Physicochemical Study of Calcium-binding Properties of Chemical Substances as Inhibitors against Calcium Phosphate Insolubilization

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Calcium-binding properties of some chemical substances known as inhibitors against calcium phosphate insolubilization were investigated using a calcium-ion-selective electrode. First, the calcium binding properties of citrate, a well-known inhibitor, were evaluated by both the formation constant $K_f$ and two parameters $N$ and $K$ in the Langmuir equation. It was confirmed that the parameters $N$ and $K$ were superior to the formation constant $K_f$ to describe the calcium-binding property. Second, the calcium-binding properties of several inhibitors such as poly-L-aspartate, poly-L-glutamate, and alginates were analyzed by the Langmuir equation. They were well-described by the Langmuir equation. The tested inhibitors were classified into two groups: inhibitors of which parameters were independent of their concentrations (Group 1) and those whose parameters were dependent on their concentrations (Group 2). The values of the affinity parameter $K$ for the Group 1 inhibitors were larger than those for the Group 2 inhibitors.

Insolubilized calcium phosphate is a problem in the food industry and nutrition. In the food industry, insolubilized calcium phosphate is a major component of fouling substances both on heat exchangers in milk sterilization and on a membrane in whey ultrafiltration. Nutritional, insolubilized calcium phosphate reduces the calcium absorption efficiency in the distal small intestine. Therefore, inhibitors against calcium phosphate insolubilization are of great interest among those who are engaged in work on food engineering and nutrition. However, the mechanisms involved in the action of the inhibitors are almost unknown, so they should be discovered to allow suitable use of the inhibitors.

Many inhibitors have acidic functional groups. In a previous paper, we reported that alginates, which have acidic carboxyl groups, inhibited calcium phosphate formation (insolubilization), suggesting that the interaction between the alginate and calcium ion was important in the inhibition. We also showed by X-ray analysis that citrate, a well-known inhibitor that chelates calcium ion, altered the crystalline forms of calcium phosphate during the amorphous-to-crystalline (hydroxyapatite) transition, suggesting that citrate lowered the effective calcium concentration in the solution. In our preceding work, by using synthesized oligo-L-glutamic acids as the inhibitors, we reported that the inhibitory activity was dependent on the molecular weight of oligo-L-glutamic acids, suggesting that the appearance of the inhibitory activity of oligo-L-glutamic acids was strongly related to the calcium-binding property of oligo-L-glutamic acids. Thus, it is probable that the interaction between acidic groups and calcium ion is a key factor for the appearance of the inhibitory activity. However, little quantitative analysis of the interaction between calcium ion and the inhibitors has been done.

Calcium-binding properties of polysaccharides and proteins that had not been recognized as inhibitors have been analyzed by a metal-indicator method or by an equilibrium dialysis method. In the former case, there has been a problem that a metal indicator that is an auxiliary ligand might influence the calcium-binding properties of the polysaccharides and the proteins in the system. The equilibrium dialysis method requires much time and is not applicable to calcium-binding substances of low molecular weight because of a limitation of molecular weight cut-off of the dialysis membrane.

A calcium-ion-selective electrode responding to calcium ion activity is expected to be useful for a calcium-binding study. It permits a calcium binding experiment in a shorter time than equilibrium dialysis, and calcium-binding properties of low molecular weight substances are easily measured. In addition, no additives are required for calcium-ion-selective electrode measurement. But there have been few studies using calcium-ion-selective electrodes.

In this study, calcium ion activity was measured in the presence of the inhibitors using a calcium-ion-selective electrode, and calcium-binding properties of the inhibitors were physicochemically analyzed.

Materials and Methods

Materials. Citrate, fructose 1,6-bisphosphate (FBP), poly-L-aspartate, alginates, and poly-L-glutamate were used as known inhibitors. Two types of sodium alginates, G11 (mannuronate/guluronate (M/G) ratio is 0.53, the viscosity of 1% aqueous solution at 20°C was 0.022 [Pa·s]) and M1 (M/G ratio is 2.4, the viscosity of 1% aqueous solution at 20°C was 0.018 [Pa·s]), were obtained from Kimitsu Chemical Industries Co. Sodium poly-L-glutamate (Product No. P-4636: weight average molecular weight 10,600; DP = 70), sodium poly-L-aspartate (Product No. P-5387: weight average molecular weight 14,400; DP = 105), and FBP were obtained from Sigma Chem. Co. (The characteristic data had been evaluated from viscosity measurements by Sigma Chem. Co.) All the other chemicals were of reagent grade.

Measurement of apparent amount of calcium bound to the inhibitors. The electromotive force $E$, measured with a calcium-ion-selective electrode, is related to calcium ion activity as well as to a free calcium ion concentration $C$ as shown in the following equation.

$$E = E^\circ + \frac{RT}{2F} \ln a$$

$$E^\circ + \frac{RT}{2F} \ln \gamma + \ln C$$

(1)

where $E^\circ$ is the standard potential; $R$, the gas constant; $T$, the temperature; $F$, Faraday's constant, and $\gamma$, the activity coefficient. If $E$ is measured at
constant $\gamma$, the value of $C$ is evaluated from a calibration curve obtained by using standard calcium solutions.

Free calcium ion concentration was measured by using an ion meter (IM-40S, TOA Electronics Ltd., Japan) connected to a calcium-ion-selective electrode (CA-135B, TOA Electronics Ltd.) and to a double junction reference electrode (HS-305DS, TOA Electronics Ltd.). The ion meter was calibrated with standard calcium solutions. The concentration of the standard solution was taken as the free calcium ion concentration. Solutions were prepared with 30 mM, 80 mM, and 130 mM Tris- HC1 buffers (pH 7.4). For keeping $\gamma$ constant, the ionic strength was adjusted to 50 mM, 100 mM, and 150 mM with a buffer solution containing KCI. In the case of the ionic strength of 0.2 [m], the adjustment of the ionic strength was needless because the change in $\gamma$ was almost negligible upon the addition of both the inhibitor and calcium ion for measuring free calcium ion concentration. All the experiments were done at 25.0 ± 0.5°C.

We measured the free calcium ion concentration $C$ in the presence of an inhibitor at the total calcium concentration in the system, $C_r$. The parameter, $\beta$, is defined by the following equation:

$$C = \beta C_i$$  \hspace{1cm} (2)

By assuming that the decrease in $C$ by adding an inhibitor was due to the binding of calcium ion to the inhibitor, the apparent amount of calcium bound to the inhibitor, $r$, is expressed by the following equation:

$$r = (1-\beta)C/C_i$$  \hspace{1cm} (3)

where $C_i$ is the inhibitor concentration.

**Analytical**

1. **Formation constant**. The formation constant $K_r$ has been used for analyzing binding properties of a ligand to metal ions.\(^\text{(30,31)}\) In this study, the formation constant for citrate was calculated from Eq. (4), on the assumption\(^\text{(30,31)}\) that one citrate molecule binds to one calcium ion, to compare the experimental data with the experimental data\(^\text{(30)}\) obtained by the metal-indicator method.

$$K_r = [\text{Citrate-Ca}^2][\text{Ca}^{2+}]/[\text{Citrate}^3]$$  \hspace{1cm} (4)

2. **Langmuir equation**. Binding properties of all the inhibitors used in this study were analyzed by the following Langmuir equation\(^\text{(30)}\).

$$r = NKC/(KC+1)$$  \hspace{1cm} (5)

where $N$ [mole-calcium bound/mole-inhibitor] is the maximum amount of bound calcium ion, and $K$ [1/M] is the affinity parameter for calcium ion. As shown by Eq. (5), the parameters $N$ and $K$ are estimated from the intercept of ordinate and the slope by plotting $1/r$ versus $1/C$. The parameters and their standard errors were estimated by linear regression analysis.

**Results**

Figure 1 shows the dependence of the formation constant $K_r$ of citrate on the free calcium ion concentration. As the free calcium ion concentration was increased, the value log $K_r$ decreased at the ionic strength of 0.2 [M] from 3.10 to 2.34 (Fig. 1(A)), increasing from 3.29 to 4.55 at the ionic strength (I) of 0.05, 0.10, and 0.15 [M] (Fig. 1(B)). At I=0.15 [M] and the total citrate concentration of 3.0 [mm], the log $K_r$ values were 3.33 and 3.38 when total calcium concentrations were 1.0 [mm] and 2.0 [mm], respectively (pH 7.4). These two values (3.33 and 3.28) were close to that (log $K_r=3.30$) obtained at the total calcium concentration of 1.5 [mm] (pH 7.35) by Ettori and Scoggan\(^\text{(59)}\) who used the metal-indicator (murexide) method. It was confirmed that the data obtained by using a calcium-ion-selective electrode in this study were comparable with that by using a calcium indicator, murexide. The binding properties of ligands has been conventionally analyzed by $K_r$. However, as mentioned above, for evaluating $K_r$, the binding ratio of calcium ion to the inhibitor must be known $a$ priori. Since the calcium-binding mechanisms for the inhibitors used in this study are unknown, it is difficult to evaluate $K_r$ for the inhibitors. Therefore, in the following sections in this study, calcium-binding properties for inhibitors are analyzed by the Langmuir equation (Eq. (5)).

In Fig. 2, calcium binding isotherms (A) and the plots of

![Fig. 1. Dependence of Formation Constants $K_r$ for Calcium Citrate on Free Calcium Ion Concentration.](image1)

![Fig. 2. Calcium Binding Isotherms (A) and the Plots of $1/r$ versus $1/C$ (B) for Citrate.](image2)
1/r versus 1/C (B) for citrate are shown. As shown in Fig. 2(A), the amount of bound calcium r was smaller at larger ionic strengths, but r seems to be independent of citrate concentration at constant ionic strength. As shown in Fig. 2(B), a linear relationship was observed at each ionic strength, indicating that the calcium-binding isotherms for citrate was described by the Langmuir equation.

Figure 3 shows the citrate concentration dependence of the parameters N and K obtained by fitting the data in Fig. 2 to Eq. (5). The parameter N had a value between 1.0 and 1.5 at each ionic strength. The affinity parameter, K, had a value above 10^3 [1/m] and decreased as the ionic strength increased. Both N and K seemed to be independent of citrate concentration. Now that, by using the Langmuir equation, the calcium-binding property of citrate was evaluated as the parameters N and K at several ionic strengths. To study the difference among the inhibitors, several known inhibitors were applied to the analysis of the calcium-binding property at a specific ionic strength (I = 0.05 [M]) in the subsequent part of this study.

Figure 4 represents calcium-binding isotherms (A) and the plots of 1/r versus 1/C (B) for fructose 1,6-bisphosphate (FBP), an inhibitor of low molecular weight, at the ionic strength of 0.05 [M]. As shown in Fig. 4(A), the value of r decreased as FBP concentration was increased at fixed free calcium ion concentration. A linearity of the plots in Fig. 4(B) indicates that each isotherm in Fig. 4(A) is described by the Langmuir equation.

Figure 5 shows FBP concentration dependences of the parameters N and K obtained by fitting the data in Fig. 4 to Eq. (5). The parameter N was similar to stoichiometric equivalent value 2 and decreased as FBP concentration was increased. The parameter K was smaller than 10^3 [1/m], and increased as FBP concentration was increased. Thus, both the parameters N and K were dependent on FBP concentration in the case of FBP.

In Fig. 6, the dependence of parameters N and K on the concentration of alginate (GIL), the concentration of alginate (GIL) being represented as the concentration of building monomer (L-asparsate). Both the parameters N and K seemed to be independent of the poly-L-asparsate concentration. The values of K were larger than 10^3 [1/m].

Figure 7 shows the dependence of parameters N and K on the concentration of alginate (GIL), the concentration of alginate (GIL) being represented as the monomer (mangonulate or guluronate: uronate residue) concentration. Both the parameters N and K seemed to be independent of alginate (GIL) concentration. The value of K were larger than 10^3 [1/m].
Figure 8 represents the dependence of parameters $N$ and $K$ on the poly-$l$-glutamate concentration, which is represented as the monomer ($l$-glutamic acid residue) concentration. As the poly-$l$-glutamate concentration increased, the parameter $N$ decreased and the parameter $K$ increased.

Figure 9 shows the dependence of parameters $N$ and $K$ on the concentration of alginate (M1L), the concentration of alginate being represented as the monomer (mannuronate or guluronate: uronate residue) concentration. As the alginate concentration increased, the parameter $N$ decreased and the parameter $K$ increased. The value of $K$ for alginate (M1L) is smaller than that for alginate (G1L), alginate rich in guluronate, as shown in Fig. 7. The affinity for calcium of guluronate is stronger than that of mannuronate,\(^\text{23}\) the value of $K$ reflecting this tendency.

**Discussion**

The metal indicator method has been used for analyzing calcium binding properties for ligands. Metal-indicator in itself binds calcium and has the formation constant of a metal-indicator calcium complex. For example, Ettori and Scoggan\(^\text{20}\) reported that murexide, a calcium indicator, had the log $K_2$ value of 2.43 (pH = 7.35, $I = 0.15 \text{[m]}$) for calcium murexide complex. As can be seen from Fig. 1, the value of log $K_2$ for calcium citrate complex varied from 2.34 to 4.55 under various conditions. Therefore, the calcium complex formation of a metal indicator would influence the interaction between inhibitors and calcium ions, especially for inhibitors with a weak affinity for calcium (log $K$ value is small). On the other hand, the calcium-ion-selective electrode method does not require any additives such as metal indicators, being suitable for obtaining detail information on the calcium-binding properties of inhibitors.

Among a few workers studying the calcium binding properties of macromolecules by using calcium-ion-selective electrodes, Glesson et al.\(^\text{28}\) evaluated the calcium-binding properties of bile acids by the formation constant $K_f$. Mathuthu et al.\(^\text{29}\) evaluated the interaction between calcium ion and fulvic acids by a complex formation function. In these studies, however, the dependence of the parameters on calcium concentration or calcium-binding substance concentration was not investigated. In addition, such parameters as the formation constant and complex formation function were evaluated by assuming specific reaction formulas, that is, binding mechanisms, although the value of the parameters depends on the reaction formulas assumed. On the other hand, the Langmuir equation, used in this study, requires no specific reaction formulas. Therefore, the calcium-binding behavior of any particular inhibitor at specific condition was described by only two parameters $N$ and $K$. As can be seen from Figs. 2 to 9, the calcium binding properties of inhibitors used in this study were well described by the Langmuir equation.

The inhibitors used in this study are classified into two groups:

Group 1: The inhibitors of which parameters $N$ and $K$ are independent of the inhibitor concentration (citrate in Fig. 3, poly-$l$-aspartate in Fig. 6, and alginate (G1L) in Fig. 7).

Group 2: The inhibitors of which parameters $N$ and $K$ are dependent on the inhibitor concentration (FBP in Fig. 5, poly-$l$-glutamate in Fig. 8, and alginate (M1L) in Fig. 9). The values of the affinity parameter $K$ for the inhibitors
in Group 1 are larger than $10^3 [1/\text{M}]$ (see Figs. 3, 6, and 7), and those for the inhibitors in Group 2 are smaller than those for the inhibitors in Group 1 (see Figs. 5, 8, and 9). It is known from a measurement of polyelectrolyte solution viscosity that the extension size of a polyelectrolyte is dependent on the polyelectrolyte concentration.\(^{33}\) Owing to an electrostatic force, the affinity of a functional group in the polyelectrolyte for calcium ion would become stronger as the distance between the functional groups and calcium ions are shorter. Based on Langmuir theory,\(^{30}\) a calcium-inhibitor binding could not be affected by other calcium-inhibitor bindings. Since the affinity of the inhibitors in Group 1 for calcium was strong ($K \geq 10^3 [1/\text{M}]$), the electrostatic force might be strong enough and the distance between calcium and an inhibitor might be short enough for the parameters $N$ and $K$ to be independent of the inhibitor concentration, but for the inhibitors in Group 2, $\textit{vice versa}$.

As shown in this study, the Langmuir equation is suitable for describing the calcium-binding behavior of inhibitors. Further investigation on the binding study using the other metal ions and the other metal-binding substances is necessary not only for describing the metal-binding behavior of metal-binding substances but also for understanding the mechanisms involved in the action of the inhibitors against calcium phosphate insolubilization.

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**References**