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Original article

Solubilisation of calcium and magnesium from the marine red algae *Lithothamnion calcareum*

Yi-Shen Zhu,[1,2] Alan Connolly,[1] Anders Guyon[1] & Richard J. FitzGerald[1]*

1 Department of Life Sciences, University of Limerick, Limerick, Ireland
2 College of Biotechnology and Pharmaceutical Engineering, Box No 77, PuZhuNanLu No 30, Nanjing University of Technology, Nanjing 211816, China

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Summary Marine minerals are a potential source of calcium and magnesium for nutritional supplementation. This study analysed the solubilisation of calcium and magnesium from the skeletal remains of *Lithothamnion calcareum*. Scanning electron microscopy analysis demonstrated a nonporous microstructure. Spectrophotometric determination showed that the calcium and magnesium contents were 30.01 and 6.22% (w/w), respectively. Solubilisation of calcium and magnesium was highly pH dependent. The temperature-dependent solubilisation of calcium fitted the shrinking core model. The apparent activation energy for calcium solubilisation was 28.6 kJ mol^{-1}. Inclusion of caseinophosphopeptides (CPPs), casein-derived mineral binding peptides, during the solubilisation of calcium and magnesium appeared to decrease the extent of calcium solubilisation at pH 6.0 and 8.0. The results herein have implications for the choice of optimal pH conditions for the sustained release of calcium and magnesium from marine mineral sources.

Keywords Apparent activation energy, caseinophosphopeptides, marine minerals, pH-stat titration.

Introduction

The skeletal remains of *Lithothamnion calcareum* are a natural source of calcium and magnesium. The red marine algae, *Lithothamnion calcareum* (Pallas), also known as *Phymatolithon calcareum* (Pallas) (Adey & McKibbin, 1970), thrive in the cold Atlantic waters off the southwest coast of Ireland and the northwest coast of Iceland. Minerals from seawater are accumulated in the algal frond over the lifespan of the organism. Eventually, the mineral-rich fronds break off the living organism and fall to the ocean floor. When harvested, these fronds which are rich in calcium and magnesium and have potential as a dietary source of these important minerals when used, for example, as a food supplement.

Calcium, the most abundant mineral in the living body, has attracted much attention due to its various functions, for example, in the regulation of blood pressure (McCarron & Reusser, 2001), the secretion of hormones and enzymes and in sending messages through the nervous system (Glade, 1997). Over 99% of bodily calcium is stored in bone and teeth. The remainder is found in blood, muscle and extracellular fluid (Laine *et al*., 2008).

*Correspondent: Fax: +353 61 331490;
e-mail: dick.fitzgerald@ul.ie

Magnesium, the second most abundant intracellular cation, is also an essential mineral required for a range of physiological functions in living organisms. Leroy (1926) and Kruse *et al*. (1933) first described the essentiality of magnesium to animals. Magnesium is a cofactor in more than 300 enzymatic reactions including enzymes involved in energy metabolism and nucleic acid synthesis. It is also required for effective absorption of calcium. Magnesium has a protective effect against a number of diseases, for example, magnesium deficiency impairs glucose tolerance and is a risk factor for the cardiovascular complications associated with diabetes (Ma *et al*., 1995). The daily magnesium intake in the Western diet while sufficient is not enough to maintain high normal serum magnesium concentrations. Therefore, increased dietary magnesium intake may reduce the risk associated with various diseases, for example coronary artery disease and osteoporosis (Vormann, 2003).

The solubility of calcium and magnesium is important for their bioavailability as dietary minerals. Casein-derived bioactive peptides are potential modulators of various physiological functions (Meisel & FitzGerald, 2003). Caseinophosphopeptides (CPPs) have been extensively studied for their potential mineral bioavailability enhancement properties. Phosphoseryl residues associated with CPPs have been shown *in vitro*

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to inhibit the formation of insoluble calcium phosphate complexes. The application of CPP–amorphous calcium phosphate (ACP) complexes as agents in the prevention/treatment of dental caries demonstrate that CPPs can act as efficient mineral carriers in oral care and dental products (Cross *et al*., 2007; Reynolds *et al*., 2008; Nongonierma & FitzGerald, 2012). A potential explanation for the role of CPPs in promoting dietary calcium uptake/absorption has been outlined (Gravaghi *et al*., 2007). The *in vivo* role of CPPs in mineral bioavailability enhancement is still unclear. The role of CPPs in mineral solubilisation from *Lithothamnion calcareum* has not been previously investigated.

The objective of this study was to characterise the dissolution properties of calcium and magnesium from the fronds of *Lithothamnion calcareum* between pH 2.0 and 8.0 at temperatures between 20 and 50 °C. The role of CPP inclusion on calcium and magnesium solubilisation was also studied.

Materials and methods

Materials

The commercially available marine mineral test sample, Acid Buf™ (Batch Nos: 270706 and 081011), derived from the fronds of the red algae *Lithothamnion calcareum* was supplied by Celtic Sea Minerals Ltd., (Carragiline, Cork, Ireland). During harvesting, the mineralised fronds were separated from extraneous materials, sterilised, dried and milled under ISO, FEMAS and GMP B2+ certification. Sodium caseinate [NaCN, 85.9% (w/w) protein] was from Arrabawn Co-op Society Ltd. (Tipperary, Ireland). Syringe filters (15 mm, 0.2 μm regenerated cellulose membranes) were from Phenomenex Inc. (Cheshire, UK). All other analytical grade reagents were supplied by Sigma-Aldrich (Dublin, Ireland).

Microscopic and spectroscopic characterisation

Scanning electron microscopy (SEM) images of the mineral sample were obtained using a Hitachi SU-70 SEM (Hitachi High-Tech Co., Tokyo, Japan) operated at an acceleration voltage of 10 kV. Chemical composition analysis was performed using an energy-dispersive X-ray (EDX) spectroscope fitted with an XMAX SDD Detector (FE-SEM SU-70; Hitachi High-Technologies Europe GmbH, Berkshire, UK) having a 50 mm^2 window.

Calcium and magnesium solubilisation

The mineral sample (2.5 g) was suspended in 150 mL distilled water in a 600 mL beaker. The pH of the solution was adjusted from neutral pH to pH 2.0 using 1.0 M HCl, to pH 4.0 and 6.0 using 0.1 N HCl, and to pH 8.0 using 0.1 N NaOH in a pH-stat (Titrino 718 pH-Stat; Metrohm, Herisau, Switzerland). The pH-stat was configured to maintain the required pH with a deviation limit of 0.01 pH units. The volume of HCl used to titrate the suspension to the required pH was recorded. The titration was continuously controlled and monitored when test sample pH reached the designed value, that is, pH 2.0–8.0. Test sample suspension was continuously stirred with a magnetic stirrer (Metrohm, Herisau, Switzerland) at speed setting 2 using a 4 cm diameter (7 g) stirring bar. Aliquots (2.0 mL) of the suspensions were taken in triplicate at time zero and various times during the titration process. These samples were filtered using 0.20-μm syringe filters; the filtrates were stored at room temperature prior to subsequent analysis. Distilled water (2 mL) was added to the test suspension after each sampling event to maintain the original test volume.

Spectrophotometric determination of calcium

Calcium was determined in duplicate for each test sample using an o-cresolphthalein complexone (o-CPC) reagent (Moorehea & Biggs, 1974; Cohen & Sideman, 1979) adapted to microplate reader format (Synergy™ HT, Biotek, Leeds, UK). Ten μL of appropriately diluted sample (the concentration of calcium in the test sample was in the range of the calcium concentration in the standards) and 200 μL of 2-amino-2-methyl-1-propanol (AMP) were added to each well in the microplate. Following a 15-s shake at medium level, the absorbance at 580 nm was determined in triplicate. Linear standards were prepared for each plate using Ca^{2+} concentrations ranging from 0.01 to 0.05 g L^{-1}. The o-CPC solution (80 μL, 100 mg L^{-1}) was then added. The plate was gently tapped to mix the solution and was then incubated at room temperature for 2 min after which the A_{580} was determined in triplicate. Distilled water (10 μL) was used as blank. The Ca^{2+} concentration in the test samples was calculated from the standard curve. The weight of dissolved Ca^{2+} in the test samples was calculated using eqn 1:

$$W_{Ca^{2+}} = ((A - A_0) - b)/a * d * V_{Total})/1000 \qquad (1)$$

where W was the weight of Ca^{2+} in grams, V_{Total} was the total volume (mL) of suspension during the titration, d was the dilution ratio, a (slope) and b (interception) were the parameters obtained from the linear equation of the Ca^{2+} standard curve, while A and A_0 were the absorbance values at 580 nm of sample and blank, respectively.

Spectrophotometric determination of magnesium

Magnesium was determined in duplicate for each test sample according to the procedure of Abernethy and

Fowler (1982), which was again adapted to 96-well microplate reader format. Linear standards were prepared for each plate using Mg^{2+} concentrations ranging from 0.001 to 0.030 g L^{-1}. Fifteen μL of appropriately diluted sample was added to each well along with 230 μL of magnesium reagent. The magnesium reagent consisted of 100 mL of stock reagent A, 100 mL of stock reagent B and 10 mL of stock reagent C which were gradually added to 800 mL of distilled water. This reagent should be at pH 11.5 and have an absorbance at 500 nm equal to 0.50 units. (Stock reagent A: 1.2 g of calmagite dissolved in 2 L of water, this solution was stored in the dark. Stock reagent B: 19 g of 2-amino-2-methyl-1-propanol dissolved in 1500 mL water. Subsequently, 3.04 g of ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacitic acid (EGTA), 67 mL of Empigen BB (300 g L^{-1}) and 1 mL of triethanolamine (TEA) were added and dissolved in this solution. The solution was then diluted to 2 L followed by adjustment to pH 11.5 using 1 M potassium hydroxide. Stock reagent C: 1.28 g of strontium chloride hexahydrate dissolved in 100 mL distilled water.) The plate was gently tapped to mix the solution and was then incubated at room temperature for 2 min after which the A_{500} was determined in triplicate. Distilled water (15 μL) was used as blank. The weight of dissolved Mg^{2+} in the test samples was calculated using eqn 2:

$$W_{Mg^{2+}} = ((A - A_0) - b)/a * d * V_{Total})/1000 \qquad (2)$$

where W was the weight of Mg^{2+} in grams, V_{Total} was the total volume (mL) of solution in titration, d was the dilution ratio, a (slope) and b (interception) were the parameters from the linear equation of the Mg^{2+} standard curve, while A and A_0 were absorbance values at 500 nm of sample and blank, respectively.

Determination of total calcium and magnesium contents

The total calcium and the magnesium contents were detected in independent triplicates. The mineral sample (about 0.1 g) was suspended in 1 mL of 37% (v/v) HCl followed by agitation for 10 min. These samples were then allowed to stand overnight at room temperature to bring about complete solubilisation of the calcium and magnesium. Following overnight reaction, test samples were adjusted to pH 7.0 using 30% (w/v) NaOH. The sample solution was then transferred to a 25 mL volumetric flask and diluted with distilled water to the mark. Diluted samples were then tested in triplicate for calcium and magnesium as previously described herein. The total weight of calcium and magnesium in the test samples was calculated using eqn 1 and 2, respectively.

Kinetic analysis

The solubilisation rates of calcium and magnesium at pH 6.0 and at 20, 30, 33 and 50 °C were determined in duplicate using the shrinking core model (SCM) essentially as previously described (Siagi & Mbarawa, 2009). In brief, the SCM is applied to characterise the reaction of a nonporous solid with a fluid. The SCM assumes that the reaction starts at the particle outer surface and then progressively continues towards the centre of the particle. The rate constant for the solubilisation process was determined using eqn 3:

$$1 - (1 - X_B)^{1/3} = kt \qquad (3)$$

where X_B was the fraction of dissolved calcium or magnesium, k was the rate constant and t was the reaction time in seconds.

The temperature effect on the solubilisation rate constant k was estimated using the Arrhenius equation to calculate apparent reaction energy according to eqn 4:

$$k = k_0 e^{-E/RT} \qquad (4)$$

where k_0 represents the pre-exponential factor, E: the apparent activation energy, R: the universal gas constant and T: the absolute temperature (°K), both E and k_0 were calculated from the linear plot of ln k vs. $1/T$ in eqn 4.

Preparation of caseinophosphopeptides

Sodium caseinate (NaCN, 10% w/v) was reconstituted in 500 mL distilled water at 37 °C. The pH was adjusted to 8.0 using 1.0 N NaOH. TPCK-treated trypsin [0.2% (w/w) of NaCN powder] was dissolved in 1.0 mL distilled water and added to the above solution with constant stirring. A pH-stat charged with 1.0 N NaOH was used to maintain the pH at pH 8.0 during the course of the 3-h reaction at 37 °C. After 3 h incubation, the hydrolysate was adjusted to pH 4.6 with 1.0 N HCl and centrifuged at 4000 ***g*** (Universal 320R; Hettich Zentrifugen, Tuttlingen, Germany) for 20 min to remove any remaining partially hydrolysed CN in the precipitate (McDonagh & FitzGerald, 1998). The clarified supernatant was aggregated with $CaCl_2$ (10% w/v) at pH 7.0 and then precipitated using an equal volume of 99.8% (v/v) ethanol (Adamason & Reynolds, 1995). Following centrifugation at 6000 ***g*** for 10 min, the precipitate was suspended in distilled water, freeze-dried (Freezone® 4.5; Labconco Co., Kansas, MO, USA) and subsequently stored at −20 °C. Decalcified CPPs were prepared using a mixed bed ion-exchange resin (Chelex-100) according to the procedure of McDonagh and FitzGerald (1998).

Determination of the effect of CPP addition on mineral solubilisation

Designated amounts of CPPs (0.125, 0.250 and 0.500 g) or decalcified CPPs (0.0817 g, equivalent to 0.125 g of nondecalcified CPPs) were suspended in 10 mL distilled water. An aliquot (50 μL) was diluted with distilled water to 750 μL for use as a blank reference for mineral content. The remaining 9950 μL was added to 2.5 g of the marine mineral sample previously suspended in 140 mL distilled water. The pH of the suspension was adjusted to pH 6.0 and 8.0. Mineral solubilisation at these pHs was followed using a pH-stat as previously described herein.

Results and discussion

The SEM image in Fig. 1 shows that the test sample has a nonporous irregular-shaped structure having diameters ranging from 1 to 40 μm. Sodium, magnesium, calcium, silicon and chlorine were detected following EDX analysis. Calcium and magnesium constituted 93.85% (w/w) of these elements. The EDX determined calcium-to-magnesium (w/w) ratio was 5:1 (n = 13). The results from the spectrophotometric determination of the acid-treated test sample showed that the percentage of solubilised calcium and magnesium was 30.01% (w/w, n = 3, CV: 2.30%) and 6.22% (w/w, n = 3, CV: 1.4%), respectively. This gives a calcium/ magnesium ratio of 4.8:1 which is in good agreement with the ratio determined by EDX. To our knowledge, no previous studies report on the concentration of calcium and magnesium in Acid Buf™ using these techniques. The high levels of calcium and magnesium present indicate that the test material is a good marine mineral source.

As expected, the rate and extent of acid uptake decreased as the pH increased from pH 2.0 to 8.0. Figure 2a shows the rate and extent of HCl uptake during titration of the test sample at pH 2.0, while Fig. 2b shows HCl uptake during titration at pH 4.0, 6.0 and 8.0. In the solubilising of calcium and magnesium at pH 2.0, it was necessary to utilise 1.0 N HCl during titration as the rate of solubilisation was very rapid. It was possible to utilise 0.1 N HCl for titrations carried out at pH 4.0, 6.0 and 8.0, where the rate and extent of solubilisation decreased with increasing pH. The titration profiles shown in Fig. 2 were typical for those obtained with different batches of Acid Buf™. The rumen/stomach provides an acidic environment, in which calcium and magnesium can be quickly released. On the contrary, the intestine provides a neutral pH environment, in which calcium and magnesium may be released much slowly. The results presented in Fig. 2 provide an indication of the potential rate of solubilisation of the test sample during gastrointestinal transit.

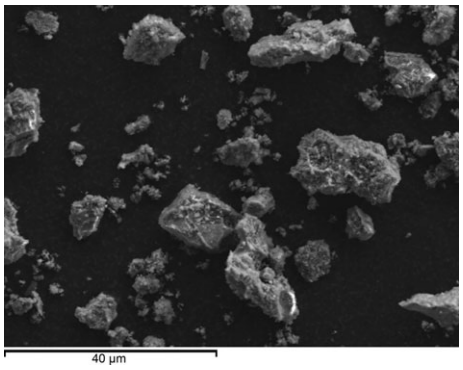

Figure 1 Scanning electron microscopy (SEM) image of the skeletal remains of *Lithothamnion calcareum*.

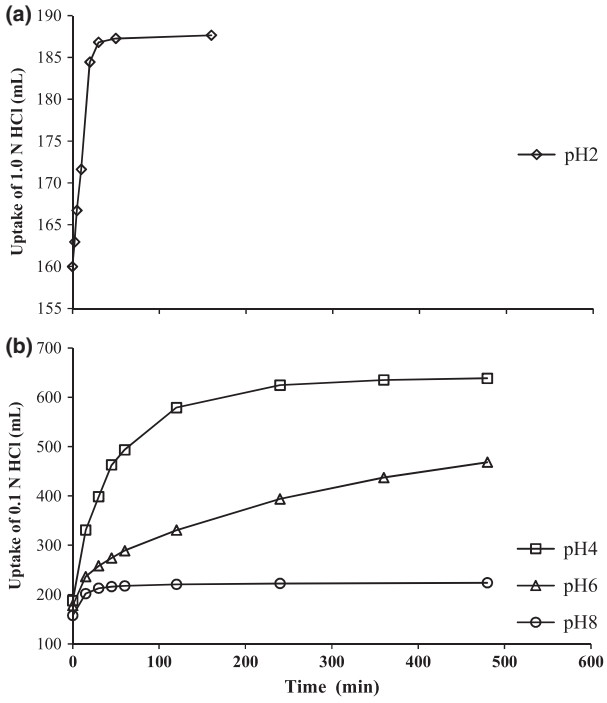

Figure 2 (a) Uptake of 1.0 N HCl as a function of solubilisation time by 2.5 g of test sample at pH 2.0 and (b) uptake of 0.1 N HCl as a function of solubilisation time by 2.5 g of test sample at pH 4.0, 6.0 and 8.0.

Spectrophotometric determination (performed with test batch 081011) demonstrated that the release of calcium increased from 3.2% (w/w) at pH 8.0 to >95% (w/w) at pH 2.0 (Fig. 3a). Furthermore, spectrophotometric determination demonstrated that magnesium release increased from 46% (w/w) at pH 8.0 to 91% (w/w) at pH 2.0 (Fig. 3b). These results were in agreement with the findings on the solubilisation of

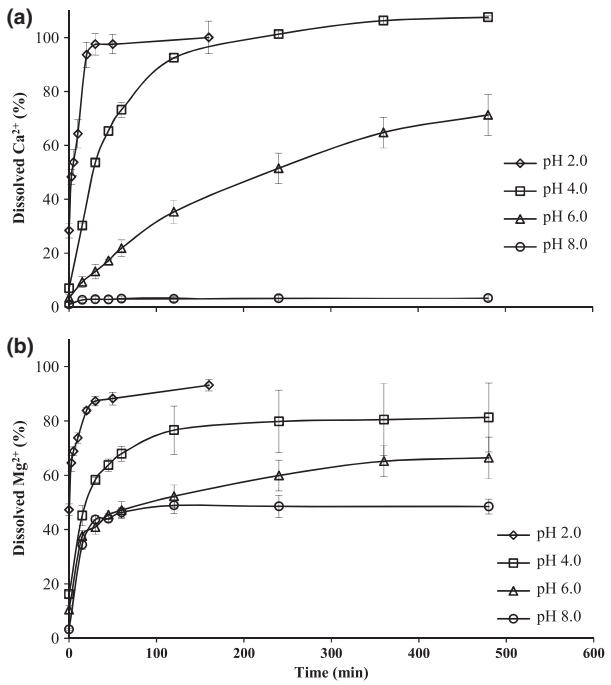

Figure 3 (a) Dissolved Ca^{2+} as a percentage of total Ca^{2+} in test sample as a function of time at pH 2.0, 4.0, 6.0 and 8.0; (b) dissolved Mg^{2+} as a percentage of total Mg^{2+} in test sample as a function of time at pH 2.0, 4.0, 6.0 and 8.0. Data points represent the mean of triplicate determinations assayed in triplicate for individual test samples at given time points.

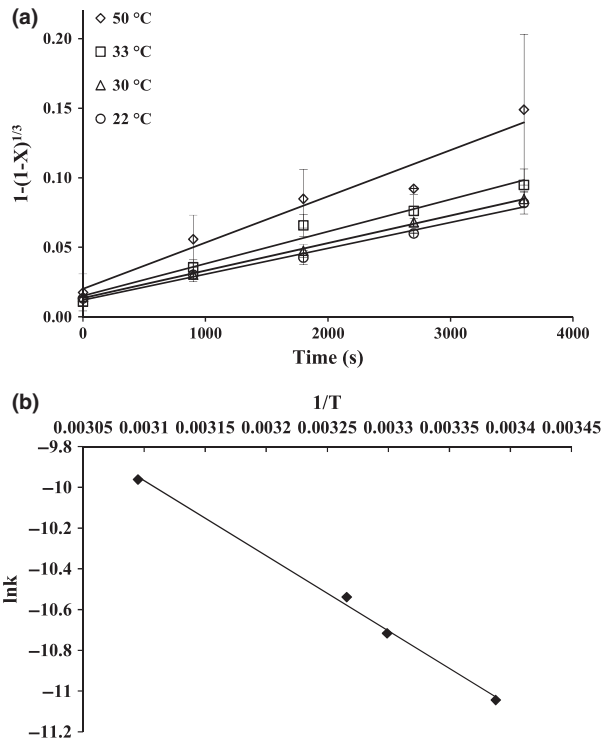

Figure 4 (a) Plot of $1\text{-}(1\text{-}X)^{1/3}$ (from the shrinking core model) as a function of calcium solubilisation time at four temperatures for the marine mineral test sample (20 °C: $y = 2 \times 10^{-5}x + 0.0121$ $r = 0.991$; 30 °C: $y = 2 \times 10^{-5}x + 0.0135$, $r = 0.988$; 33 °C: $y = 2 \times 10^{-5}x + 0.015$, $r = 0.974$; 50 °C: $y = 3 \times 10^{-5}x + 0.02$, $r = 0.951$) and (b) Arrhenius plot for calcium solubilisation. Data points represent mean ± SD of two determinations.

magnesium from magnesium hydrate (Guo *et al.*, 2011). It also appeared that the rate of magnesium solubilisation was faster than that for calcium particularly at pH 6.0 and 8.0 (Fig. 3). In comparison with the acid uptake results (Fig. 2), it was shown that the rate and extent of calcium and magnesium solubilisation increased with decreasing titration pH (Fig. 3). Calcium and magnesium are mainly released in the acidic environment. This indicates that the minerals should be solubilised during the gastric stage of digestion.

The influence of temperature on the solubilisation rate of calcium and magnesium was investigated between 20 and 50 °C. It was shown that the temperature-dependent solubilisation of calcium fitted well with the shrinking core model (Fig. 4a). The results indicate that eqn 3 is valid for description of the dissolution of calcium from the test sample, and that the pH-dependent solubilisation is the rate-limiting step for the dissolution process. The Arrhenius plot in Fig. 4b was drawn from the constants derived from the slopes in Fig. 4a. The calculated apparent activation energy for calcium solubilisation was 28.6 kJ mol^{-1} (Fig. 4b). A wide range of activation energies have been reported in literature for calcium dissolution, that is, from 26.1 kJ mol^{-1} (Siagi & Mbarawa, 2009) to 19 kJ mol^{-1} (Alkattan *et al.*, 1998). The ratio of hydrogen ion activity at the surface vs. hydrogen ion activity in solution has been shown to be strongly dependent on pH and temperature. It is generally accepted that the pH-dependent dissolution of minerals is also strongly dependent on temperature (Aydogan *et al.*, 2007). The results obtained herein are therefore in general agreement with those previously reported in related studies on mineral solubilisation. As already outlined, the test sample contains about 6% (w/w) magnesium and the rate of magnesium solubilisation was faster than that for calcium. Ideally, sampling intervals should be per minute. However, it was not possible to take samples at such short intervals using the manual experimental protocol employed. Therefore, the apparent activation energy for magnesium solubilisation could not be determined. To our knowledge, no previous studies report on the rate and extent of calcium and magnesium solubilisation from the skeletal remains of *Lithothamnion calcareum*.

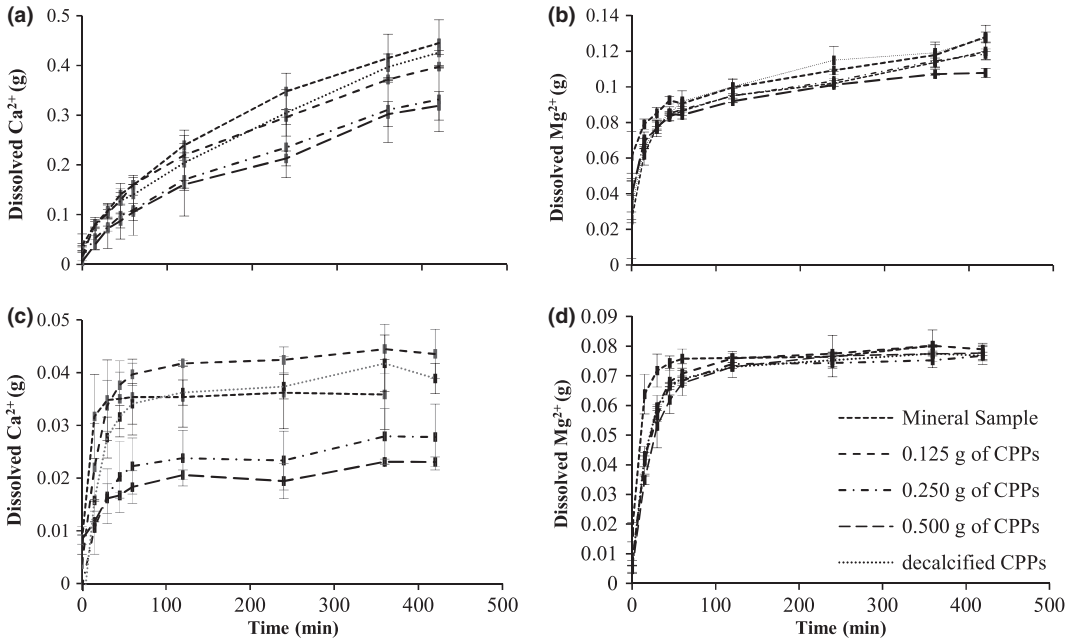

Figure 5 Weight of dissolved (a) calcium and (b) magnesium at pH 6.0 along with weight of dissolved (c) calcium and (d) magnesium at pH 8.0 using 2.5 g of test sample and also test sample incubated with 0.125 g caseinophosphopeptides (CPP), 0.250 g CPP, 0.500 g CPP and decalcified CPPs as a function of solubilisation time. Data points represent the mean of triplicate determinations assayed in triplcate for individual test samples at given time points.

The effect of CPP addition on mineral solubilisation was determined at pH 6.0 and 8.0 as these conditions are relevant to those in the distal ileum where maximal passive absorption of calcium is thought to take place (FitzGerald, 1998). Surprisingly, the inclusion of CPPs appeared to decrease the amount of calcium dissolved from 2.5 g of the test sample at pH 6.0 (Fig. 5a) and pH 8.0 (Fig. 5c). Furthermore, it appeared that decalcified CPPs resulted in a lower decrease in calcium solubilisation than nondecalcified CPPs. At pH 6.0, the weight of calcium solubilised from the test sample, test sample with decalcified CPPs and test sample with CPPs 0.125, 0.250 and 0.500 g was 0.47, 0.43, 0.39, 0.37 and 0.30 g, respectively. The weight of calcium solubilised from the test sample, test sample with 0.125 g CPPs and test sample with decalcified CPPs at pH 8.0 was similar, that is, about 0.04 g of soluble calcium per 2.5 g of test sample, while the weight of calcium solubilised from the test sample with 0.250 and 0.500 g CPPs at pH 8.0 was 0.032 g and 0.023 g of soluble calcium per 2.5 g, respectively. There were no marked differences observed in the extent of magnesium solubilised from the test sample on inclusion of CPPs between 0.125 and 0.500 g and decalcified CPPs at the equivalent to 0.125 g per 2.5 g at either pH 6.0 or 8.0 (Fig. 5b,d). The weight of magnesium solubilised from the samples with and without CPPs ranged between 0.10 and 0.12 g at pH 6.0 and was about 0.08 g at pH 8.0.

The reduced level of dissolved calcium observed from the mineral sample (Fig. 5a,c) may be as a direct result of CPP addition. As we already know, CPPs form complexes with calcium through phosphorylated seryl residues (Nongonierma & FitzGerald, 2012). Therefore, nondecalcified CPP addition increased the calcium concentration of the original test solution which, in turn, may have led to reduced calcium solubilisation from the mineral sample. This finding is further demonstrated by the results obtained for the decalcified CPPs (Fig. 5a,b). Addition of the decalcified CPPs resulted in a reduction in calcium concentration of the original test solution, while also demonstrating an increase in dissolved calcium when compared to nondecalcified CPP addition at equivalent levels (i.e. 0.125 g). In terms of dissolved magnesium (Fig. 5b,d), both the CPPs and decalcified CPPs did not complex with magnesium; therefore, magnesium content was not affected.

The data described herein were obtained with two discrete batches of test material. While batch-to-batch variation is inevitable, the trends in the data obtained herein should be reflective of those expected in different batches of this marine mineral ingredient.

Conclusion

The skeletal remains of the red algae *Lithothamnion calcareum* display a nonporous microstructure as determined using SEM. It contains 30.01 and 6.22% (w/w) calcium and magnesium, respectively. The rate and extent of calcium and magnesium solubilisation from the marine mineral source was pH dependent. Higher rates and extents of solubilisation occurred as the pH was decreased. The rate and extent of calcium solubilisation increased with increasing temperature within the range 20–50 °C. These results have implications for the sustained release and potential enhanced bioavailability of calcium and magnesium from marine mineral sources when formulated within food and beverage products particularly at acidic pH, that is, pH 2.0. At pH 6.0 and 8.0, less calcium and magnesium could be released. The results suggest that the gastric phase of digestion may further enhance the release and bioavailability of calcium and magnesium from the skeletal remains of *Lithothamnion calcareum*. The inclusion of CPPs, in both their decalcified and nondecalcified form, appeared to decrease the extent of calcium solubilisation from the test sample at pH 6.0 and 8.0. Inclusion of CPPs appeared to have no effect on magnesium solubilisation.

Acknowledgments

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