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# Variation of insoluble calcium salts in protein adsorption and suspension stability when dispersed in sodium caseinate solutions

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ABSTRACT

The objective of this study was to determine how protein adsorption is affected by insoluble calcium salts when the salts are dispersed in sodium caseinate solution and its effect on suspension stability. Calcium carbonate ($CaCO_3$), hydroxyapatite (HA) and tricalcium phosphate (TCP) showed different adsorption capacities. The saturation of protein with $CaCO_3$ was the lowest of the three. The adsorption preference of α-casein (α-CN) and β-casein (β-CN) protein fractions was different depending on the calcium salt. For $CaCO_3$, α-CN was preferentially adsorbed over β-CN, while β-CN was preferred for HA and a similar decrease trend of α-CN and β-CN for TCP. The protein affected the stability of the calcium suspension depending on the protein concentration. The critical concentration of protein that needed to stabilize suspension varied among calcium salts.



## 1. Introduction

Asian diet is prevalently deficient in calcium, one of the major components responsible for bone development and health. Proteins, taken 50% of bone tissue by volume, also have a positive effect on the growth of bone. A recent research on the interaction of protein with calcium showed that the bone status improved with protein intake when calcium was supplemented, suggesting a synergistic positive effect of calcium and protein on bone health (Heaney, 2002).

Insoluble calcium supplements, such as calcium carbonate ($CaCO_3$), hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) and tricalcium phosphate (TCP, $Ca_3(PO_4)_2$), have been widely used in calcium-fortified formula milk and dairy beverage. However, problems of poor solubility for formula protein powder and precipitation for formula dairy beverage still occur. The common corrective action of adding stabilizers does not fully restore suspension of the Ca particles and tends to increase the cost and reduce the taste. It was found that the protein adsorption significantly improved the suspension stability of HA particles (Tercinier, Ye, Anema, Singh, & Singh, 2013; Tercinier, Ye, Anema, Singh, & Singh, 2014; Tercinier, Ye, Singh, Anema, & Singh, 2014).

The commercially used insoluble calcium supplements, including HA, $CaCO_3$and TCP, have their unique properties on topography, composition and potential, which might result in the variation in interactions with proteins, which could impact suspension stability. Thus, the objective of the present study was to explore the variation in protein adsorption and suspension stability of different insoluble calcium salts when dispersed in sodium caseinate solution.

## 2. Materials and methods

### 2.1. Materials

Sodium caseinate (SC) was purchased from Sigma–Aldrich (St. Louis, MO, USA). The protein content was approximately 84.6% (w/w wet basis) by the Kjeldahl method, in which a conversion factor of 6.38 was used.

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HA powder and TCP powder were obtained from Chemische Fabrik Budenheim (Budenheim, Germany), and $CaCO_3$ powder was obtained from Maruo (Akashi, Japan). The particle size of HA, TCP and $CaCO_3$ powders was determined using Dynamic Laser Scattering (Microtrac S3500, Microtrac, Montgomeryville, PA, USA). The median particle sizes $d_{(50)}$ of $CaCO_3$, HA and TCP powders were 1.30, 2.06 and 1.93 μm, respectively. The specific surface areas of calcium salt powders were determined by Brunauer–Emmett–Teller method, and the values for $CaCO_3$, HA and TCP powders were 13.8, 88.6 and 67.2 $m^2/g$, respectively.

### 2.2. Preparation of calcium salt suspensions

The suspensions of insoluble calcium salts in protein aqueous solutions were prepared by the following two different methods modified from Tercinier and co-workers Tercinier et al. (2013).

The first method was to investigate the process of protein adsorption and identify the saturated adsorption plateau. To achieve this goal, the amount of calcium salts was fixed with the concentration of proteins being adjusted. The powder of SC was dissolved in Milli-Q water to achieve a concentration of 40 mg/mL by gentle stirring for 4 h using the C-MAG HS7 magnetic stirrer (IKA, Staufen, Germany) and then storing overnight at 4 °C. Protein solution (40 mg/mL) was diluted to different concentrations in the range of 0.04–40 mg/mL. Then 0.5 mL of diluted protein solution was added into a 1.5 mL Eppendorf tube (Eppendorf, Hamburg, Germany) containing 20 mg calcium salt dispersed in 0.5 mL of Milli-Q water, and mixed using a Genius 3 Vortex (IKA). Thus the initial concentration in the suspensions ranged from 0.02 to 20 mg/mL.

The second method was to detect the adsorption preference of different protein components in sodium caseinate. The amount of protein was kept constant at 0.5 mL, 4 mg/mL with the concentrations of calcium salts ranging from 2 to 450 mg in 0.5 mL of Milli-Q water. Samples were handled the same way as method 1.

All suspensions from above were incubated for 2.5 h at room temperature in a rotary incubator to form the SC–Ca complexes which is sufficient for completion of the reaction based on previous work (Johnsson, Richardson, Sallis, & Nancollas, 1991; Tercinier et al., 2013; Wassell, Hall, & Embery, 1995; Yin, Liu, Zhan, Ding, & Yuan, 2002). The suspensions were then centrifuged for 7 min at 7000 rpm (Sorvall Legend Micro 17R, Thermo Fisher, Waltham, MA, USA), and the supernatants were collected and then analyzed for the residual protein content measuring using Biuret or Lowry method and the protein composition analyzed using SDA-PAGE. The insoluble calcium salt pellets were rinsed twice with Milli-Q water to remove the loosely bound proteins and used for turbidity test.

The zeta-potential of suspensions (0.05% w/w) of $CaCO_3$, HA and TCP and the SC solution (5 mg/mL) were measured with Malvern Zetasizer Nano-ZS instrument (Malvern Instruments Ltd., Malvern, UK). The pH of suspensions (2% w/w) of $CaCO_3$, HA and TCP and the SC solution (5 mg/mL) were measured with Seven Easy pH meter (Mettler-Toledo, Greifensee, Switzerland).

### 2.3. Characterization of protein adsorption

#### 2.3.1. Surface protein concentration

Biuret method (Gornall, Bardawill, & David, 1949) was used to determine the protein content in supernatants from HA–SC and TCP–SC suspensions, while the Lowry method (Lowry, Rosenbrough, Farr, & Randall, 1951) was used for supernatant from $CaCO_3$–SC suspension where the initial concentration of SC was less than 1 mg/mL. The content of adsorbed protein on insoluble calcium particle was defined as the difference between the initial protein content of the suspension and the protein content in the supernatant (Tercinier et al., 2013). The adsorbed protein concentration was equal to the adsorbed protein content divided by the mass of added calcium particles.

#### 2.3.2. Adsorption preference of different casein components

To determine the adsorption preference of different casein components with the calcium particles, protein patterns in the supernatant from suspension prepared with the second method were analyzed with SDS-PAGE according to the procedure of Laemmli (1970). A mixture of 0.5 mL of supernatant and 0.5 mL of sample buffer (0.5 M Tris, 4.0% w/v SDS, and 0.01% w/v bromophenol blue) was incubated for 3 min in a boiling water bath. A 20 μL aliquot of solution was loaded on to SDS gels (12% separating gel and 4% stacking gel) and separated on a Bio-Rad Mini-PROTEAN Tetra (Bio-Rad, Hercules, CA, USA). The protein bands were stained using a solution of Coomassie Brilliant Blue R-250 (Sinopharm Chemical Reagent Co. Ltd, Shanghai, China) and destained in a 7.5% acetic acid and 5% methanol solution. The intensities of protein bands were determined using Bio-Rad Gel Doc EZ (Bio-Rad). The amount of protein remaining in the supernatant was expressed as the ratio of protein content in the supernatant to that in the control solution without calcium salts (Tercinier et al., 2013).

### 2.4. Turbidity measurement

The SC–Ca complexes were resuspended in Milli-Q water to obtain a 0.125% (w/w) concentration for turbidity measurements, using a UV-1200 spectrophotometer (Meipuda instrument Co., Shanghai, China). The changes in absorbance at a wavelength of 900 nm (Tercinier et al., 2013) within 120 min were monitored. The reduction in absorbance was calculated using the following equation:

$$\text{Absorbance reduction } (\%) = (A_t/A_0) \times 100 \quad (1)$$

where $A_t$ is the absorbance at time t and $A_0$ is the initial absorbance.

## 3. Results and discussion

The zeta-potential of suspensions (0.05% w/w) of $CaCO_3$, HA and TCP and the SC solution (5 mg/mL) were about 7.4, −2.7, −2.3 and −27.1 mV, respectively. The change of the zeta-potential was an indicator of the adsorption of SC onto the calcium particles (Yu, Hu, Pan, Yao, & Jiang, 2006), which showed in supplementary information (Fig. S1). The confocal micrographs also confirmed the adsorption (Fig. S2). The pH of suspensions (2% w/w) of $CaCO_3$, HA and TCP and the SC solution (5 mg/mL) were ~9.2, ~7.3, ~7.1 and ~7.0, respectively. The protein adsorption in buffer solutions with various pH values was also studied in the investigation of WPI and different calcium salts (Fig. S3), where the solution pH values were adjusted to pH 7, pH 8 and pH 9 for $CaCO_3$ with WPI; pH 6, pH 7 and pH 8 for HA and TCP with WPI. It showed that the binding protein amount was decreased when pH increased, which was consistent with the study of Tercinier et al. (2014a), (b). In addition, a more significant contribution from salt type to protein adsorption was observed. For example, the saturated adsorption of WPI for $CaCO_3$ was ~0.3 $mg/m^2$ at pH 7, but for HA and TCP the amounts were ~1.0 $mg/m^2$ and ~1.5 $mg/m^2$, respectively. On the other hand, the saturated adsorption of WPI for $CaCO_3$ changed for ~0.3 $mg/m^2$ to ~0.15 $mg/m^2$ within pH 7 to pH 9. This result suggested that the variations among different calcium salts were more important. Considering the importance of the variations among calcium salts, we expected to concentrate on the variation caused by three calcium salts themselves without pH adjustment in the preparation of