

Theoretical molecular aspects of colloidal calcium phosphate in bovine milk

Jongwoo Choi^{1*}

¹Department of Food Science, The Pennsylvania State University, 322 Food Sci Bldg, University Park PA 16802

우유 속에 존재하는 칼슘과 인의 복합체에 대한 이론적인 분자학적 특성

최종우^{1*}

¹펜실베이니아 주립대학교 식품가공학과

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Abstract : A simplified model for the colloidal calcium phosphate (CCP) nanocluster was developed from an active role of phosphate in the precipitation of casein (CN)/Ca mixtures and the composition of casein micelles (CM). The possible shape of the CCP nanocluster was selected as a tetrahedron, and we estimated that 4 CN molecules were involved in crosslinking a single CCP nanocluster. Similar values were obtained for the number of CN molecules involved in stabilizing the nanocluster when the number of CNs attached onto each nanocluster surface was deduced from the composition of CM. If one phosphoserine cluster consisted of 3 phosphoserine residues, the theoretical molecular weight and volume for the nanocluster were estimated to be 4,898 g/mol and 2.88 nm³, respectively. It was also shown that the position of Ca present in our model were reasonably located to accommodate the serine phosphate in CN molecule.

Key words : Colloidal calcium phosphate, Phosphoserine cluster, Casein micelle

I. Introduction

The principal components of bovine milk include water (87.1%), fat (4.0%), protein (3.3%), lactose (4.6%), and mineral substances (0.7%) (Walstra et al., 1999). Fat and casein (CN) are not distributed as individual molecules in solution; rather, they exist in large, complex associated structures, which take certain physical forms. For example, most of milk fat is in separate small globules and CN appear as casein micelle (CM), which are spherical complexes of CN and inorganic compounds. CNs exist in a stable colloidal suspension of aggregates known as CM. CM are largely composed of water and CN but it also contains a small but essential amount of inorganic constituents. On

the dry matter basis, CM is composed of ~6.6% salts and 93% CN. The most important inorganic compounds are Ca and phosphate, which is given the term colloidal calcium phosphate (CCP) or insoluble Ca phosphate as it is also called. CCP consists of Ca, P, and trace amounts of citrate and Mg. We will largely ignore the low quantities of other salts when the theoretical molecular weight (MW) of CCP is estimated. The molar ratio of Ca/P_i and Ca/P_(i+o) in CCP is reported as 3:2 and 1:1 (Schmidt, 1982; Holt et al., 1989), respectively, which is a vital piece of information for estimating the theoretical MW of CCP.

Several roles of CCP have been discovered. CCP nanoclusters contribute to the textural properties of dairy products (Choi et al., 2008). Lee and Lucey (2004) showed that the timely solubilization of CCP during the yogurt manufacturing process could improve physical

*Corresponding author: Tel: +1-814-863-4958
E-mail address: juc44@psu.edu

properties of these gels (i.e. whey separation). O'Mahony et al. (2005) reported that partial solubilization of the CCP associated with the *para*-CN matrix of curd is responsible for most of the early textural changes during Cheddar cheese ripening. From nutritional point of view, CCP is a highly bioavailable source of Ca and P that is needed for bone and teeth formation (Holt and Sawyer, 1988). The CCP nanocluster also plays a pivotal role in the formation and the stability of CM (Horne, 1998).

In previous article (Horne et al., 2007), the possible shape of CCP, and theoretical MW of CCP were briefly reported. In this paper, we report more details about molecular aspects of CCP nanocluster that was theoretically developed (Horne et al., 2007), and thus some of aspects reported in Horne et al. (2007) are further elaborated.

II. Methods used for development of theoretical CCP model

1. Possible Shapes of CCP

Two independent studies [i.e. the role of phosphate in Ca-precipitated CN (Horne, 1982) and thermodynamics and kinetics of the growth of CCP (Holt, 1997) provide a plausible mechanism for the formation of CCP. Serine residues on CN are usually phosphorylated (this P is called organic P, P_o). 3 or 4 phosphoserine residues are found to be flanked or grouped (clustered) in the amino acid sequence of the CNs. The phosphoserine

cluster has a seemingly contradictory dual role, i.e. initiation of CCP growth as well as its termination. In brief, the cluster as a nucleation site attracts Ca and P (this P is termed inorganic P, P_i) from solution into the phosphoserine center. This "seed" is then allowed to mature, but at some point further growth would be frustrated when the incoming phosphoserine cluster is attached to the last available plane for the entry of Ca and P onto that surface. We think fully formed CCP result when no further Ca and P take part in that "maturation" process. This suggests that the number of serine clusters attached to the surface of CCP would determine the size, possibly MW for CCP.

The detailed crystal structure of CCP will dictate the positions that Ca and phosphate ions take up as they join the growing crystal. This growth is going to be frustrated by additional CN molecules coming with its phosphoserine clusters to take up the phosphate moiety positions on that structure. With the adsorption of phosphoserine cluster to the last available face on the crystal, growth is stopped and we have the final CCP structure in place. We think that these faces on the CCP crystal are largely planar and of relatively small size, not much more than the unit cell of the crystal. The minimum faces that would satisfy this requirement would be 4 faces for a tetrahedron, or 6 faces for a bipyramid and a rhombohedron (Fig. 1). We are going to use these geometries for our discussion.

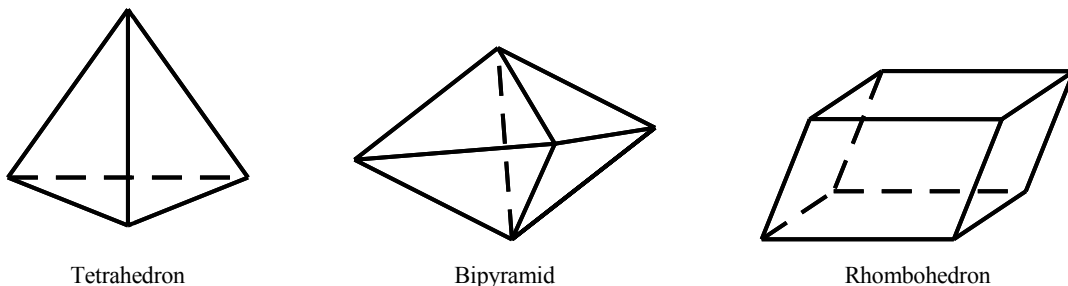


Fig. 1. Possible shapes of CCP nanocluster.

2. Calculation of Molecular Weight

The size of a CCP nanocluster can be calculated from the number of faces we allow for the nanocluster. We favor either the tetrahedron (4 faces), or the bipyramid (6 faces). If we then argue that the phosphoserine cluster (Fig. 2) has to have 3 phosphoserine residues (Ser P) to initiate/inhibit growth, then the tetrahedral nanocluster will contain 12 ester phosphates (4 faces \times 3 Ser P; Fig. 3). We can make use of the observation that in CCP the ratio of Ca to P_i and that of Ca to $P_{(i+)}$ are 3:2 and 1:1, respectively (Schmidt, 1982; Holt et al., 1989). This would mean that the CCP would contain 36 Ca ions, 24 P_i and 12

ester P_o for a tetrahedral structure. If 4 Ser P are required to do the same job, then 48 calcium ions, 32 P_i and 16 ester P_o are needed for the same shape (tetrahedron) above. If the CCP could also take the shape of a bipyramid, a tetrahedron combined with another, the six phosphoserine clusters would be bound to each surface. The number of Ca ions, P_i , and P_o will be twice as many as a single tetrahedron. The molecular weight of CCP would be either 4,897.8 (36 $CaHPO_4$), 6,530.4 (48 $CaHPO_4$), or 9,756.6 (72 $CaHPO_4$) g/mol. We believe that MW of CCP in milk could range from 4,897.8 to 9,756.6 g/mol. This number was experimentally confirmed (Choi et al., 2011).

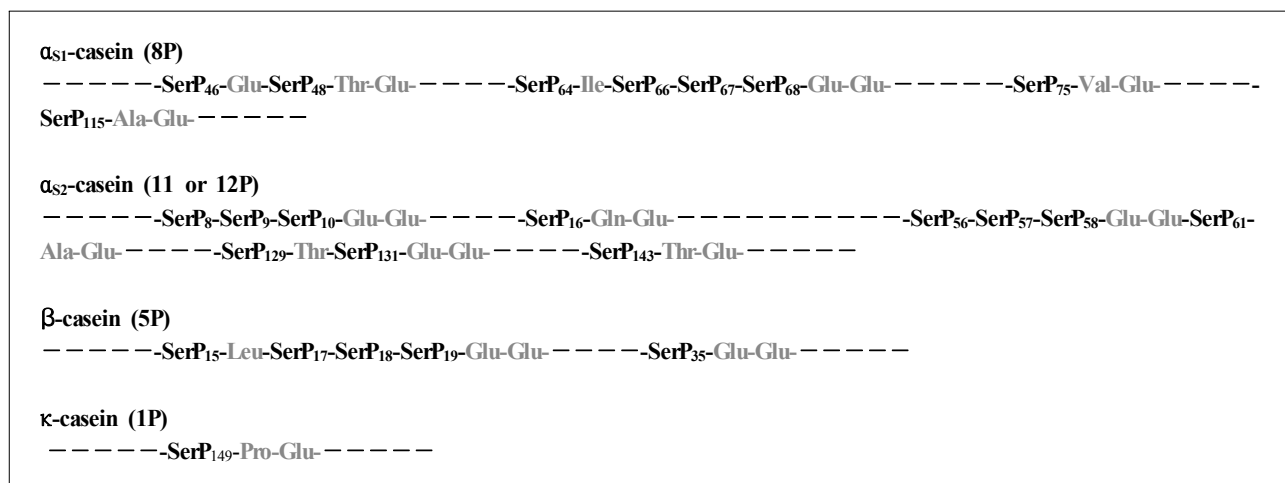


Fig. 2. The positions of phosphate residues in the bovine caseins, indicating the phosphoserine residues in bold (Horne, 2006).

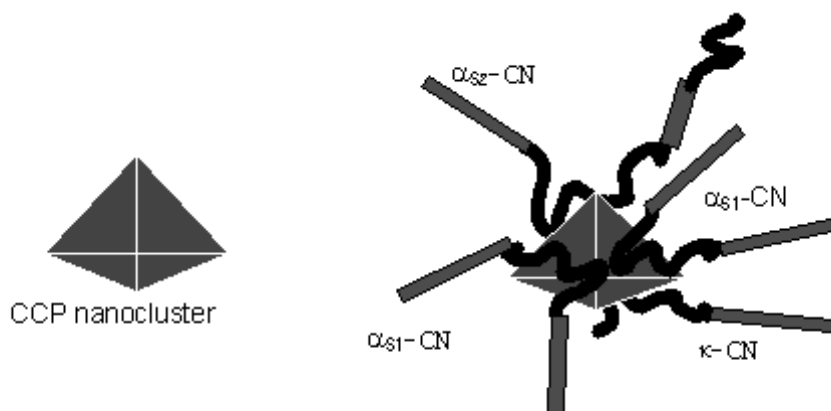


Fig. 3. Possible model for a CCP nanocluster with 4 caseins bound on tetrahedron surfaces. We do not know exactly which caseins are involved, and thus, we arbitrary assigned possible caseins on each surface.

3. Volume occupied by CCP

Dicalcium phosphate anhydrous has triclinic unit cell ($a = 6.910(1)$, $b = 6.627(2)$, and $c = 6.998(2)$ Å; $\alpha = 96.34(2)$, $\beta = 103.82(2)$, and $\gamma = 88.33(2)$ at 25°C; see Fig. 4 for the structure of unit cell), and one unit cell contains 4 ($Z = 4$) molecules of CaHPO_4 (Mathew and Takagi, 2001). If we take tetrahedron as a possible shape for CCP and assume that 3 Ser P are in a single phosphoserine cluster, the tetrahedron would then consist of 9 unit cells of dicalcium phosphate anhydrous (DCPA) (36 Ca, 36 P = 12 P_o + 24 P_i). For the sake of volume calculation of a tetrahedron, its unit cell is approximated to be cubic (Fig. 4). An average length of each edge in cube is then 0.6845 nm, $(0.6910 + 0.6627 + 0.6998)/3$. A cube that consists of 27 cubic unit cells has 2.0535 nm, 0.6845×3 , length of each edge. Total volume of cube is $2.0535^3 = 8.6593 \text{ nm}^3$. Volume of a tetrahedron is one third of cube volume, which is $(1/3) \times 8.6593 \text{ nm}^3 = 2.88 \text{ nm}^3$ (Fig. 4). If the bipyramid shape is taken, the volume of bipyramid would be $2.88 \times 2 = 5.76 \text{ nm}^3$.

4. Length between Ser P and Ser P residues in phosphoserine cluster

A length of each edge of tetrahedron is $\sqrt{(2 \times 0.0535^2)}$

= 2.90 nm which is close to the experimentally observed size of a nanocluster 2.5 nm (McGann et al., 1983). The distance between a Ca site and the next Ca site is $2.90/3 = 0.96$ nm. The distance between a Ca site and another Ca site with one Ca site in between would be $0.96 \times 2 = 1.92$ nm. The length from one Ser P residue to the next Ser P would be 2.094 nm when the phosphoserine cluster is fully stretched. Therefore, each Ser P would even bind Ca sites away from each other by a Ca in between.

5. The number of phosphoserine clusters per CCP nanocluster

We could also arrive at the number of phosphoserine clusters per CCP nanocluster. Total inorganic material in a CM is about 6.6% (dry weight basis), and whole CN makes up the rest of 93.3% (Schmidt, 1982). Normal milk usually contains about 2.5% protein as CN. The amount of minerals would be $(6.6\%/100) \times 2.5\% = 0.165\%$ or 0.165 g/100 mL milk. The number of CCP cluster per 100 ml of milk is dependent on MW of CCP nanocluster. MW of CCP was estimated to be 4,897.8, 6,530.4, or 9,795.6 g/mol. If we take 9,795.6 as the MW, the number of CCP would be $0.165 \text{ g}/100 \text{ ml} \times 9,795.6 \text{ g/mol} \times 6.02 \times 10^{23}$. The number of CN chains per 100 ml of milk would be $[(2.5/25,000 \text{ g/mol})/$

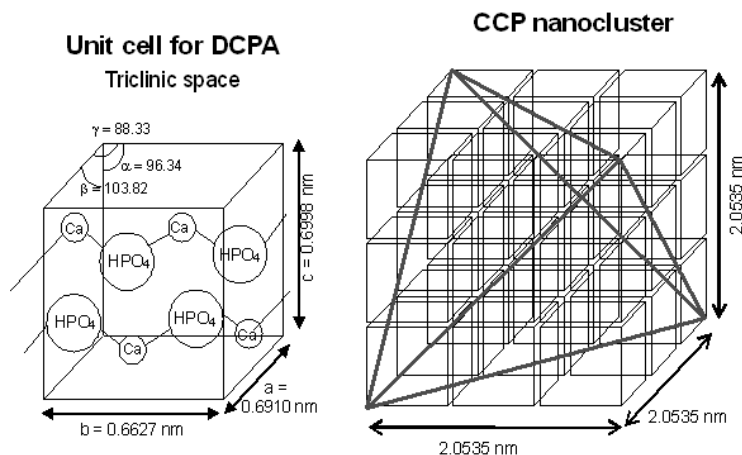


Fig. 4. Structure of unit cell for dicalcium phosphate anhydrous (DCPA), and shape of a CCP nanocluster. Unit cell was drawn from description of MacLennan and Beevers (1955) and CCP nanocluster was redrawn from Horne et al. (2007).

100 ml] $\times 6.02 \times 10^{23}$. The ratio of the number of nanoclusters to the number of chains is 1/6.05, which means 1 nanocluster for 6 chains. If we take the MW of CCP nanocluster to be 4,897.8 and 6,530.4 g/mol, then there would be 1 nanocluster for 3 and 4 CN chains, respectively.

6. The number of phosphoserine cluster per casein chain

From the mole ratio of each CN molecule ($\alpha_{s1}:\alpha_{s2}:\beta:\kappa = 4:1:4:1$; Davies and Law, 1980) in CM and if three phosphoserine residues that are in close proximity are considered to be one phosphoserine cluster (Fig. 1), then 10 CN molecules ($4\alpha_{s1} + 1\alpha_{s2} + 4\beta + 1\kappa$) would have, on average, 10 phosphoserine clusters ($4 \times 1 + 1 \times 2 + 4 \times 1$). Therefore, there is on average one phosphoserine cluster per chain, and so one CCP nanocluster would accommodate the same number of phosphoserine clusters as the number of CN chains; each face on the tetrahedron would have one phosphoserine cluster.

However, if we take 2, 3, and 1 as the number of phosphoserine clusters for α_{s1} -, α_{s2} -, and β -CN (Swaisgood, 1992), respectively, then the total number of phosphoserine clusters would be 15, (4×2) + (1×3) + (4×1). Therefore, there are 15 phosphoserine clusters for every 10 (average) molecules of CN, and so 4 CN molecules would have 6 phosphoserine clusters. If one CCP nanocluster only needs 4 phosphoserine clusters for "saturation", it means 2 phosphoserine would be left free, or ~ 2 average molecules of pro-tein, or $\sim 20\%$ of $\alpha_{s1} + \alpha_{s2} + \beta$ -CN.

III. Discussion

In developing a model for bovine CCP nanocluster, our modeling process was very similar to solving a complex "jigsaw puzzle". Each piece of separate information was small, often oddly shaped, and interlocking,

but when complete, our jigsaw puzzle produced a "reasonable" picture of bovine CCP nanocluster as shown in Fig. 3. The shape of CCP nanocluster in our model is tetrahedron or possibly bipyramid. Lyster et al. (1984) and Holt et al. (1989) found that CCP nanocluster has short-range order. This could indicate that CCP nanoclusters are very small crystallites. The tetrahedron has the least number of surfaces and edges among possible geometries, and could be the best choice as a likely shape of a nanocluster. Our shape, however, contrasts with the spherical shape reported by Holt et al. (1989). It is very likely that CCP nanoclusters could be grown through either surface or edge, and thus a spherical shape is hard to cause a termination in nanocluster growth. As seen in Fig. 1, the 3 phosphoserine residues in α_{s1} -, α_{s2} -, and β -CN are found to occur side by side (in a cluster). Thus, we thought that at least 3 phosphoserine residues may constitute a single phosphoserine cluster for full participation in the growth of CCP nanocluster. This number is in agreement with that reported by Aoki et al. (1992). The final piece of our "jigsaw", which further narrowed down the possible MW of CCP nanocluster, was the ratio of Ca to P_i or $P_{(i+o)}$. This value was used to conclusively decide that a single phosphoserine cluster (supplied possibly by a CN chain) crosslinked onto each surface, led to the involvement of 4 CN chains in a single CCP nanocluster and thus the theoretical MW of CCP nanocluster. Our theoretically-derived MW was in the range from 4,900 to 9,800 g/mol. The volume of CCP (2.88 nm^3) should be experimentally verified. We also did some calculation (length between Ser P and Ser P) to show that Ser P in the phosphoserine cluster was geometrically fitted into the model.

IV. Conclusions

We estimated the volume of CCP, length between Ser P and Ser P residues in phosphoserine cluster, and

the number of phosphoserine cluster per casein chain, These estimations reasonably supported the model of CCP nanocluster.

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