Inhibitory Activity of Alginates against the Formation of Calcium Phosphate

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The effect of alginates, a dietary fiber, on calcium phosphate formation was investigated, and compared with that of the well-known inhibitors, poly-L-glutamate and citrate. First, the amount of calcium phosphate precipitate was measured at various alginate concentrations. An alginate delayed the formation of the calcium phosphate precipitate as well as poly-L-glutamate could. Second, the induction time (i.t.), when the transition of amorphous calcium phosphate to the crystalline form would occur, was measured in base titration experiments. Alginates delayed i.t., again as well as poly-L-glutamate could. These results indicate that alginates were an inhibitor of calcium phosphate formation. The plot of i.t. vs. alginate concentration was sigmoidal, which is similar to that of i.t. vs. poly-L-glutamate concentration and different from that of i.t. vs. citrate concentration. The induction time increase was slight at molar ratios of calcium to uronate above approximately 1/2. This indicates that the interaction between the uronate residue and calcium ion played an important role in the inhibition of calcium phosphate formation by alginates.

The formation of calcium phosphate often causes problems in the food industry. Calcium phosphate precipitated on the plate of heat exchangers inhibits heat transfer and lowers the efficiency.1) During ultrafiltration of milk and whey, calcium phosphate precipitation leads to clogging of the pores of the membrane, resulting in a lower flux and reduced efficiency.2) Moreover, the formation of a calcium phosphate precipitate is also important from a physiological point of view. The formation of calcium phosphate would reduce calcium absorption in the intestine3) and cause undesirable biological calcification.4)

Therefore, effective inhibitors of calcium phosphate formation are of great value. Schmidt et al.5) have suggested that an inhibitor isolated from a natural product such as milk could be used in the food industry. Schibler et al.6) tested pyrophosphate as an inhibitor of undesirable biological calcification. Many inhibitors have been reported, such investigations being important not only for clarifying the mechanism for calcium phosphate formation but also for utilizing such inhibitors in various situations. The inhibitors already reported are as follows: peptides or proteins like poly-L-glutamate,7) casin phosphopeptides (CPPs),7) phosvitin8)–10) and phosvitin like Mg2+11)–13) and Zn2+12), acidic compounds of low molecular weight like citrate,13) pyrophosphate,14) ATP1)1,1.2,1.3,1.4) and other macromolecules like polyacrylate,15) chondroitin sulfate,16) and proteoglycan.17) Williams and Sallis18) have suggested that the inhibitory mechanism for macromolecules would be different from that for the other inhibitors, but only a few macromolecular inhibitors have been reported, especially regarding polysaccharides.

Alginates, a dietary fiber, could be expected to inhibit the formation of calcium phosphate, since it possesses acidic groups as well as inhibitors, like poly-L-glutamate, CPP and proteoglycan do. In this study, the effect of alginates on calcium phosphate formation was investigated and compared with that of well-known inhibitors. The inhibitory activity was evaluated by two conventional methods, one involving measurement of the amount of calcium phosphate precipitated, and the other an evaluation of the induction time in a base titration experiment. In the base titration experiment, the effects of the mannuronate/guluronate ratio (M/G ratio) and of the molecular size of the alginate were also investigated.

Materials and Methods

Alginates. Four kinds of alginates (sodium salts) were supplied by Kimitsu Chemical Industries Co. As shown in Table I, they were classified by viscosity and M/G ratio. Since the molecular weight of alginates cannot be accurately measured, the viscosity of a 1% alginate solution at 20°C is taken as the index of molecular size.

Control inhibitors. Poly-L-glutamate with a mean molecular weight of 13,500 (a molecular weight distribution range from 2,000 to 15,000) was obtained from Sigma Chem. Co. Citrate was purchased from Kanto Chemical Co.

Reagents. Calcium chloride dihydrate, anhydrous sodium dihydrogen phosphate, anhydrous disodium hydrogenphosphate, potassium dihydrogenphosphate, potassium chloride, citrate and nitrate (conc.) were obtained from Kanto Chemical Co. The potassium hydroxide solution (1N) for base titration experiments was obtained from Wako Pure Chemical Industries, and sodium azide was obtained from Nacalai Tesque.

Evaluation of the inhibition of calcium phosphate formation by measuring the amount of calcium phosphate precipitated. The method of Hay et al.19) was slightly modified. In this study, 20 mM phosphate buffer (NaH2PO4 & Na2HPO4) at pH 7.40 was used as the phosphate source. A half milliliter

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<th>Table I. Alginates Used in This Study</th>
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<td>M/G ratio</td>
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<td>0.53</td>
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of the phosphate solution, 0.2 ml of 200 mM KCl to control the ionic strength, and 0.2 ml of 15 mM NaCl as an antiseptic were mixed in 1.5 ml polypropylene tubes. An alginate (2.4 M kg ratio with higher viscosity) or poly-L-glutamate was added to the solution at specified concentrations. Immediately after 0.1 ml of 40 mM CaCl₂ had been added to the solution, it was incubated in a shaker at 20.0 ± 0.5°C for 2, 8 or 24 hr. Each solution then centrifuged at 15,000 rpm for 10 min, and the supernatant was diluted with a 0.1 N nitrate solution and stored. The calcium concentration in the diluted supernatant solution was measured by inductively-coupled plasma spectrophotometry (Seiko Electronic Industries Co.), being calibrated with a CaCl₂ standard solution (Kanto Chemical Co.). In the experiments, all solutions were prepared with deionized and distilled water.

Evaluation of the inhibition of calcium phosphate formation by the base titration method. The pH of a solution decreases during calcium formation. Williams and Sallis have measured the amount of a base to be added to keep pH constant, and evaluated the induction time as an index for the inhibitory activity against calcium phosphate formation. This method was slightly modified in this study. Fig. 1 showing the experimental apparatus. An alginate, poly-L-glutamate or citrate were dissolved into 95.0 ml of water at the specified concentration in a reaction vessel at 25.0 ± 0.5°C. After adding 2.5 ml of 120 mM KH₂PO₄, the pH of the solution was adjusted to 6.00 with 1 N KOH or 1 N HCl. Then, 2.5 ml of 200 mM CaCl₂ was gradually added to the solution. The pH value was maintained at 5.9 ± 0.1 with 1 N KOH during the addition of CaCl₂, and reached 6.00 by the end of the addition. In order to induce spontaneous precipitation, a sufficient amount of 1 N KOH was added over a 60-sec period to adjust the pH to 7.40. The mixture was maintained at pH 7.40 throughout the reaction by using a pH-stat (TOA Electronics), and the amount of 1 N KOH added was continuously recorded. During the experiment, the solution temperature was kept at 25.0 ± 0.5°C, and nitrogen gas was bubbled to exclude CO₂ from the solution. All solutions were prepared with deionized and distilled water.

The induction time was estimated by the method of Meyer and Eanes from a base titration curve, as shown in Fig. 2. After the pH value had reached 7.4, the amount of the base added gradually increased as the reaction proceeded (see part b in Fig. 2), and then suddenly increased (see part c in Fig. 2). This sudden increment of the base added was caused by the transition from amorphous to crystalline calcium phosphate. As illustrated in Fig. 2, the linear parts of b and c in the base titration curve were extrapolated, and the time at the intersection of the two lines was taken as the induction time.

Results
1) Evaluation of the inhibition of calcium phosphate formation by measuring the amount of calcium phosphate precipitated

Figure 3 shows the dependence of calcium concentration in the supernatant on the monomeric concentration of poly-L-glutamate, a well-known inhibitor of calcium phosphate formation. The calcium concentration in the supernatant was almost constant with low concentrations of poly-L-glutamate, but suddenly increased at a certain concentration of poly-L-glutamate. As the incubation time increased, the calcium concentration in the supernatant decreased. This result indicates that poly-L-glutamate delayed, that is, inhibited rather than prevented the calcium phosphate formation as reported by Hay et al. and Termine and Posner. Figure 4 shows the dependence of the calcium concentration in the supernatant on the alginate concentration in the supernatant of the solutions. The concentration of poly-L-glutamate is expressed by the monomer (L-glutamate) concentration. The total concentrations of calcium and phosphate in the system were 4 mM and 10 mM, respectively.
centration in the supernatant on the monomeric concentration of the alginate. The concentration dependence in Fig. 4 is similar to that for poly-L-glutamate in Fig. 3, and the calcium concentration in the supernatant also decreased as time elapsed. This result indicates that the alginate also delayed rather than prevented the calcium phosphate formation.

2) Evaluation of the inhibition of calcium phosphate formation by the base titration method

Figure 5 shows the dependence of induction time on the poly-L-glutamate concentration, as well as the effect of citrate, a well-known inhibitor of low molecular weight, on the induction time. The double logarithmic plot of poly-L-glutamate concentration versus induction time is sigmoidal. On the other hand, as the citrate concentration increased, the induction time increased, the plot being concave upwards. These results indicate that poly-L-glutamate and citrate both delayed calcium phosphate formation above a certain concentration, although the concentration dependence was different.

Figure 6 shows the dependence of induction times on the alginate concentration. Alginates delayed calcium phosphate formation. The double logarithmic plots of alginate concentration versus induction times are sigmoidal, as well as that for poly-L-glutamate in Fig. 5. The M/G ratio influenced considerably the concentration dependence of the induction time, although the molecular size of the alginates had a little effect on the induction time above an alginate concentration of 10^3 μM.

Discussion

In this study, the inhibitory activity of alginates against the formation of calcium phosphate was investigated, and compared with that of the well-known inhibitors, poly-L-glutamate and citrate.

Naito has reported that the calcium concentration in the supernatant was linear with the logarithm of poly-L-glutamate concentration. As shown in Fig. 3 in the present study, thresholds of the poly-L-glutamate concentration for inhibitory activity were observed, although the phosphate solution pH and the ionic strength were different from those in the work of Naito. In this study, the calcium concentration was measured over a wider range of poly-L-glutamate concentration, probably causing a different dependence of calcium concentration on the poly-L-glutamate concentration between Naito's and this work.

Williams and Sallis have reported that citrate was a poor inhibitor over the citrate concentration range tested (10—200 μM). As can be seen from Fig. 5, however, the inhibitory activity of citrate was strong enough compared with that of poly-L-glutamate at concentrations above 10^3 μM. In Fig. 5, the double logarithmic plot of citrate concentration versus induction time was concave upwards. On the other hand, in Figs. 5 and 6, the double logarithmic plots of alginate or poly-L-glutamate concentration versus induction times were sigmoidal. These results suggest that the inhibitory mechanism of macromolecular inhibitors is different from that of low molecular weight inhibitors.

As can be seen from Figs. 3 and 4, the effect of alginates on the calcium concentration was similar to that of poly-L-glutamate. In addition, citrate, poly-L-glutamate and the alginates delayed the induction time in Figs. 5 and 6. Therefore, it was confirmed that alginates are an inhibitor of calcium phosphate formation.

In Fig. 6, the induction time increase was slight at inhibitor concentrations above approximately 10^4 μM, where the molar ratio of calcium to uronate (mannuronate or
guluronate) was 1:2. Angyal has suggested that one calcium ion would bind to two uronate residues in an alginate. According to Nishiyama, 21) 1 mol of calcium ion bound to 2—3 mol of uronate residue. From this information, the induction time increase would be slight when all the calcium in the system was bound to uronate in the alginates, suggesting that the interaction between the uronate residue and calcium ion played an important role in inhibiting calcium phosphate formation. As can be seen from Fig. 6, the induction time was longer for lower M/G ratios at higher alginate concentrations. Haug, 22) and Haug and Smidsrød 23,24) have reported that alginates rich in guluronate had a greater affinity for divalent ions than those rich in mannnuronate. This greater affinity of guluronate for calcium ions would have caused the longer induction time at higher alginate concentrations in Fig. 6. At alginate concentrations from $5 \times 10^2$ to $5 \times 10^3 \mu M$, however, the induction time was longer for higher M/G ratios, the reason for this being unknown at present. The polymer structure caused by the difference in M/G ratio might have influenced the induction time.

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