 m^2/g, respectively. These CHAp's will be designated as C(1.1), C(2.7), C(5.8), or C(8.1)

2.2 Adsorption and desorption behavior of protein

Bovine serum albumin (BSA; Sigma-Aldrich Co. A4503) and egg white lysozyme (LSZ; Sigma-Aldrich Co. L6876) were used as model protein drugs. **Table 1** shows their physicochemical properties, where BSA molecule is longer than LSZ by three times and the isoelectric points (*pI*) were 4.8 for BSA and 11.2 for LSZ, respectively. Adsorption and desorption behavior of protein on CHAp was monitored with our developed-flow system. The apparatus was composed of a control unit attached to six electric valves for automatic switching of solution, a monitor, a sample-loaded column, a peristaltic pump to introduce the solution into the column, a UV detector, and a recorder. 1.0

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g-protein/dm^3-phosphate-buffered solution (PBS) was flown into the column at a flow rate of 1 cm^3/min for following dynamic adsorption behavior. The loaded amount of CHAp powder was 0.28–0.59 g with a constant loading layer 1.25 cm thick to obtain constant apparent interface area between the solution and the CHAp as much as possible. The solution was successively switched to 5 mM PBS and 20 mM PBS as the eluent of protein and dynamic desorption behavior was monitored. The adsorbed and desorbed amount was estimated from graphical integration of adsorption and desororpton curves. The detailed experimental and analytical procedure was described in a previous manuscript.[6)]

3. Results and discussion

Figures 1 and **2** show adsorption and desorption behavior of BSA and LSZ, respectively. The concentration of BSA reached plateau within 4 h in step A, indicating completion of adsorption for all the samples. For the C(1.1), the induction time for rise of the concentration was observed, which clearly suggested the adsorbed amount was larger than three other samples. In 5 mM PBS (step B), BSA monotonously decreased. Switching to 20 mM PBS in step C accelerated desorption of BSA: the initial steep increase followed by the gradual decrease for all the samples. In contrast to BSA, adsorption of LSZ for the C(1.1) reached saturation faster than other samples shown by step A in Fig. 2, leading to the least adsorbed amount of all the samples. In 5 mM (step B), the C(1.1) showed a different behavior; it took 4 h to stop desorption while 6 h for other three samples. This indicates that binding strength of LSZ to the surface of the C(1.1) is the lowest. In 20 mM PBS (step C), the similar responses to those of BSA were observed: initial large amount of desorption which was followed by gradual decrease.

Table 1. Chemical and physical properties of proteins used in this study

	Bovine serum albumin (BSA)	Egg white lysozyme (LSZ)
Isoelectric point: pI	4.8	11.2
Molecular weight (g·mol^{-1})	66,000	14,300
Molecular size (nm)	4 × 4 × 14	3 × 3 × 4.5
The number of amino residues	580	129
Morphology	Ellipsoidal	Ellipsoidal

The adsorbed amounts of BSA and LSZ were estimated from graphical integration of the adsorption curves in step A and shown in **Figs. 3** and **4**, respectively, where the left and the right coordinates were mg-protein per unit sample surface area [mg–protein·(m^2-sample)$^{-1}$] and mg-protein per unit sample mass [mg–protein·(g-sample)$^{-1}$], respectively. As had expected from the adsorption curve (step A) in Fig. 1, the C(1.1) showed the most amounts of BSA for both in Fig. 3. Adsorption is a surface phenomenon and therefore, the adsorbed amounts per unit sample surface area were compared. The amounts of BSA decreased much as the increase of the contents of the carbonate ion from 1.1 to 2.7 mass %. More increase to 5.8 and 8.1 mass % did not change the amounts so much. With a batch system in our previous result, the adsorbed amounts decreasesd with the content of the carbonate ion. This difference for two system was possibly due to much shorter contact time of BSA to the CHAp in the flow system than 24 h in the batch system. For LSZ, the adsorbed amounts per unit sample surface area were not different so much for the four CHAp shown by Fig. 4. This dependency of the adsorbed amounts of LSZ on the content of the carbonate ion were similar to those with our batch system.[7)]

In order to examine the difference of binding strength between the proteins and the surfaces of the samples, the desorption ratio (r_{de}) of BSA and LSZ in 5 mM (step B in Figs. 1 and 2) and 20 mM-PBS (step C) was compared among the samples of various contents of the carbonate ion in **Figs. 5** and **6**. In 5 mM-PBS (step B), the r_{de}'s of BSA were comparable, 0.15–0.19

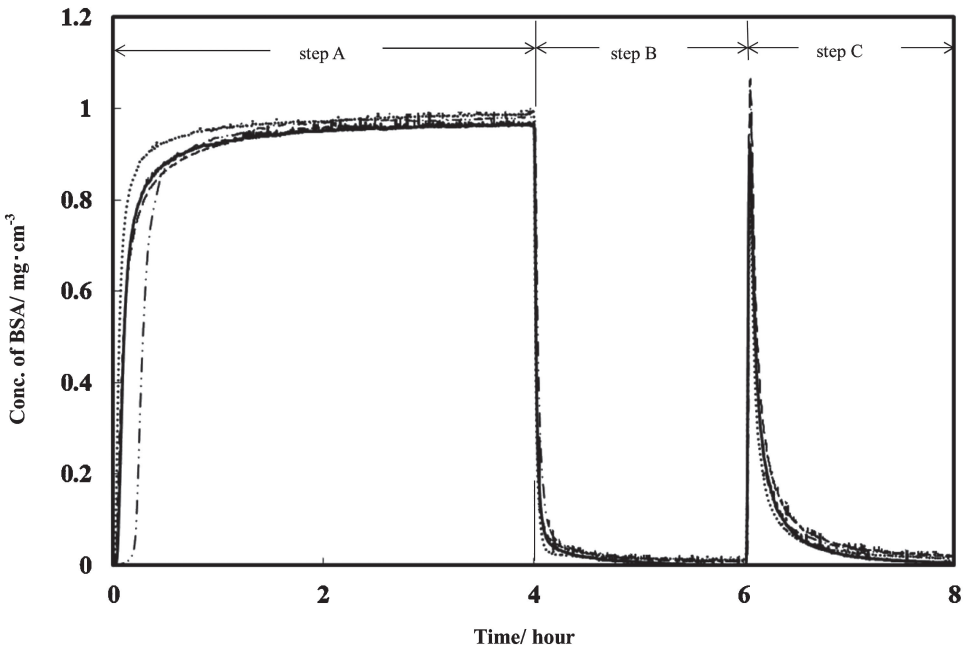

Fig. 1. Adsorption and desorption behavior of LSZ on apatite of various contents of the carbonate ion.
—·— C(1.1); ——— C(2.7); ····· C(5.8); – – – – C(8.1); step A 1g-BSA/dm^3-5 mM PBS; step B 5 mM PBS; step C 20 mM PBS; flow rate 1 cm^3/min.

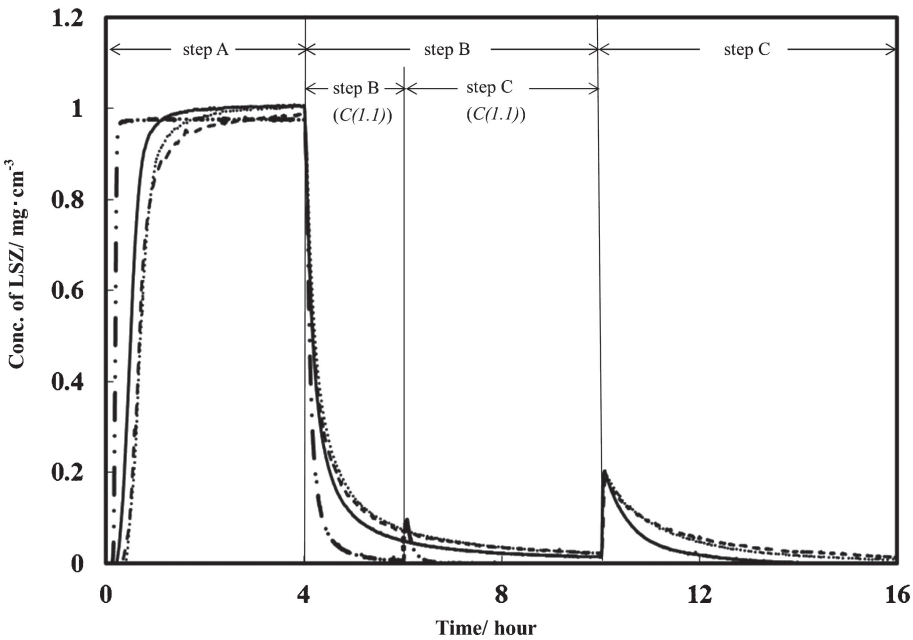

Fig. 2. Adsorption and desorption behavior of LSZ on apatite of various contents of the carbonate ion.
—·— C(1.1); ——— C(2.7); ····· C(5.8); – – – – C(8.1); step A 1 g-LSZ/dm^3-5 mM PBS; step B 5 mM PBS; step C 20 mM PBS; flow rate 1 cm^3/min.

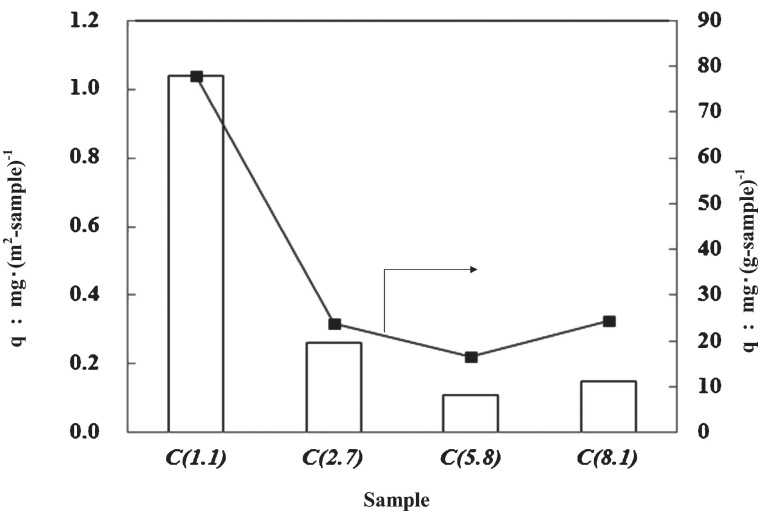

Fig. 3. Adsorbed amount of BSA on apatite of various contents of the carbonate ion. Bar (□) mg-BSA·(m^2-sample)$^{-1}$; ■ mg-BSA·(g-sample)$^{-1}$.

shown by filled bars in Fig. 5. Those of LSZ in 5 mM-PBS were much higher for all the samples, 0.59–0.83, than BSA as shown by filled bars in Fig. 6. This suggests that binding strength between BSA and the surface are larger than LSZ. Adsorption of protein on apatite occurs with electrostatic force[8] and this result may be therefore attributed to more numbers of binding sites such as positively- or negatively-charged amino residues for BSA due to larger size of BSA than LSZ as shown by Table 1. Furthermore the ionized *C* or the *N terminal* of protein also definitely contributes to binding: the carboxyl anion ($-COO^-$) for BSA and the amino cation ($-NH_3^+$) for LSZ are present because the isoelectric point (*pI*) of the former and the latter are 4.8 and 11.2, respectively.

In 20 mM PBS (Step C), both the proteins desorbed again after completion of desorption in 5 mM PBS for all the samples. This suggests that ions of four time concentration in PBS (Step B) substitutively adsorbed in more amount on the surface and promote desorption of the proteins. Let us focus effect of the conent of the carbonate ion on the r_{de}. The r_{de}'s for BSA increased with the content of the carbonate ion in 20 mM PBS. This reflects that the incorpration of the carbonate ion leads to weaker binding strength of BSA and the surface. The difference of binding strength indicates crystallographic difference of each CHAp: incorporation of the carbonate ion into HAp structure inhibits crystal growth along the *c axis* and decreases the ratio of the exposed area of the *a and b faces* of HAp on which the primary adsorption site of BSA, the *c site* (calcium ion) is.[9] This leads to weaker binding between BSA and the surface and increases the r_d. In Fig. 3, the adsorbed amount of BSA decreased as the increase of the content of the carbonate ion,

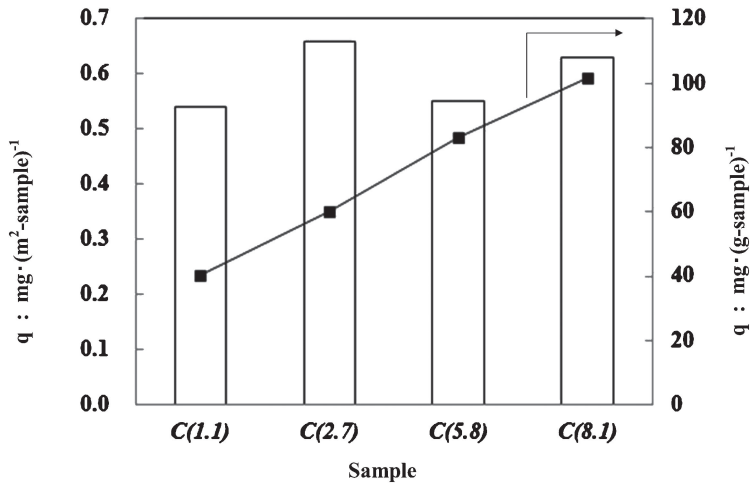

Fig. 4. Adsorbed amount of LSZ on apatite of various contents of the carbonate ion. Bar (□) mg-LSZ·(m^2-sample)$^{-1}$; ■ mg-LSZ·(g-sample)$^{-1}$.

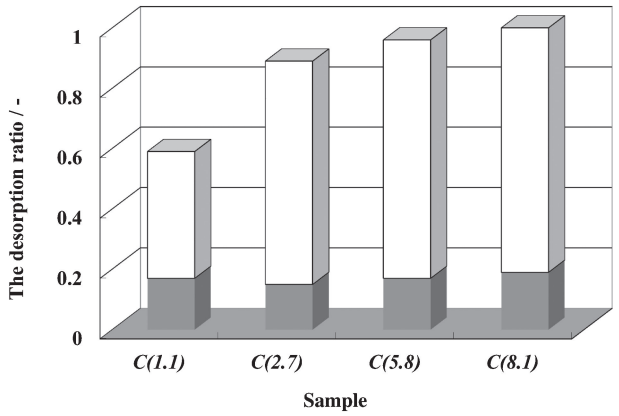

Fig. 5. The desorption ratio of BSA on the carbonate ion-containing HAp. ■ in 5 mM; □ 20 mM-PBS.

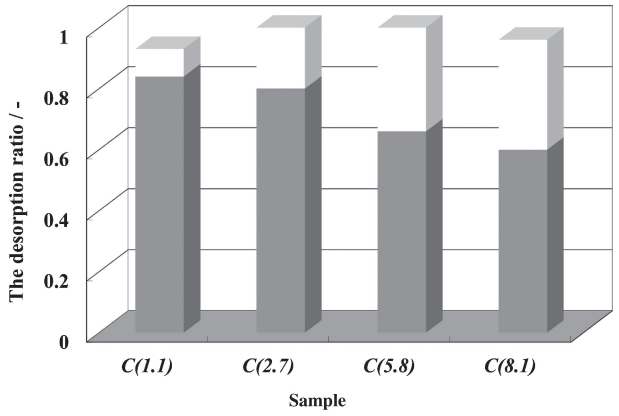

Fig. 6. The desorption ratio of LSZ on the carbonate ion-containing HAp. ■ in 5 mM; □ 20 mM-PBS.

which was also ascribed to the decrease of the exposed ratio of the *c site*.

On the contrary, the r_{de} of LSZ decreased from 0.83 to 0.59 in 5 mM PBS with increase of the content as shown by filled bars in Fig. 6, leading to stronger binding between LSZ and the surface. LSZ adsorbs primarily on the *p site* (phosphate ion) on the *c face*.[10] The incorported carbonate ion increases the numbers of this site due to change in crystallographic morphology as mentioned before, leading to decrease of the r_{de}. In contrast to BSA, the adsorbed amount of LSZ did not depend on the content of the carbonate ion as shown in Fig. 4 in spite of the change of the r_{de}. Luo and Andrade[8] mentioned that the binding strength of the negative charge of PO_4^{3-} at *the p site* with NH_3^+ in proteins is not so much high as that of the positive charge of Ca^{2+} at *the c site* with COO^- in proteins. Their statement and our result may coincidentally suggest that the adsorption of LSZ on the surface is not so much selective as that of BSA.

Conclusively, with our flow system, the diffference of binding strength of BSA and LSZ to the surface of carbonate-containing apatite was clarified: the desorption ratios of BSA and LSZ in the flow of PBS showed reverse tendency.

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