Inhibitory Activity of Oligo- and Poly-l-glutamic Acids against Calcium Phosphate Insolubilization and Calcium Binding with Special Relevance to Their Molecular Weight Dependence

Kazutaka YAMAMOTO, Hitoshi KUMAGAI, Atsuko SUZAKI, and Soichi ARAI

Department of Applied Biological Chemistry, Division of Agriculture and Agricultural Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Received March 23, 1994

The inhibition by oligo- and poly-l-glutamic acids of calcium phosphate insolubilization was investigated with special attention to their molecular weights. The inhibitory activity was evaluated by the induction time from base titration experiments. The inhibitory activity of oligo-l-glutamic acids increased as their molecular weights (residue numbers) increased, and that of poly-l-glutamic acid (20 mer) was almost the same as that of poly-l-glutamic acids (70 mer or 340 mer). Calcium binding properties of oligo-l-glutamic acids were measured by using a calcium-ion-selective electrode. The apparent amount of calcium bound to oligo-l-glutamic acids tended to increase as their molecular weights increased. The calcium-binding isotherm of oligo-l-glutamic acid (20 mer) was almost identical with that of poly-l-glutamic acid (70 mer). Moreover, the effect of poly-l-glutamic acid on the crystalline forms of insolubilized calcium phosphates was investigated by X-ray diffraction analysis, with the result that CaHPO$_4$·2H$_2$O (DCPD), a precursor of hydroxyapatite, was not observed.

In the food industry, insolubilized calcium phosphate is a bothersome factor as a fouling substance that reduces heat transfer of a heat exchanger in milk sterilization, and mass transfer of a membrane in ultrafiltration of whey. Physiologically, it is known that insolubilized calcium phosphate in a diet decreases the absorption of soluble calcium in the distal small intestine. The inhibitors are also interesting substances involved in biological calcification. Therefore, the inhibitors against calcium phosphate insolubilization have been attracting much attention both in food engineering and in nutrition. Many inhibitors of calcium phosphate insolubilization have been reported, but the mechanisms involved in the action of the inhibitors are little known. In addition, the relationship between molecular structure of the inhibitor and its inhibitory activity is little known. Such studies are important for obtaining a compass to the chemical design of the inhibitors as well as for clarifying the calcification in the inhibitors participate.

Many known inhibitors have acidic functional groups. In our previous paper, alginate, a natural polyelectrolyte composed of both mannuronate (M) and guluronate (G) with carboxylic groups, was found to be an inhibitor, and the inhibitory activity of alginate was compared with that of poly-l-glutamate, a well-known inhibitor. In that paper, we also suggested that the interaction between the uronate residue of the alginate and calcium ion was important in the inhibition of calcium phosphate formation. On the other hand, we confirmed by an X-ray study that citrate, which is a typical inhibitor and a chelating reagent for calcium, influenced the crystalline forms of insolubilized calcium phosphates, suggesting that citrate interacted with calcium ion to lower the effective calcium concentration. Thus, the interaction of an acidic group with calcium ion, e.g. chelation, may be a key factor for the inhibitory activity.

The molecular weight of the inhibitors, besides the acidic functional groups, may also influence the inhibitory activity. However, the effects of molecular weight of inhibitors on the inhibitory activity have not been investigated extensively. Amjad reported the influence of molecular weight of polyacrylic acid (PAA), which is polydisperse (non-homogeneous in molecular weight), on the inhibitory activity against calcium phosphate insolubilization. Such polydisperse polymers as PAA are commercially available. However, a polydisperse polymer is not a suitable material for studying in detail the molecular weight effect on the inhibitory activity, and it is necessary to use a monodisperse polymer.

In this study, we synthesized monodisperse peptides composed of l-glutamic acid by the solid phase peptide synthesis method in which R. B. Merrifield has developed and measured the inhibitory activity of the peptides against calcium phosphate insolubilization. Furthermore, we evaluated the interaction between calcium ion and the peptides by the calcium-ion-selective electrode method. In addition, the crystalline forms of insolubilized calcium phosphates were analyzed by X-ray diffraction so as to get information on the mechanisms involved in the crystallization of calcium phosphates in the presence of an inhibitor.

Materials and Methods

Reagents. Poly-l-glutamic acids (Product No. P-4636: weight average molecular weight 10,600, Degree of polymerization 70, Sodium salt; Product No. P-4886: weight average molecular weight 51,300, Degree of polymerization 340, Sodium salt; these characteristics data had been evaluated by viscosity) were obtained from Sigma Chem. Co. All other chemicals were of reagent grade.

Preparation of oligo-l-glutamic acids. Oligo-l-glutamic acids were manually synthesized by the solid-phase method using the 9-fluorenylethoxy carbonyl (Fmoc) strategy. p-Benzylxoxbenzyl alcohol resin was used for synthesizing oligo-l-glutamic acids. The side chain of l-glutamic acid was protected by a t-butyl group. Dicyclohexycarbodiimide (DCC) was used as a coupling reagent in the presence of 1-hydroxybenzotriazole (HOB). Dimethylformamide (DMF) was used as the primary
solvent for deprotection, coupling, and washing. The completion of couplings was monitored by the Kaiser test.\textsuperscript{21} Cleavage and deprotection of the synthesized peptide-resins were done using trifluoroacetic acid (TFA). The deprotected peptides were purified by repeated ether extraction. The identity of the resulting peptide was checked by both reversed phase high pressure liquid chromatography (RP-HPLC) and mass spectrum. Reversed-phase HPLC was done on a FinePak SIL C\textsubscript{18}S column (4.6 mm ID x 150 mm, Jasco, Japan) with a 60-minute linear gradient of 0-40% CH\textsubscript{3}CN in 0.1% TFA at a flow rate of 1.0 ml/min. Secondary Ion Mass Spectroscopy (SIMS) was done on JMS-SX102A/102A (JEOL, Japan).

Evaluation of the inhibitory activity against calcium phosphate insolubilization by a base titration method. The induction time from the base titration experiment was taken as an index for the inhibitory activity against calcium phosphate insolubilization. Details have been described in our previous paper.\textsuperscript{15}

Measurement of calcium binding properties of oligo-L-glutamic acids. A calcium-ion-selective electrode was used for measuring free calcium ion concentration. The electromotive force, \( E \), is related to calcium ion activity \( a \) and to free calcium ion concentration \( C \) as shown in Eq. (1).

\[
E = E^{0*} + (RT/2F) \ln a = E^{0*} + (RT/2F) (\ln \gamma + \ln C)
\]  
(1)

where \( E^{0*} \) 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 at 25.0 \pm 0.5 °C 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. All the solutions were prepared with 30 mM Tris-HCl buffer (pH 7.4). For controlling \( \gamma \) to be constant, the ionic strength was adjusted to 50 mM by using the buffer solution containing KCl.

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

\[
C = \beta C_t
\]  
(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 bound calcium to the inhibitor, \( r \), is expressed by the following equation:

\[
r = (1 - \beta) C_i/C_t
\]  
(3)

where \( C_i \) is the inhibitor concentration.

X-ray study of calcium phosphates insolubilized during the base titration experiments was done as in our preceding study.\textsuperscript{18} Immediately after the solution in the base titration experiment was harvested at a selected time, it was frozen with liquid nitrogen at a harvesting time and freeze-dried. The resulting powder was washed and then analyzed by X-ray diffraction. Details of this procedure are described in our preceding paper.\textsuperscript{18}

Results

1) Monodispersity of synthesized peptides

Figure 1 shows typical RP-HPLC charts of synthesized peptides. Each peptide was observed as a single peak in the RP-HPLC chart and was ascertained to be highly monodisperse. The identity of each synthesized peptide was confirmed by SIMS. Each synthesized peptide was used without further purification both for the inhibitory activity measurement and the calcium binding study.

2) Evaluation of the inhibitory activity against calcium phosphate insolubilization by the induction time

Figure 2 shows the dependence of the induction time on the concentrations of oligo- and poly-L-glutamic acids. The concentrations of oligo- and poly-L-glutamic acids were represented as the monomer (L-glutamic acid residue) concentration. The total concentrations of calcium and phosphate in the system were 5 mm and 3 mm, respectively.
with that of poly-l-glutamic acids, suggesting that inhibitory activity of oligo-l-glutamic acids (more than 20mer) was almost the same as that of poly-l-glutamic acids.

3) Calcium binding properties of oligo-l-glutamic acids

Figures 3, 4, and 5 show the dependence of \( r \) on free calcium concentration, the calcium binding isotherms, for oligo- and poly-l-glutamic acids. At each constant concentration of l-glutamic acid residue, the value \( r \) tended to increase as the peptide chain of oligo-l-glutamic acids was elongated. Moreover, the plot of \( r \) versus the free calcium concentration, the calcium binding isotherm, for oligo-l-glutamic acid (20mer: molecular weight 2598) was almost identical with that of poly-l-glutamic acid (70mer). The values \( r \) of oligo- and poly-l-glutamic acids tended to decrease as the monomer concentrations increased.

4) X-ray study of insolubilized calcium phosphates in the presence of poly-l-glutamic acid

The base titration curve in the presence of poly-l-glutamic acid (70mer) is shown in Fig. 6(A), and the X-ray diffraction curves of calcium phosphates insolubilized during the base titration experiments in the presence of poly-l-glutamic acid are shown in Fig. 6(B). Each diffraction curve was measured by using the insolubilized calcium phosphate which was frozen at each harvesting time shown in Fig. 6(A). The concentration of poly-l-glutamic acid was selected at 0.8 mm so that the induction time was about 58 min, which was the same value as that in the experiment using citrate at a concentration of 0.5 mm in our preceding paper. The characteristic peaks for HAp were observed at representative diffraction angles of 25.9 and 31.8° 20 among the broad band of amorphous calcium phosphate after the induction time. However, characteristic peaks for

![Fig. 3. Calcium-binding Isotherms of Oligo- and Poly-L-glutamic Acids. The monomer (l-glutamic acid residue) concentrations of oligo- and poly-l-glutamic acids were 1.0 mM.](image)

![Fig. 4. Calcium-binding Isotherms of Oligo- and Poly-L-glutamic Acids. The monomer (l-glutamic acid residue) concentrations of oligo- and poly-l-glutamic acids were 1.5 mM.](image)

![Fig. 5. Calcium-binding Isotherms of Oligo- and Poly-L-glutamic Acids. The monomer (l-glutamic acid residue) concentrations of oligo- and poly-l-glutamic acids were 2.0 mM.](image)

![Fig. 6. Calcium Phosphates Insolubilized during the Base Titration Experiment in the Presence of Poly-L-glutamic Acid (0.8 mM l-glutamic Acid Residue). (A) Base titration curve; (B) X-ray diffraction curves.](image)
Discussion

Molecular weight influence of the inhibitor on the inhibitory activity has never been investigated for a monodisperse system. In this study, we synthesized monodisperse oligo-L-glutamic acids and used them both in the inhibitory activity measurements and in the evaluation of calcium binding properties. As can be seen from Fig. 2, the inhibitory activity of each monodisperse oligo-L-glutamic acid increased as their molecular weights increased. Furthermore, the inhibitory activity of oligo-L-glutamic acid of molecular weight 2598 was almost the same as those of poly-L-glutamic acids with molecular weights of higher than 10,000.

Effects of molecular weight on the inhibitory activity have been investigated by using polydisperse polycrylic acids (PAA). Amjad (13) has reported that PAA has the maximal molecular weight (about 2000) for inhibiting calcium phosphate insolubilization. According to Okamoto’s work, PAA had the maximal molecular weight around 4000 for the inhibition of calcium carbonate insolubilization. In addition, Okamoto (21) stated that the chelating ability of PAA for calcium ions increased and the gelation of PAA by calcium ions was facilitated as the molecular weight was increased. He also discussed how the maxima might be observed because of the competitive functions of the chelation and the gelation, concluding that polymers with high inhibitory activity can be designed by controlling the molecular weight so that the polymers have both high chelating ability and low gelation tendency. As can be seen in Fig. 2, the maximal molecular weight, that is, the maximal residue number, was not found for the inhibitory activity against calcium phosphate insolubilization. Since oligo- and poly-L-glutamic acids have no gelation tendency, the maxima might have disappeared.

As can be seen from Figs. 3, 4, and 5, the apparent amount of bound calcium to oligo-L-glutamic acid, r, tended to increase as both their molecular weights and concentration become larger, and the isotherm of oligo-L-glutamic acid (20 mer) was almost the same as that of poly-L-glutamic acid. This behavior was similar to that of the induction time shown in Fig. 2. These results indicate that the calcium binding properties of oligo- and poly-L-glutamic acids strongly influence the appearance of the inhibitory activity against calcium phosphate insolubilization.

As also shown in Figs. 3, 4, and 5, the value r of each oligo- and poly-L-glutamic acids tended to decrease as each monomer concentration increased. It is known that an extension size of a polyelectrolyte depends on the polyelectrolyte concentration in a viscosity measurement.24 As for oligo-L-glutamic acids in this study, the concentration dependence of polyelectrolyte might influence the calcium binding behavior.

To investigate the effects of poly-L-glutamic acid, which binds calcium ion as shown in Figs. 3, 4, and 5, on the crystalline forms of insolubilized calcium phosphate, we also did X-ray diffraction measurements with calcium phosphates insolubilized in the presence of poly-L-glutamic acid. In our preceding paper, CaHPO₄*2H₂O (DCPD) was not observed before the induction time (58 min) in the presence of citrate. As shown in Fig. 6(B), DCPD was not observed before the induction time (58 min), while DCPD was observed as a precursor of HAp before the induction time (17 min) in the absence of inhibitors as reported in our preceding paper.18 These results indicate that poly-L-glutamic acid might reduce the effective calcium concentration and lower the supersaturation degree of DCPD by its calcium binding ability.

In conclusion, oligo-L-glutamic acid with the molecular weight of 2598 (20 residues) had almost the same inhibitory activity against calcium phosphate insolubilization as poly-L-glutamic acids. The appearance of the inhibitory activities of oligo- and poly-L-glutamic acids against calcium phosphate insolubilization are strongly related to the calcium binding properties of oligo- and poly-L-glutamic acids. It was suggested that the crystalline forms of calcium phosphates insolubilized in the presence of poly-L-glutamic acid might be altered by the calcium binding ability of poly-L-glutamic acid.

Acknowledgments. This work was financially supported by Meiji Milk Products Co., Ltd (Japan). We express our thanks to Dr. H. Aoki, Dr. M. Akao, Dr. M. Ogaki, and Dr. S. Nakamura of Division of Inorganic Materials, Institute for Medical & Dental Engineering, Tokyo Medical & Dental University and to Mr. T. Ikomasa and Dr. S. Kano of Waseda University for their help with X-ray diffraction measurements. We also express our thanks both to Dr. J. Nakayama for the help with SIMS measurements and to Dr. H. Kuniyoshi for technical advice about synthesizing peptides.

References


Molecular Weight Dependent Properties of Oligo-L-Glu

1665

precursors of HAp, like DCPD, were not observed.