Interaction between hydroxyapatite and proteins by liquid chromatography using simulated body fluids as eluents

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Hydroxyapatite (HAp) column chromatography for proteins using simulated body fluids (SBFs) as eluents was investigated in order to mimic the flow environment of human body fluid on the HAp surface *in vivo*. Acidic plasma proteins such as albumin, γ-globulin, and fibrinogen having carboxyl groups with a negative charge were suggested to adsorb at positively charged sites (Ca sites) on the HAp surface. These acidic plasma proteins were not even eluted at higher inorganic ion molarities of human body fluid. Whereas basic proteins such as papin, cytochrome *c*, and lysozyme were easily eluted at lower inorganic ion molarities of human body fluid due to existing Na^+, Ca^{2+}, Mg^{2+} and $(CH_2OH)_3CNH_4^+$ ions in the eluents. The elution behavior of proteins using SBF in the presence or absence of glucose did not change. From these considerations, bone matrix proteins as acidic and neutral proteins would be considered an advantageous condition in order to adsorb on HAp *in vivo*.

1. Introduction

Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] (HAp) is the main inorganic component of hard tissues such as bone and tooth, and is utilized in biomaterials such as coating layers of artificial bones [1, 2], bone fillings [3] and drug carriers due to its excellent bioaffinity. If these materials were implanted *in vivo*, plasma proteins would adsorb immediately on the surfaces. The main plasma proteins is albumin (3300–4000 mg/dL), globulin (880–3530 mg/dL), and fibrinogen (340–430 mg/dL) [4]. These plasma proteins effect the dissolution [5], nucleation and crystal growth of HAp [6, 7], as well as cell adhesion on HAp [8, 9]. Therefore, it is important to elucidate interfacial phenomena between HAp and proteins *in vivo*.

Liquid chromatography using HAp as an adsorbent has already been utilized in purification and separation of biological molecules such as nucleic acids and proteins [10–12]. Potassium phosphate buffer and sodium phosphate buffer (NaP) are mainly used as eluents. The separation mechanism of proteins on a HAp column could be interpreted by considering two kinds of adsorbing sites, i.e. positively charged sites (Ca sites) on HAp which can adsorb negatively charged sites such as carboxyl groups of proteins, and negative charged sites (P sites) which can adsorb positively charged sites such as amino or guanidinyl groups [12]. The elution behavior of proteins using various inorganic ion eluents [13–15] and the effect of pH on proteins adsorption [16] has also been studied. However, HAp chromatography mimicked human body environment did not reported.

The authors mimicked the flow environment of human body fluid by the HAp chromatography using simulated body fluids (SBFs) as eluents in order to evaluate the interactions between HAp and proteins. Acidic plasma

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proteins (albumin, γ-globulin, and fibrinogen) and basic proteins (papain, cytochrome *c*, and lysozyme) were use as adsorbates. The effect of SBF containing glucose on the elution behavior of proteins was also examined because human fluid contains 45–95 mg/dL glucose.

2. Materials and methods

2.1. Adsorbent

The HAp adsorbent was synthesized by a hydrolysis process. Briefly, α-$Ca_3(PO_4)_2$ was prepared by heating a 2 : 1 molar mixture of $CaHPO_4 \cdot 2H_2O$ (reagent grade; Kanto Pure Chemical Industries, Japan) and $CaCO_3$ (reagent grade; Kanto Pure Chemical Industries) at 1000 °C for 9 h, and subsequently 1200 °C for 1 h. The hydration reaction of α-$Ca_3(PO_4)_2$ was carried out in water at pH 9 and 90 °C for 1 h. The pH was maintained constant with NaOH solution. The resulting powder was identified by X-ray powder diffraction (M03X-HF; MAC Science, Japan), and examined by scanning electron microscopy (S-2380N; Hitachi, Japan). The specific surface area was determined using the BET method (2100-01; Shimadzu, Japan). HAp powder was identified as a single phase, which was an aggregated from consisting of needle-like crystals. The specific surface area was 17 m^2/g.

2.2. Adsorbates

Albumin from bovine serum (> 95%), fibrinogen from bovine plasma (> 80%), γ-globulin from human serum (> 95%), cytochrome *c* from horse heart (> 90%), and lysozyme from egg white were purchased from Wako Pure Chemical Industries (Japan). Papain from papaya latex was purchased from Sigma Chemical (USA). These proteins were used without further purification.

2.3. Liquid chromatography

The experimental system for the chromatography studies was assembled using an ion exchange chromatograph (2000i/sp; Dionex, Japan) from which the suppressor had been removed. The HAp adsorbent was used without granulation. Columns were packed with adsorbent by introducing the suspension into a column made of polyether ether ketone (I.D. = 4 mm, $L = 250$ mm). The adsorbent in the column was exchanged with fresh adsorbent at daily intervals to avoid changes of the chemical surface properties of the crystals.

First, in order to clarify the adsorption sites on the HAp surface and the adsorption groups of the proteins, we used the inorganic ion molarity gradient method with an equimolar mixture of Na_2HPO_4 and NaH_2PO_4 (NaP, pH = 6.8), and NaCl. The NaP molarity is given as the phosphate ion molarity (sodium ion molarity in NaP is 1.5 times the phosphate ion molarity). A solution of NaP (10 mM) was used as initial eluent and for stabilizing the pH (6.8) during the gradient elution of NaCl. The molarity gradient of NaP and NaCl was 4.9 and 5.0 mM/mL, respectively.

Thereafter, the adsorption behavior of proteins using SBF as eluent was investigated. The inorganic ion composition of SBF is listed in Table I, corresponding with inorganic ions molarities in human body fluid. Reagent grade NaCl, KCl, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, $NaHCO_3$, K_2HPO_4, and Na_2SO_4 were used for preparation the eluent solutions. The inorganic ion concentration ratio varied lineally between 0 and 1.2 using normalized inorganic ion concentration ratios (1.0SBF) in Table I. A solution of Tris-buffer (20 or 75 mM) was used as initial eluent, and the same concentration Tris-buffer co-existed during the molarity gradient elution of SBF eluents. The elution behavior of proteins in SBF containing glucose was examined using 500 mg/dL glucose co-existing in chromatographic experiments because human body fluid contains 45–95 mg/dL glucose.

The concentration and amount of proteins injected were adjusted to 1–10 mg/mL and 50–100 μL, corresponding to the absorbance and solubility of proteins, respectively. The proteins eluted were monitored by ultraviolet absorption at 280 nm. All experiments were performed at a flow rate of 0.5–2.0 mL/min at room temperature. The inorganic ion molarities of the eluents were determined using an Abbe refractometer. The elution molarity of proteins is defined as the inorganic ion molarity of the NaP or NaCl eluent, or the inorganic ion concentration ratio of the SBF eluents at the main peak maximum of the chromatograms. The elution molarity in Table II is given as the mean values of repeated runs ($n = 2$–3), and has been reported previously to be independent of flow rates [17].

TABLE I Ion compositions of SBF eluents

Eluent	Ion concentration (mM)								
	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}	Tris-buffer[a]
Human body fluid	142.0	5.0	2.5	1.5	103.0	27.0	1.0	0.5	—
No. 1	142.0	5.0	2.5	1.5	147.8	4.2	1.0	0.5	75
No. 2	142.0	5.0	2.5	1.5	147.8	4.2	1.0	0.5	20
No. 3	142.0	5.0	2.5	1.5	151.0				20
No. 4	142.0	5.0			147.0				20
No. 5	142.0				142.0				20

[a] Tris-buffer: solutions were buffered at pH 7.25 with trishydroxymethyl-aminomethane [$(CH_2OH)_3CNH_2$] and HCl. Initial eluents of No. 1 and Nos. 2–5 used 75 and 20 mM Tris-buffer. Inorganic ion concentrations in Nos. 1–5 have been defined as the ion concentration ratios compared to human body fluid.

TABLE II SBF ion concentration ratios of proteins

No.	Protein (origin)	pI[a]	No. 1	No. 2	No. 3	No. 4	No. 5
1	Albumin (bovine serum)	4.7–4.9	> 1.2	> 1.2	> 1.2	> 1.2	> 1.2
2	Fibrinogen (bovine plasma)	5.5	> 1.2	> 1.2	> 1.2	> 1.2	> 1.2
3	γ-Globulin (human serum)	5.3–7.3	> 1.2	> 1.2	> 1.2	> 1.2	> 1.2
4	Papain (papaya latex)	8.8–9.5	0	0.1	0.1	0.2	0.2
5	Cytochrome *c* (horse heart)	10.1	0	0.2	0.2	0.4	0.4
6	Lysozyme (egg white)	11.2	0	0	0	0	0

[a] Isoelectric point.

Inorganic ion concentration ratios in the table were normalized inorganic ion concentrations of SBF eluents as given in Table I. Data are represented as the mean values of repeated runs ($n = 2$–3).

3. Results

3.1. HAp column chromatography using NaP and NaCl

Fig. 1 shows chromatograms of albumin, fibrinogen, γ-globulin, papain, cytochrome *c*, and lysozyme using the NaP eluent. All proteins were eluted below 0.3 M NaP. Fig. 2 shows chromatograms using first an NaCl molarity gradient and a successive NaP molarity gradient. Cytochrome *c*, lysozyme, and papain were eluted using an NaCl molarity gradient, but albumin, fibrinogen, and γ-globulin were not eluted even at 0.3 M NaCl. These proteins were easily eluted by a subsequent NaP molarity gradient. The elution behavior of mixed proteins has been in agreement with individual elution behavior of proteins.

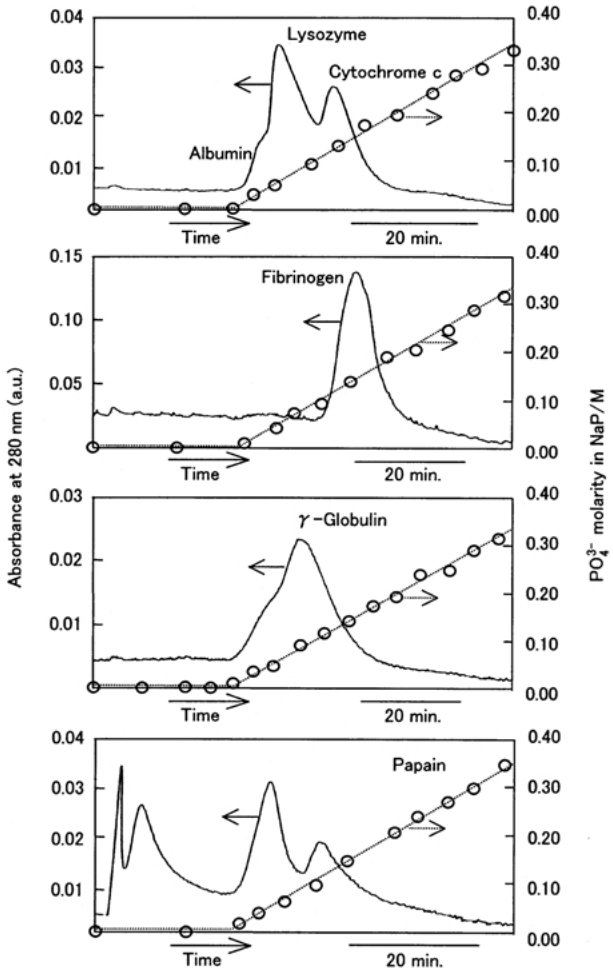

Figure 1 Chromatograms of albumin, γ-globulin, fibrinogen, papain, lysozyme, and cytochrome *c* using the NaP molarity gradient method.

3.2. HAp column chromatography using SBF

Fig. 3 shows chromatograms for cytochrome *c* using various SBF eluents. Cytochrome *c* was eluted in 75 mM Tris-buffer used as an initial eluent (No. 1), but was not eluted in 20 mM Tris-buffer. When carrying out a gradient elution of SBF using No. 2 solution, cytochrome *c* was eluted at 0.2 SBF ion concentration ratio. Eliminating HCO_3^{2-}, HPO_4^{2-} and SO_4^{2-} ions from No. 2 solution (No. 3), the elution molarity and the peak shape of the chromatogram did not change. However, when excluding Ca^{2+} and Mg^{2+} ions from No. 3 solution (No. 4), the elution molarity increased from 0.2 SBF to 0.4 SBF, and the chromatogram shape

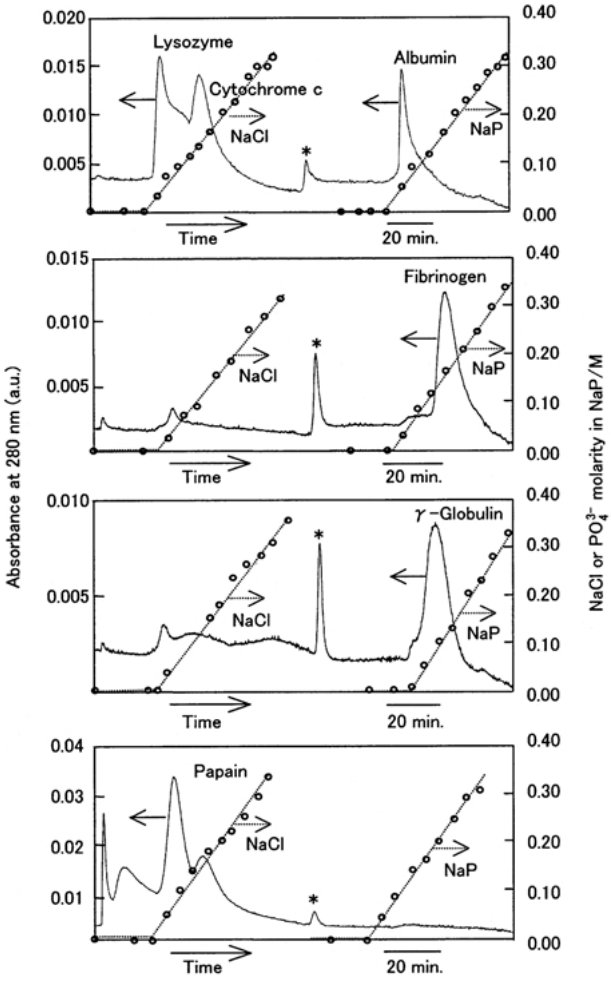

Figure 2 Chromatograms of albumin, γ-globulin, fibrinogen, papain, lysozyme, and cytochrome *c* using the NaCl–NaP double molarity gradient method. The peaks marked with an asterisk (*) are due to the changing of eluent.

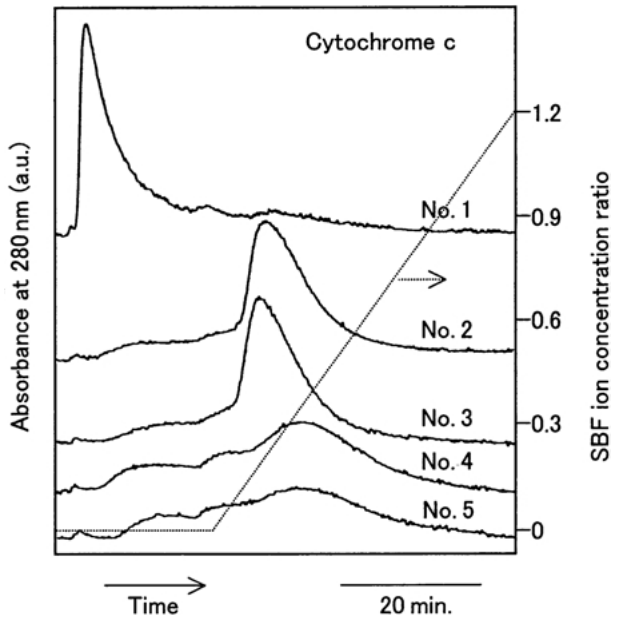

Figure 3 Chromatograms of cytochrome *c* using SBF eluents.

broadened. When removing K^+ ions from No. 4 solution (No. 5), no significant difference between Nos. 4 and 5 chromatograms is observed.

Table II lists the elution molarities of the proteins, albumin, fibrinogen, γ-globulin, papain, cytochrome *c*, and lysozyme using SBF eluents with various inorganic ion compositions. Albumin, fibrinogen, and γ-globulin were not eluted even at 1.2 SBF inorganic ion concentration ratios in all eluents, whereas papain, cytochrome *c*, and lysozyme were easily eluted below 1.0 SBF.

3.3. HAp column chromatography using SBF containing glucose

Fig. 4 shows chromatograms of papain using No. 2 SBF eluent with and without glucose. The elution molarity and shape of the chromatogram were not influenced by glucose. Albumin, fibrinogen, and γ-globulin were not eluted below 1.2 SBF using SBF containing glucose.

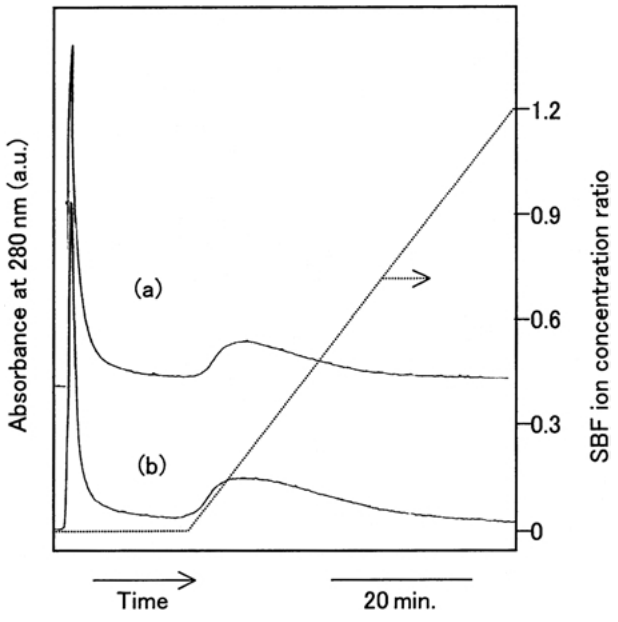

Figure 4 Effect of glucose in SBF (No. 2 in Table I) on the papain adsorption onto HAp. (a) No. 2 SBF eluent with 500 mg/dL glucose, (b) No. 2 SBF eluent without glucose.

4. Discussion

HAp column chromatography for various proteins has been already investigated by using inorganic ion eluents, and the adsorption mechanism can be explained as follows [12]. Acidic proteins (pI < 7) having carboxyl (or phosphate) groups with a negative charge mainly adsorb at positively charged sites (Ca sites) on the HAp surface and elute with increasing anion molarity such as phosphate ions in the eluents. Basic proteins (pI > 7) possessing amino or guanidinyl groups with a positive charge mainly adsorb at negatively charged sites (P sites) and elute with increasing cation molarity such as sodium, potassium, calcium, and magnesium ions in the eluents. Exceptionally, acidic proteins are not eluted using chloride ions. The elution ability of Ca^{2+} and Mg^{2+} ions for basic proteins is considerably higher than that of Na^+ and K^+ ions. Therefore, the adsorption behavior of plasma proteins such as albumin, fibrinogen, and γ-globulin, as shown in Figs. 1 and 2, was found to be classified as acidic proteins. Whereas the elution behavior of basic proteins such as papain, cytochrome *c*, and lysozyme was found to be classified as basic proteins.

The elution behavior of proteins using SBF as eluents could be explained by the above described mechanism. Acidic proteins such as albumin, fibrinogen, and γ-globulin were not eluted even at 1.2 SBF, as shown in Table II. This indicates that the ion concentrations of HCO_3^{2-}, HPO_4^{2-}, and SO_4^{2-} in human body fluid do not enough to elute the acidic proteins. On the other hand, basic proteins such as papain, cytochrome *c*, and lysozyme were easily eluted below 1.0SBF in all eluents. The elution of basic proteins considered to be caused by positively charged Na^+, K^+, Ca^{2+}, Mg^{2+}, and trishydroxymethyl-aminomethane $[(CH_2OH)_3CNH_4^+]$ in the eluents. Ca^{2+} and Mg^{2+} ions in the eluents were particularly influenced the elution of basic proteins, as shown in Fig. 3. A difference in the elution behavior of proteins in the presence and absence of glucose was not observed, as shown in Fig. 4. Therefore, non-ionized functional groups or non-ionized biomolecules was suggested to do not affect the interaction between HAp and proteins in human body fluid.

Main bone matrix proteins (e.g., type I collagen, osteonectin [18, 19], osteocalcin [19], and, osteopontin) are acidic or neutral proteins, which would also be an advantageous condition in order to adsorb on HAp, and the adsorbed proteins may promote bone formation *in vivo*.

5. Conclusions

Plasma proteins such as albumin, γ-globulin, and fibrinogen were found to be classified as acidic proteins by liquid chromatography, and these proteins having carboxyl groups with a negative charge are suggested to mainly adsorb at positively charged Ca sites on the HAp surface. The acidic proteins were not eluted even at

higher inorganic ion molarities than human body fluid. Whereas basic proteins such as papain, cytochrome *c*, and lysozyme were easily eluted from HAp at lower inorganic ion molarities compared to human body fluid. The elution behavior of proteins using SBF in the presence and absence of glucose did not change. From these considerations, bone matrix proteins as acidic or neutral proteins would also be an advantageous condition in order to adsorb on HAp *in vivo*.

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