Inhibitory Effect of Protein Hydrolysates on Calcium Carbonate Crystallization

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Protein hydrolysates, prepared by enzymatic digestion of soybean protein and egg white albumin using several proteases, inhibited the crystal growth of calcium carbonate. Each hydrolysate showed different inhibitory activities, suggesting the key role of peptide structures in the inhibition. The deamidation of protein hydrolysates by glutaminase increased not only the inhibitory activity toward the crystal growth of calcium carbonate but also the resistance of the hydrolysates against peptic digestion. Furthermore, the addition of sodium chloride, citric acid, or lactose into the reaction mixture enhanced the inhibitory activity. The protein hydrolysates inhibited both nucleation and crystal growth of calcium carbonate and also affected the crystal morphology.

Keywords: *Protein hydrolysate; calcium carbonate; crystal growth; soybean protein; egg white albumin; glutaminase*

INTRODUCTION

Intestinal calcium absorption proceeds by two mechanisms: an active transcellular process that takes place in the duodenum and a passive paracellular process throughout the small intestine (Bronner, 1998). In either process, calcium must be in a soluble and ionized form to be absorbed by the intestine (Levenson and Bochman, 1994). Calcium absorption is influenced by various factors including oligosaccharides, sugar alcohols, dietary fibers, and amino acids (Weaver, 1998; Suzuki et al., 1998). Casein phosphopeptides (CPP), which are formed by digestion of bovine casein, have shown some promise as calcium absorption enhancers (Sato et al., 1991). The peptide's ability to form soluble complexes with calcium phosphate is responsible for the enhanced intestinal calcium absorption that has been observed even in vitamin D-deficient animals (Sato et al., 1986). CPP are characterized by their inhibitory activities toward the crystallization of calcium phosphate and calcium carbonate due to the interaction between the phosphoserine cluster and the calcium salts. Such anticariogenic peptides are potentially useful as toothpaste, mouthwash, and food additives for the prevention of dental caries (Adamson and Reynolds, 1995).

Although some acidic macromolecules, such as invertebrate lectins (Muramoto et al., 1994) and poly-(L-glutaminate) (Yamamoto et al., 1994), have been shown to have inhibitory activities toward calcium carbonate crystallization, not only the interaction between their acidic functional groups and calcium ions but also their molecular structures were key factors for the activity.

In this paper, we describe the inhibitory activities of soybean protein and egg white albumin hydrolysates toward calcium carbonate crystallization. We also attempted to enhance the inhibitory activity by deamidating the hydrolysates and by adding some food constituents to the reaction mixture. In addition, the mechanism involved in the inhibitory activity was examined to demonstrate its applicability in crystal engineering.

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MATERIALS AND METHODS

Materials. Egg white albumin was purchased from Nacalai Tesque (Kyoto, Japan), and soybean protein (Fujipro R) was obtained from Fuji Oil Co. (Osaka, Japan). Protease M from *Aspergillus oryzae*, protease N from *Bacillus subtilis*, protease P from *Aspergillus melleus*, and protease S from *Bacillus* sp. were obtained from Amano Seiyaku Co. (Nagoya, Japan). Pepsin (EC 3.4.23.1) from porcine gastric mucosa was from Merck Co. (Darmstadt, Germany). All other reagents were of analytical grade from Wako Chemicals (Osaka, Japan).

Enzymatic Hydrolysis. Each protein (2.0 g) was dissolved in 100 mL of distilled water. Protease (20 mg) was added to the protein solution after the pH was properly adjusted. Enzymatic hydrolyses were performed at pH 3.0 and 50 °C for protease M, at pH 7.0 and 55 °C for protease N, at pH 8.0 and 45 °C for protease P, at pH 8.0 and 70 °C for protease S, and at pH 2.0 and 37 °C for pepsin (Chen et al., 1995). After digestion for various periods, hydrolysates were heated in boiling water for 5 min to inactivate proteases. The hydrolysates were neutralized and centrifuged at 4000*g* for 20 min, and the supernatants were lyophilized.

Calcium Carbonate Crystallization Assay. The assay was based on the titrated volume of 0.1 N NaOH to maintain the pH (8.5) of the supersaturated calcium carbonate solution during the crystallization. The rate of crystallization was proportional to the titrant volume measured by an autotitration system (pH stat model AUT-211, Toa Electronics, Tokyo, Japan). The crystallization started with an induction period of relatively constant pH, during which time crystal nucleation occurred, and continued with a rapid decrease in pH indicating crystal growth.

Sample solution (1.0 mL) was mixed with 1.0 mL of 40 mM $CaCl_2$ and 1.2 mL of distilled water in a reaction vessel. The

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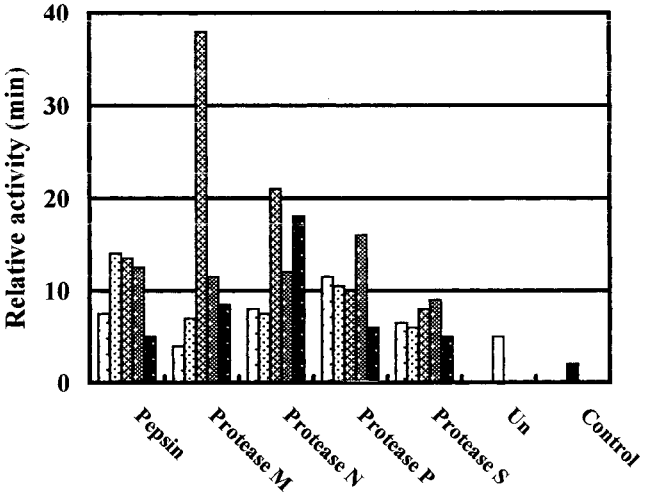

Figure 1. Inhibition of calcium carbonate crystallization by soybean protein. The protein (final concentration = 0.05%) was hydrolyzed with various proteases for indicated periods. Control: without protein hydrolysate; Un: unhydrolyzed proteins. Hydrolysis times (bars from left to right in each grouping): 15 min, 30 min, 1 h, 2 h, and 4 h.

assay was started immediately after the addition of 40 mM $NaHCO_3$ (0.8 mL) and continued for 60 min. The temperature of a water-jacketed reaction vessel was maintained at 37 °C by circulating water. The relative activity was defined to be the time required for the half-volume of 0.1 N NaOH titrated in the absence of sample.

Deamidation of Protein Hydrolysates. Soybean protein was hydrolyzed with protease M for 1 h as described above. The hydrolysate (2% solution) was treated with glutaminase (1/50 = E/S) (Amano Seiyaku) at pH 7.0 and 50 °C for indicated periods. The extent of deamidation was evaluated by measuring the amount of ammonia produced by glutaminase treatment using the Conway microdiffusion method (Kato et al., 1987).

Scanning Electron Microscopy (SEM). Calcium carbonate crystals were prepared from supersaturated calcium carbonate solution in the presence of protein hydrolysates. The crystals were air-dried and analyzed by a Hitachi S-4200 scanning electron microscope, operating at 10 kV and equipped with a Korex X-ray spectrum analyzer for elemental analysis of crystals.

RESULTS AND DISCUSSION

Inhibitory Effects of Protein Hydrolysates on Calcium Carbonate Crystallyzation. Soybean protein was hydrolyzed with five different proteases for various periods, and the inhibitory activities of the hydrolysates toward the calcium carbonate crystallization were measured. Although the protein showed only a weak inhibitory activity, the activity increased upon hydrolysis (Figure 1). Each hydrolysate showed different degrees of inhibition, revealing the key roles of the structures of generated peptides for the activity. There must be an optimal size for the activity, because the activity decreased with increasing hydrolysis time. High-performance gel filtration chromatography of soybean protein hydrolysates prepared by 1-h digestion with each protease showed various elution profiles, in which 13−18 kDa fractions dominated (data not shown). Further digestion increased the fractions of lower molecular masses accompanying a decrease of the inhibitory activity. Egg white albumin showed a similar inhibitory activity upon enzymatic hydrolysis; however, the activity was weaker than those of soybean protein

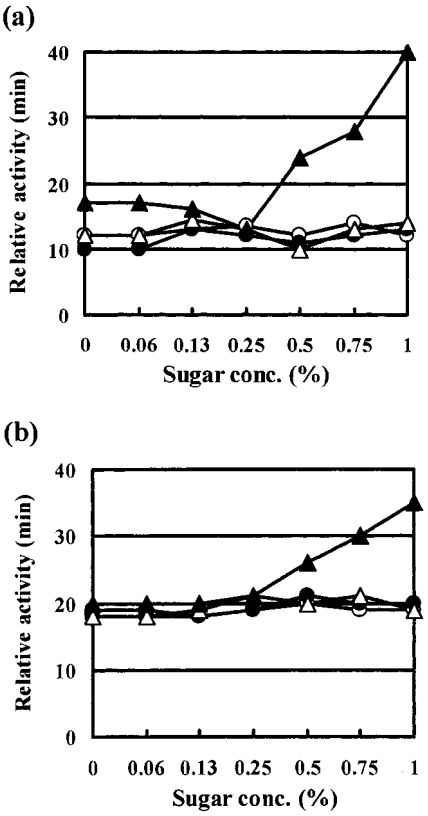

Figure 2. Effect of sugars on the inhibitory activities of protein hydrolysates toward calcium carbonate crystallization: (a) soybean protein hydrolysate (final concentration = 0.025%); (b) egg white albumin hydrolysate (final concentration = 0.125%); (○) glucose; (●) galactose; (△) sucrose; (▲) lactose. Soybean protein and egg white albumin hydrolysates were prepared by 1-h digestion with protease M and 4-h digestion with protease N, respectively.

hydrolysates (data not shown). This may be due to the high contents of acidic amino acid residues in soybean protein. The soybean protein hydrolysate obtained by protease M digestion showed the highest inhibitory activity after 1-h hydrolysis.

Effect of Additives on the Inhibitory Activity of Protein Hydrolysates. A wide variety of food constituents, such as oligosaccharides, sugar alcohols, dietary fibers, and amino acids, interact with calcium ions and affect the calcium absorption when taken as part of a meal (Weaver, 1998; Suzuki et al., 1998). Figure 2 shows the effects of several common sugars on the inhibitory activities of soybean protein (final concentration = 0.025%) and egg white albumin (final concentration = 0.125%) hydrolysates toward the calcium carbonate crystallization. D-Glucose, D-galactose, and sucrose had no effect; however, lactose enhanced the inhibitory activities of protein hydrolysates in a dose−response manner at a concentration over 0.5% lactose. Lactose is known to increase calcium absorption from the intestine. Several mechanisms have been hypothesized; these include the formation of a soluble calcium−lactose complex (Chaley and Saltman, 1963), the reduction of intestinal pH owing to acids formed after the fermentation of lactose with intestinal bacteria, and an increase in the permeability of calcium ions to the intestinal absorptive cells (Armbrecht and Wasserman, 1976). However, the actual mechanism by which lactose stimulates calcium absorption remains unclear. Lactose itself did not inhibit the crystallization in the concentration range tested in this study. This result

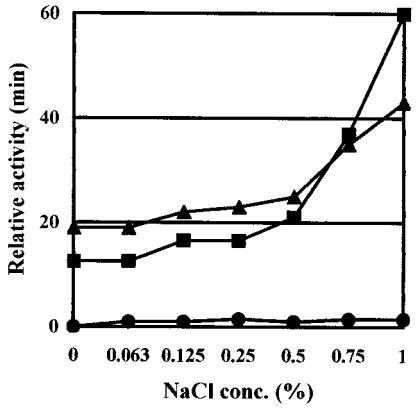

Figure 3. Effect of NaCl on the inhibitory activities of hydrolysates toward calcium carbonate crystallization: (●) NaCl alone; (■) + soybean protein hydrolysate (final concentration = 0.025%); (▲) + egg white albumin hydrolysate (final concentration = 0.125%). The protein hydrolysates were the same as in Figure 2.

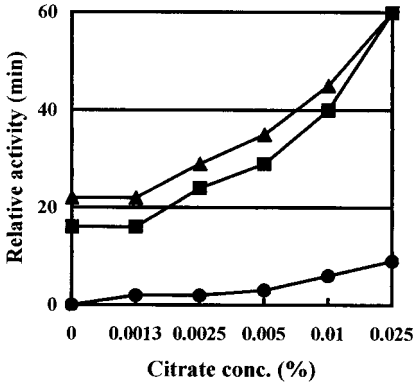

Figure 4. Effect of citrate on the inhibitory activities of protein hydrolysates toward calcium carbonate crystallization: (●) citrate alone; (■) + soybean protein hydrolysate (final concentration = 0.025%); (▲) + egg white albumin hydrolysate (final concentration = 0.125%). The protein hydrolysates were the same as in Figure 2.

indicates that the interaction between lactose and calcium ions might be mediated by peptides causing inhibition of the calcium carbonate crystallization.

The enhancing effect of sodium chloride on the inhibitory activities of protein hydrolysates is shown in Figure 3. Although sodium chloride itself had no effect, the inhibitory activity increased in the presence of protein hydrolysates by increasing the concentration of sodium chloride above 0.5%. The inhibitory activity of protein hydrolysates toward the calcium carbonate crystallization may also be affected by the ionic strength of the calcium carbonate solution.

Citrate, which can chelate with calcium ions, showed a weak inhibitory activity in this system (Figure 4). The addition of citrate to protein hydrolysates resulted in a significant inhibitory activity. These results suggest that various food constituents may potentiate the inhibitory activities of protein hydrolysates in the intestine.

Effect of Deamidation on the Inhibitory Activity of Protein Hydrolysates. A characteristic of soybean proteins is their high content of acidic amino acid residues, accounting for 30−40% of total amino acids (Yamauchi et al., 1991). However, ~50% of acidic amino acids, which may interact with calcium ions, are in the amide form. Soybean protein hydrolysates were deamidated with glutaminase, and the changes in the

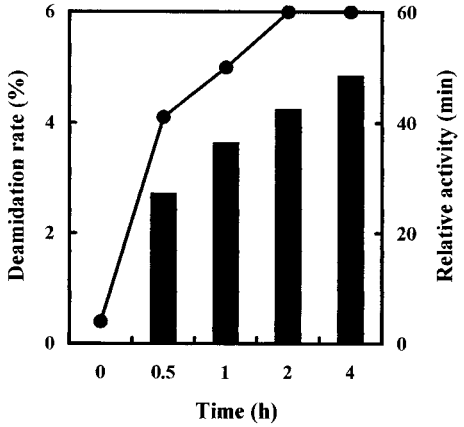

Figure 5. Effect of deamidation of soybean protein hydrolysate on its inhibitory activity toward calcium carbonate crystallization: (■) deamidation rate; (●) inhibitory activity. Soybean protein (2%) was hydrolyzed with protease M (1/100 = E/S, w/w) for 1 h and lyophilized. The hydrolysate (2%) was treated with glutaminase (E/S = 1/50, pH 7.0, 50 °C) for indicated periods and lyophilized. The concentration of hydrolysate was adjusted to 0.005% (final concentration).

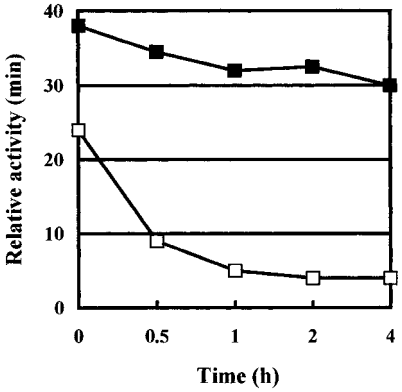

Figure 6. Effect of peptic digestion on the inhibitory activity of soybean protein hydrolysate with or without glutaminase treatment: (■) soybean protein hydrolysate (final concentration = 0.005%) with glutaminase treatment; (□) soybean protein hydrolysate (final concentration = 0.025%) without glutaminase treatment.

inhibitory activity toward calcium carbonate crystallization were examined (Figure 5). By increasing the deamidation rate, the inhibitory activity of the hydrolysate increased markedly, indicating that glutamic acid and aspartic acid residues played major roles in the inhibitory activity. In contrast, the egg white albumin hydrolysates containing lesser amounts of the amide forms of acidic amino acid residues showed only a small increase in the activity, even after deamidation.

The deamidation influenced not only the inhibitory activity of soybean protein hydrolysates toward calcium carbonate crystallization but also the resistance of the hydrolysates against peptic digestion. The protein hydrolysate (2%) prepared by 1-h digestion with protease M was treated with glutaminase (E/S = 1/50, 50 °C, 30 min) and further digested with pepsin (E/S = 1/100, 37 °C) for indicated periods. The soybean protein hydrolysate readily lost its inhibitory activity by peptic digestion, whereas the hydrolysate treated with glutaminase did not lose the activity so quickly (Figure 6). This could be advantageous for the animal system because the hydrolysates can accumulate in the small intestine without further proteolytic degradation to form soluble complexes with calcium carbonate.

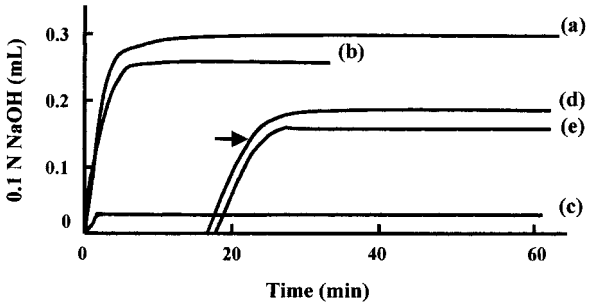

Figure 7. Calcium carbonate crystallization shown by the titration curves in the presence of soybean protein hydrolysates: (a) in the absence of protein hydrolysate; (b) in the presence of 0.013% (final concentration) protein hydrolysate; (c) in the presence of 0.125% (final concentration) protein hydrolysate; (d) in the presence of 0.025% (final concentration) protein hydrolysate; (e) additional protein hydrolysate was added to (d) at the time indicated by the arrow (final concentration = 0.150%).

Inhibition Mechanism for Protein Hydrolysates in Calcium Carbonate Crystallization. The molecular interactions, which take place at the interface between peptides contained in protein hydrolysates and calcium ions on the crystal surface, are important for the inhibition of calcium carbonate crystallization (Mann, 1988; Wheeler et al., 1981). The inhibition could be demonstrated by the addition of soybean protein hydrolysates to the supersaturated calcium carbonate solution after the nucleation or the crystal growth had begun (Figure 7). The onset of nucleation and growth were accompanied by titrant addition, and the rate of crystallization could be monitored from the recorded titrant volume added as a function of time. Figure 7a represents the titration curve without protein hydrolysates, showing that the crystallization was completed within 10 min (the control). When soybean protein hydrolysates was added to 0.0125% (final concentration) to the $NaHCO_3$ solution prior to the addition of $CaCl_2$ solution, the induction time was the same as that for the control (Figure 7b), notwithstanding very low inhibition. By the addition of soybean protein hydrolysates to 0.125% (final concentration), the pH decrease stopped and the induction time was delayed for at least 60 min, indicating the inhibition of nucleation (Figure 7c). In the presence of 0.025% soybean protein hydrolysates, the induction time was 22 min (Figure 7d). When additional soybean protein hydrolysates were added to the reaction mixture, complete inhibition of the crystal growth was observed (Figure 7e). These results indicate that soybean protein hydrolysates inhibited both the nucleation and the crystal growth of calcium carbonate.

Although this assay method using an autotitration system was tried for the calcium phosphate crystallization, the electrode failed to work because the precipitated salts covered it. The inhibitory activity of soybean protein hydrolysates toward the crystallization of calcium phosphate was examined by the precipitation test (Sato et al., 1991). Similar inhibitory activities were observed; however, the process of the crystallization could not be monitored.

SEM of Calcium Carbonate Crystals. Calcium carbonate crystals were formed in the presence of 0.025% soybean protein hydrolysates or 0.125% egg white albumin hydrolysates and were subjected to SEM (Figure 8). Most of the crystals showed a cubic or rectangular morphology in the absence of protein hydrolysates. On the other hand, the crystals grown in the presence of protein hydrolysates were much smaller in size and appeared to be extensively aggregated. The hydrolysates derived from soybean proteins and egg white albumin gave different initial morphologies: the anomalous angular shape for soybean protein hydrolysates and the rounded shape for egg white albumin hydrolysates. This may be due to the position of the carboxyl groups of the peptides contained in each protein hydrolysate. The peptides may interact with different sites of growing crystals, preventing further

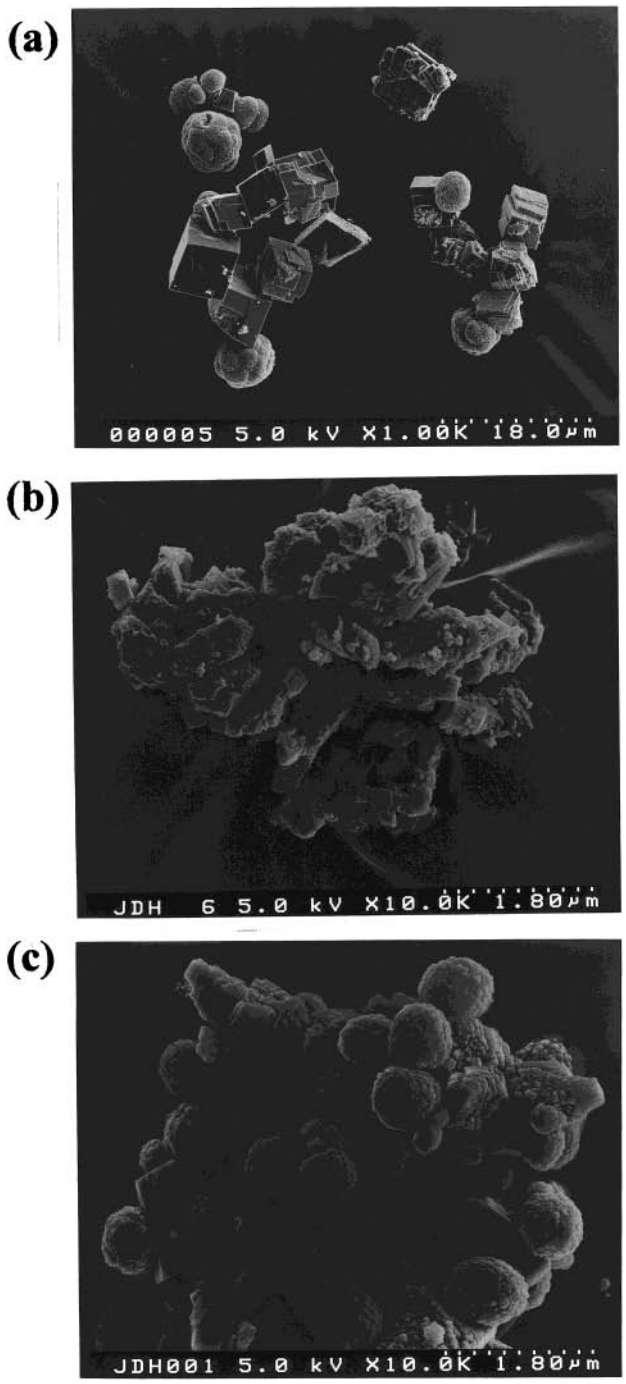

Figure 8. SEM of calcium carbonate crystals formed in the presence of protein hydrolysates: (a) crystals formed in the absence of protein hydrolysates (1000×); (b) crystals in the presence of 0.025% soybean protein hydrolysates (10000×); (c) crystals in the presence of 0.125% egg white albumin hydrolysates (10000×).

accretion of mineral, thereby changing the crystal morphology.

In conclusion, soybean protein hydrolysates inhibited calcium carbonate crystallization by preventing both nucleation and growth. The inhibitory activity increased by deamidating the hydrolysates with glutaminase. Egg white albumin hydrolysates were less effective in the inhibition, probably due to the low contents of acidic amino acid residues. Some food constituents enhanced the inhibitory activity, which may be advantageous in the improvement of calcium absorption from food ingredients in the small intestine. Furthermore, protein hydrolysates are possible regulators for the nucleation and growth of inorganic materials in crystal engineering.

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