Journal of Colloid and Interface Science 394 (2013) 458–466

Contents lists available at SciVerse ScienceDirect

Journal of Colloid and Interface Science

www.elsevier.com/locate/jcis

Adsorption of milk proteins on to calcium phosphate particles

Lucile Tercinier [a], Aiqian Ye [a,*], Skelte Anema [b], Anne Singh [b], Harjinder Singh [a]

[a] Riddet Institute, Massey University, Private Bag 11 222, Palmerston North 4442, New Zealand
[b] Fonterra Research Centre, Private Bag 11 029, Palmerston North 4442, New Zealand

ARTICLE INFO

Article history:
Received 21 September 2012
Accepted 27 November 2012
Available online 5 December 2012

Keywords:
Milk proteins
Caseins
Whey proteins
Calcium
Phosphate
Hydroxyapatite
Adsorption
Surface modification
Suspension stability

ABSTRACT

The adsorption of caseins from sodium caseinate (SC) and whey proteins from whey protein isolate (WPI) on to particles of hydroxyapatite (HA) was studied. Confocal microscopy and zeta-potential measurements showed that both caseins and whey proteins bound to HA, resulting in an increase in the absolute value of the zeta-potential of the particles. This adsorption improved the suspension stability of the HA particles in water. For both protein sources, there was a preference in the protein adsorption: in the order β-casein > α_s-casein > κ-casein for sodium-caseinate-coated particles; in the order β-lactoglobulin > α-lactalbumin for WPI-coated particles. The adsorption of caseins and whey proteins on to HA could be fitted using a simple Langmuir model, suggesting a single layer adsorption of caseins and whey proteins on to the HA surface. Possible mechanisms involved in the interaction between milk proteins and HA are discussed, in relation to the structure and the surface properties of both milk proteins and HA particles.

1. Introduction

Milk proteins and insoluble calcium salts co-exist in many dairy products, such as calcium-fortified milks, beverages and yoghurts. A common insoluble calcium salt that is used in these products is tricalcium phosphate (TCP). Most commercial TCP is powdered hydroxyapatite (HA). HA is the main constituent of human bones and teeth and is the most thermodynamically stable of the calcium phosphate phases. As HA is barely soluble in water (solubility constant at 25 °C, $K_{sp} = 4.7 \times 10^{-59}$ [1]), synthetic HA is one of the preferred calcium salts for the calcium fortification of food products. It is widely used in heat-treated calcium-fortified formulations such as UHT milks because it does not cause any heat instability, unlike soluble calcium salts [2,3]. Despite its industrial relevance, the interactions between HA particles and milk proteins have not been studied in detail, as HA is often considered to be inert in milk and is generally believed not to interact with other milk components. However, it is known that HA interacts strongly with proteins in a wide range of biological applications [4,5]. For example, HA can be used for the separation of proteins in high performance liquid chromatography techniques [6]. Also, interactions between proteins and synthetic HA crystals have been widely studied for nano-ceramics and bone replacement, as the binding of proteins to bone substitutes can have a dramatic effect on the clinical success of a bone implant [7,8].

The surface of HA particles has been shown to play a critical role in their stability and behaviour in suspension. Therefore, a number of researchers have focused on the surface modification of these particles by electrostatic or steric stabilisation using the ability of HA to adsorb other components [4,5,9,10]. A wide range of proteins or peptides has been used to modify and stabilise the surface of HA and other insoluble calcium phosphates (these include bovine serum albumin, egg lysozyme, bovine serum fibrinogen [10] and lactoferrin [11]), as well as ions (Ca^{2+}, PO_4^{3-} [12], citrate [13] and fluoride [14]) and other molecules (dodecyl alcohol [15] and silica [9]). This leads to the hypothesis that interactions may occur between milk proteins and HA particles when they are suspended in milk, resulting in the adsorption of the milk proteins on to the surface of the HA particles.

To our knowledge, the adsorption of milk proteins on to food-grade HA particles has not been reported. There are a few studies on the interactions between milk proteins and HA in relation to bone or dentistry topics [16–18]. van Kemenade and de Bruyn [16] looked at the effect of casein adsorption on the kinetics of HA precipitation whereas Ritzoulis et al. [17] used the binding property of caseins to HA to make a porous ceramic–protein composite material from sodium caseinate and HA. Devold et al. [18] also studied the in vitro

Abbreviations: ANOVA, analysis of variance; DIC, differential interference contrast; HA, hydroxyapatite; SC, sodium caseinate; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TCP, tricalcium phosphate; WPI, whey protein isolate.

* Corresponding author. Fax: +64 6 350 5655.
E-mail address: a.m.ye@massey.ac.nz (A. Ye).

0021-9797/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jcis.2012.11.058

adsorption of milk proteins on tooth enamel and showed that, at neutral pH, caseins were preferentially bound to tooth enamel, forming protein pellicles. At acid pH, bovine serum albumin and lactoferrin were preferentially bound and the caseins did not adsorb, probably because the pH was close to the isoelectric point of casein. The adsorption of milk proteins on to the surface of HA is likely to be governed mainly by electrostatic interactions, but specific interactions between the phosphoserine residues of casein molecules and the calcium from HA may also be involved.

The objective of this study was to examine the adsorption of the main proteins in milk, caseins and whey proteins, on to the surface of HA particles under a range of conditions. This will increase our understanding of the system formed when HA is suspended in milk, and could also lead to new strategies for stabilising insoluble calcium salts in a milk system.

2. Materials and methods

2.1. Materials

Sodium caseinate (SC) (Alanate 180) and whey protein isolate (WPI) (Alacen 895) powders were obtained from Fonterra Co-operative Group Limited, New Zealand. The protein content was approximately 93% w/w in the SC powder and approximately 94% w/w in the WPI powder.

Food-grade HA powder was purchased from Budenheim (TCP 53-83, Budenheim, Germany). Particle size analysis of the HA powder in water was carried out using a Malvern Mastersizer 2000 (Malvern Instruments Ltd., Malvern, Worcestershire, UK). A suspension of HA in water (1% w/w) was first prepared and introduced drop by drop into the wet dispersion unit of the Mastersizer until the desired obscuration was achieved. The particle size distribution was calculated based on a refractive index of 1.63 and an absorption factor of 0.001 and the median particle size $d_{(0.5)}$ was found to be 4.5 μm. The reported specific surface area of the powder, determined by the BET (Brunauer–Emmett–Teller) method, was 65 m^2/g. The morphologies of the powders were observed by scanning electron microscopy. The particles were roughly spherical and each particle was made up of nano-sized crystals that were aggregated together, which explains the high surface area of the powder.

2.2. Preparation of HA/protein suspensions

Two methods were used to prepare suspensions of HA in protein solutions with a range of different protein to HA ratios.

- In the first method, stock solutions (6% w/w on a powder basis) of SC or WPI in Milli-Q water were prepared and stirred for at least 1 h and were left overnight at 4 °C to allow complete hydration. Protein solutions of different concentrations (0.1–6% w/w) were prepared by diluting appropriate volumes of the stock solution of SC or WPI in Milli-Q water and stirring for at least 1 h. A constant amount of HA powder (10 g) was added to 90 g aliquots of the protein solutions of different concentrations.
- In the second method, a constant amount of protein (100 μL of 1% w/w SC or WPI) was added to Eppendorf tubes containing HA suspensions of various concentrations (0.9 mL of HA suspensions of concentration between 0.1 and 3% w/w). A control was prepared by adding 100 μL of protein solution to 0.9 mL of water.

The suspensions were stirred for 2 h at room temperature (approximately 20 °C). They were then centrifuged (3000*g* for 20 min) to separate the HA from the protein solution. The supernatants were carefully poured from the pellet, weighed and analysed for residual protein content (Sections 2.3 and 2.4). The HA pellets were rinsed twice with Milli-Q water to remove the loosely bound proteins and were put aside for analysis.

2.3. Determination of surface protein concentration

The supernatants of the suspensions prepared with a constant amount of HA and various protein concentrations were analysed for total protein using the Kjeldahl method. A factor of 6.38 was used to convert nitrogen content to protein content.

The surface protein concentration (mg protein/m^2 HA) was calculated from the surface area of the HA and the difference between the amount of protein used to prepare the suspension and that measured in the supernatant. The surface protein concentration was corrected to take into account the portion that was entrapped between particles in the centrifuged pellet but not adsorbed on to the particles (occluded solvent). The surface protein concentration was calculated using the following formula.

$$\text{Surface protein concentration} = \frac{m_i[P_i] - m_{\text{sup}} \cdot [P_{\text{sup}}] - (m_{\text{wetsed}} - m_{\text{drysed}}) \cdot [P_{\text{sup}}]}{m_{\text{drysed}} \cdot [\text{SA}_{\text{HA}}]} \times 10$$

where m_i is the mass of the initial protein solution (g), m_{sup} is the mass of the supernatant (g), m_{wetsed} is the mass of wet HA powder obtained after centrifugation (g), m_{drysed} is the mass of dry HA powder added to the initial protein solution (g), P_i is the measured protein concentration of the initial protein solution (g/100 g), P_{sup} is the measured protein concentration of the supernatant (g/100 g) and SA_{HA} is the surface area per gram of HA (65 m^2/g). The portion of the non-adsorbed protein was shown to be 15% of the supernatant proteins at the worst (for high initial protein concentrations, when a large part of the initial proteins remained in the supernatants after adsorption). However, it is believed that this figure could be even lower than 15%, as the HA pellets were quite compact and the unbound or loosely bound proteins could have been squeezed out of the occluded solvent.

2.4. Determination of surface protein composition and preferential adsorption

To identify which proteins bound to HA, supernatants prepared with a constant amount of protein solution added to different amounts of HA were analysed. The individual proteins in the supernatant were determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). A known amount of supernatant was mixed with SDS buffer (0.5 M Tris/HCl buffer, 2% w/w SDS and 0.01% w/w bromophenol blue, pH 6.8) and 5% w/w β-mercaptoethanol was added to the samples followed by heating at 95 °C for 5 min in a boiling water bath to reduce the proteins. A 10 μL aliquot of the solution was loaded on to SDS gels previously prepared on a Bio-Rad mini-gel slab electrophoresis unit (Bio-Rad Laboratories, Richmond, CA) and run at 200 V for 1 h. The protein bands were stained using a solution of Amido Black and the percentage composition of each sample was determined by scanning the bands for α_s- (α_{s1}- + α_{s2}-), β- and κ-caseins (for samples prepared with SC) or β-lactoglobulin and α-lactalbumin (for samples prepared with WPI).

The integrated intensities of the bands were determined using Molecular Dynamics ImageQuant TL software (version 7.0). The amount of protein remaining in the supernatants was expressed as a percentage of the proteins in the control (0.1% w/w SC or WPI without added HA). Each gel experiment was repeated at least three times. Variations were ~4% for α_s-casein (α_{s1}- + α_{s2}-), ~3% for β-casein, ~6% for κ-caseins and ~3% for β-lactoglobulin and α-lactalbumin.