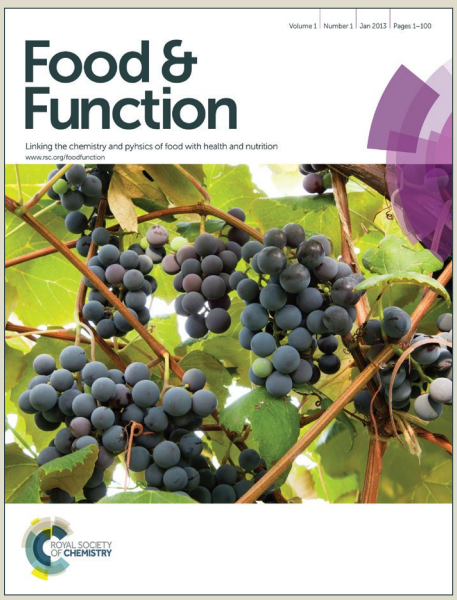
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Fabrication and characterization of the nano-composite of whey protein hydrolysate chelated with calcium

Cai Xixi, Zhao Lina, Wang Shaoyun*, Rao Pingfan

College of Bioscience and Biotechnology, Fuzhou University, Fuzhou, Fujian 350108, China

*Corresponding Author

Tel: +86-591-22866375

Fax: +86-591-22866278

E-mail address: shywang@fzu.edu.cn

Abstract

The nano-composites of whey protein hydrolysate (WPH) chelated with calcium were fabricated in aqueous solution at 30 °C for 20 min, with the ratio of hydrolysate to calcium 15:1 (w/w). The UV scanning spectra, fluorescent spectra, Fourier transform infrared spectroscopy, dynamic light scattering and atomic force microscope were applied to characterize the structure of WPH-calcium chelate. The nano-composite showed the successful incorporation of calcium into the WPH, indicating the interaction between calcium and WPH. The chelation of calcium ions to WPH caused the molecular folding and aggregating which led to the formation of WPH-calcium chelate in nanoparticle size, and the principal sites of calcium-binding corresponded to the carboxyl groups and carbonyl groups of WPH. WPH-calcium chelate exerted the excellent stability and absorbability in both acidic and basic conditions, which was beneficial to calcium absorption in gastrointestinal tract of human body. Moreover, the calcium absorption of WPH-calcium chelate on Caco-2 cells was significantly higher than those of Calcium gluconate and $CaCl_2$ *in vitro*, suggesting the possible increases in calcium bioavailability. The findings suggest that WPH-calcium chelate has the potential in making dietary supplements for improving the bone health of human body.

Key words: Whey protein hydrolysate-calcium chelate; Nanocomposite; Fabrication; Characterization

Introduction

Calcium plays an important role in the human body. The adequate intake of calcium could increase the bone density of children and it is essential among the middle-aged and the aged to prevent osteoporosis.[1,2] Calcium deficiency is proved to be associated with the development of diseases, including osteoporosis, osteopenia and arterial hypertension.[3,4] This deficiency could be controlled by maintaining the adequate calcium level through the intake of calcium. The ionized calcium has served as main calcium supplements for human beings in recent years.[5] However, the low bioavailability of dietary ionized calcium severely restricts its usage, and the inorganic calcium is inclined to form calcium phosphate deposition in basic intestine environment.[6] Bioavailability of calcium is one of the most important topics in nutrition research. The possibility to enhance calcium absorption at intestinal level and, subsequently, its availability for mineralized tissues, has attracted numerous studies at different levels, animal and/or human.[7] Chelated calcium, of which certain substances prevent its precipitation, could be effectively absorbed by the body.[8]

The bioavailability of dietary ionized calcium is affected by intestinal basic environment. Calcium-binding peptides from soybean protein hydrolysates, can form complexes with calcium to improve its absorption and bioavailability.[8] Concerning whey protein hydrolysate (WPH), our previous study has affirmed that WPH produced by controllable hydrolysis conditions could bind with calcium, and therefore form soluble WPH-calcium chelate. Nonetheless, it is still unclear about the structure and bioavailability of the nano-composite.

The objective of this study was to prepare the nano-composites of whey protein hydrolysate chelated with calcium, to characterize the WPH-calcium chelate through UV scanning spectra, fluorescent spectra, Fourier transform infrared spectroscopy, dynamic light scattering and atomic force microscope. In order to test calcium uptake efficiency *in vitro,* celluar uptake of calcium of WPH-calcium chelate on Caco-2 cell line was experimented. The study could provide basic theories for the function and activity evaluation of the potential calcium supplements.

Materials and methods

Materials

Whey protein was kindly provided by Hilmar Corporation (USA). The commercial protease, Flavourzyme (EC. 3.4.11.1, 2×10^6 U g^{-1}) and Protamex (EC. 3.4.24.28, 1.5×10^6 U g^{-1}) were purchased from Novo (Novozymes, Denmark). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were the product of Gibco BRL Life Technologies. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (USA). All the other chemicals and solvents were of analytical grade.

Fabrication of whey protein hydrolysate-calcium chelate

In order to fabricate whey protein hydrolysate-calcium chelate, whey protein hydrolysates was first prepared. 5 % (w/v) whey protein concentrate solution was heated at 80 °C for 20 min to get full dissolution. The sample was hydrolyzed using Flavourzyme and

Protamex (2:1, w/w) with substrate/ enzyme ratio of 25:1 (w/w) at 49 °C and pH 7.0, the optimized temperature and the pH conditions. Sample was collected at different times (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h) and immediately heated at 100 °C for 10 min to inactivate the proteases and centrifuged at 16,000 g for 20 min, then the supernatant named whey protein hydrolysate (WPH) was collected for the determination of the calcium-chelating capacity. The degree of hydrolysis (DH) of hydrolyzed protein was determined using a formaldehyde titration method.[9] The lyophilized WPH was dissolved in distilled water, and $CaCl_2$ solution was subsequently added to make the ratio of hydrolysate to calcium 1:1, 5:1, 10:1, 15:1, 20:1, 25:1, 30:1, 35:1 (w/w), respectively. The reaction was carried out in a shaker at 30 °C for different time (10, 20, 30, 50, 90, and 120 min, respectively). Then absolute ethanol was added continuously until the final concentration was up to 85 % to allow the chelate deposited, and then centrifugated at 10,000 × *g* for 10 min. The obtained composite was collected, designated as whey protein hydrolysate-calcium chelate, for the determination of properties.

Calcium-binding capacity assay

The calcium-binding capacity was defined as the content of calcium (μg) bound with peptide (mg) after chelating reaction. Lyophilized whey protein hydrolysate was dissolved in deionized water to be 1.0 mg mL^{-1}, and mixed with 5 mmol L^{-1} of $CaCl_2$ in 0.2 mol L^{-1} of sodium phosphate buffer (pH 8.0). The solution was stirred at 37 °C for 2 h and pH was maintained at 8.0 with a pH meter. The reaction mixture was centrifuged at 10,000 *g* at the room temperature for 10 min in order to remove insoluble calcium phosphate salts. Deionized water instead of WPH mixed with 5 mmol L^{-1} of $CaCl_2$ in 0.2 mol L^{-1} of

sodium phosphate buffer was set as WPH-free control experiments. The calcium contents in the supernatant were determined using a colorimetric method with ortho-cresolphthalein complexone reagent.[10] The absorbance at 570 nm was determined after adding the working solution to the sample. The experiments were performed in triplicate, and values were expressed as mean ± standard deviation (SD).

Ultra-violet spectroscopy

The ultraviolet (UV) spectra of whey protein hydrolysate and its calcium complex were recorded over the wavelength range from 190 to 400 nm by a UV-Vis spectrophotometer (UV-2600, UNICO Instrument Co. Ltd., Shanghai, China) as the method described by Chen *et al*.[11]

Fluorescence spectroscopy

Fluorescence spectra were measured to monitor conformational changes in the WPH induced by calcium chelation using a Hitachi F-4600 fluorescence spectrophotometer (Hitachi Co., Japan). The excitation wavelength was 295 nm and emission wavelengths between 310–400 nm were recorded.

Fourier transform infrared spectroscopy

Freeze-dried sample (1 mg) mixed with 100 mg of dried KBr was loaded on the Fourier transform infrared spectroscopy (FTIR) spectrograph. All FTIR spectra were recorded using an infrared spectrophotometer from 4000 to 400 cm^{-1} (360 Intelligent, Thermo Nicolet Co., USA). The peak signals in the spectra were analyzed using OMNIC 8.2 software (Thermo Nicolet Co., Madison, WI, USA).

Size distribution

The size distribution of the WPH-calcium chelate was determined by dynamic light scattering (Zetasizer 3000HS, Malvern, UK) at room temperature and recorded for a time determined by the zetasizer program in the automatic mode. The samples were dispersed in deionized water (RI=1.330) and passed through 0.45 μm filter membrane before experiment.

Atomic force microscopy

Atomic force microscopy (AFM) measurements were carried out on a TM-AFM 5500 (Agilent Technologies, USA) at room temperature. 2 μL of WPH /WPH-calcium chelate was deposited on a freshly cleaved mica surface, spread and dried with a stream of nitrogen. The images were obtained in tapping mode, using micro-fabricated silicon cantilevers tips with a resonance frequency of 145 kHz and a spring constant of 5 N/m.

Calcium releasing percentage

WPH-calcium chelate and $CaCl_2$ were dissolved in deionized water to a concentration of 10 μg mL^{-1} respectively, and the calcium-releasing percentage was analyzed at various pH values, including 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0. After incubation in a shaking water bath at 37 ℃ for 2 h, the solutions were centrifuged in a refrigerated centrifuge at 10,000 × *g* for 10 min. The calcium content of the supernatant and the total calcium in the solution were measured using a colorimetric method with ortho-cresolphthalein complexone reagent.[12] The Calcium-releasing percentage was calculated as follows:

Calcium-releasing (%) = Calcium in supernatant / total Calcium in solution × 100

Effect of WPH–calcium chelate on celluar uptake of calcium

Cytotoxicity test The human colon adenocarcinoma, Caco-2, cell line provided by

Bioengineering Institute of Fuzhou University was used between passage number 20 and 40. Caco-2 cells were grown in DMEM supplemented with 20 % (v/v) FBS, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin and maintained at 37 °C in a humidified atmosphere of 5 % CO_2 - 95 % air atmosphere. For subculturing, the cells were removed enzymatically (0.25 % trypsin-EDTA, 10 min, 37 °C) and split at a ratio of 1:3 when confluence was reached. Cells used in cytotoxicity test were seeded on 96-well plastic cell culture clusters at a density of 1×10^4 cells cm^{-2}. For 24 h before the experiment, the cell medium was free of fetal bovine serum. For the uptake studies, cells were seeded at a density of 5×10^4 cells cm^{-2} onto 6-well plastic cell culture clusters and grown for 14 days. Cytotoxicity test was assessed by the MTT assay. Briefly, culture medium was removed and cells were pre-incubated with the tested compounds in DMEM at 37 °C for 24 h. Then 20 μL MTT (5 mg mL^{-1} in PBS) was added to each well and the cells were incubated for another 4 h. The solution was carefully aspirated and the formazan produced by mitochondrial dehydrogenase activity dissolved in 150 μL DMSO. Absorbance at 490 nm was measured and results were expressed in % of control.

Cellular uptake experiment The cell layers were washed twice with PBS to completely remove the medium. 1.5 mL of different concentration of WPH-calcium chelate was applied for 1 h at 37 °C, 5 % CO_2. Then the medium was removed and the cells were washed three times with PBS. The cells were placed on ice and then solubilized with 1 mL 0.1 % (v/v) Triton-X (in 5 mmol L^{-1} Tris-HCl, pH 7.4) for 30 min. The lysates were transferred into 1.5 mL Eppendorf tubes and centrifuged at 10,000 × *g* at 4 °C for 5 min. The supernate was collected and the total protein content and calcium content were

measured by the bicinchoninic acid (BCA) method and atomic absorption method[13] respectively. Results of calcium uptake were expressed in μg mg^{-1} protein.

Statistical Analyses

All data are presented as means (standard deviations, SDs) of three independent experiments. Statistical analysis was done using Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

Results and discussion

Fabrication of WPH -calcium chelate

Whey proteins are globular proteins that are difficult to access enzymes. The heat denaturation results in unfolding of structure and full exposure of reactive sites to enzyme, which would facilitate enzymatic hydrolysis[14,15]. The whey proteins were pre-heated at 80 °C for 20 min to get full denaturation.

The Ca-chelating capacity of whey protein hydrolysate (WPH) obtained at different hydrolysis periods ranged from 23.9 to 34.9 μg mg^{-1} (Fig. 1). These results indicated that the degree of enzyme treatment influences the Ca-chelating activity of the obtained WPH. The Ca-chelating capacity increased with the time of hydrolysis. The hydrolysate with a DH of 25.9 %, obtained at the hydrolysis time of 7 h, possessed the Ca-chelating capacity of 33.9 μg mg^{-1}. If the hydrolysis time was prolonged, there was no significant further increase in Ca-chelating ability ($p < 0.05$), which meant that hydrolysis time played an important role in the chelating reaction between WPH and Ca ions. A similar result was reported that yak casein hydrolysate had highest Zn-chelating capacity at DH 22.8 % and

if the hydrolysis time was too long, the Zn-chelating ability decreased.[12] In order to get the WPH with the highest Ca-chelating capacity, whey protein hydrolysate was prepared with DH of 25.9 %. The obtained hydrolysate was freeze-dried, pulverized, placed in sealed bags, and stored at 4 °C for further chelate preparation.

In order to fabricate whey protein hydrolysate-calcium chelate, the effects of chelating time and mass ratio of WPH to calcium were evaluated. The chelation of WPH and calcium was a fast reaction that chelating time did not seem to affect the calcium-binding capacity of WPH in the selected range (Fig. 2A), whereas the influence of mass ratio of WPH to calcium was significant (Fig. 2B). The increase in the WPH/calcium ratio showed an increasing trend for calcium-binding capacity of WPH, when the ratio reached up to 15:1 (w/w), the trend slowed down (Fig. 2B). Therefore, 20 min and 15:1 (w/w) were determined to be the optima chelating time and mass ratio of WPH to calcium for the fabrication of WPH-calcium chelate. The lyophilized WPH was dissolved in distilled water, and $CaCl_2$ solution was subsequently added to make the ratio of hydrolysate to calcium 15:1 (w/w) and the reaction was carried out in a shaker at 30 °C for 20 min. Then absolute ethanol was added continuously until the final concentration was up to 85 % to allow the chelate deposited, and then centrifugated at 10,000 × *g* for 10 min. The obtained composite was collected, designated as WPH-calcium chelate.

Ultra-violet (UV) spectroscopy

The UV absorption spectra of $CaCl_2$, WPH and WPH-calcium chelate showed obvious band shifts (Fig. 3). With the addition of Ca ions, the UV absorption spectra of WPH obviously shifted/changed both in band and intensity in the area of 230 to 300 nm. The

maximum absorption peak of WPH shifted from 272 to 277 nm, which meant that the oxygen atom of the carbonyl group in peptides bound with Ca ions.[16] The spatial structure with the chirality of the chromospheres (C=O, -COOH) and auxochromes (-OH, $-NH_2$) of peptides changed after binding with calcium,[17,18] which induced intensity changes and red shift in the UV spectra. Both the band shift and intensity change suggest that WPH could bind with calcium ions and therefore form WPH-calcium chelate.

Fluorescence spectroscopy

Fluorescence spectroscopy can be used to identify the interactions among protein and other molecules in composites.[19] Decreased florescence intensity is a classic indicator for peptide folding, the calcium ions caused peptides folding, and led to the formation of calcium-WPH chelate.[20] WPHs containing different concentrations of $CaCl_2$ were analyzed by a fluorescence spectrometer, respectively. With increasing content of calcium chloride of 0, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 mmol L^{-1}, the fluorescence intensity of samples decreased in proper sequence, and the absorption peak of the samples shifted from 351 to 357 nm (Fig. 4). The fluorescence intensity decreased with increasing $CaCl_2$, which might also be due to fluorescence quenching effect of metal ions. The aromatic amino acids phenylalanine, tyrosine, and tryptophan can generate endogenous fluorescence at the appropriate excitation wavelength. The fluorescence absorption band at 320 nm decreased as the calcium concentration increased. In particular, the endogenous fluorescence decreased dramatically when 1 mmol L^{-1} of $CaCl_2$ was added to the WPH solution (Fig. 4). With the calcium ion concentration increased, the extent of endogenous fluorescence extinction reduced. In general, calcium ion may cause fluorescence

quenching of calcium-binding WPH, which likely contributed to the decrease in the fluorescence intensity.[21]

Fourier transform infrared spectroscopy

Changes in the characteristic FTIR absorption peaks of carboxylates and amides in proteins can reflect the interaction of metal ions with organic ligand groups in the protein.[20] The FTIR spectra of WPH and WPH-calcium chelate are shown in Fig. 5. After binding with Ca^{2+}, some shifts occurred in the chelate complex, such as the wave number shift from 3,411 to 3,407 cm^{-1}, from 1,634 to 1,646 cm^{-1}, from 1,519 to 1,552 cm^{-1}, and from 1,405 to 1,401 cm^{-1}. The spectra of WPH-calcium chelate also exhibited strong bands at wave number of 1,646 cm^{-1} (amide I), corresponding to C = O and 1,401 cm^{-1} (amide II), corresponding to C-N and N-H. Moreover, the band at 3,411 cm^{-1} shifted to become two absorption peaks, which was caused by NH^{3+} of peptide bond or side chain losing a proton to exist as NH_2. The bond of NH_2 was coordinated to Ca^{2+} inducing N-H stretch and hydrogen bonds replaced by Ca-N bonds, two peaks appeared at 3,407 and 3,230 cm^{-1}. The results demonstrate carboxyl oxygen and amino nitrogen atoms are the interaction sites between calcium and WPH. These changes of bands indicate that some bonds in WPH bound with calcium ions and form WPH-calcium chelate.

Size distribution

Particle size, one of the important physical characteristics of composites, depends on the volume of the particles. .Fig. 6 shows typical size distribution of the nanoparticles of WPH and WPH-Calcium chelate. The mean radius of WPH and WPH-Ca chelate were 104.9 ± 0.9 nm and 86.9 ± 0.4 nm, respectively. The data are presented as means of three

independent experiments and correspond to the particle size distributions in Fig. 6. Statistical analysis was done using Student's *t* test. The particle size of WPH-Ca chelate decreased when compared with unchelated WPH, which might be due to structure folding between WPH and calcium ions during the chelation processing. It was in good agreement with the results of fluorescence spectrometer, suggesting the presence of calcium ions caused peptide folding and aggregating which led to the formation of WPH-calcium chelate complex. Moreover, the particle size distribution directly proved that the WPH-calcium chelate belongs to the compact nano-composite.

Atomic force microscopy

Tapping mode AFM images yielded information about the surface features of WPH and WPH-calcium chelate. Only water instead of sample was used as control experiment. Considering that the water background using water instead of sample was only a blank image, it wasn't collected. Nevertheless, the images of WPH and WPH-Ca chelate could obviously exhibit the particle morphology (Fig. 7). As shown in Fig. 7A, the WPH demonstrated shuttle-shaped structures and sizes of peptides were concentratedly distributed. The presence of calcium ions led to forming point-like domains placing irregularly in the visualized area (Fig. 7B). The particle sizes of WPH and WPH-Ca chelate from AFM were 200-250 nm and 160 nm (length) respectively, which matched up with the results of DSL. These results suggested that a strong interaction existed between WPH and calcium ions. The morphology images from AFM were in good agreement with the results of fluorescence spectrometer and particle size distribution.

Calcium releasing percentage

The calcium-releasing percentages of WPH-Ca chelate and $CaCl_2$ at various pH values were shown in Fig. 8. The solubility between WPH-Ca chelate and $CaCl_2$ were apparent different. For both of them, the calcium-releasing percentage varied with the increase of pH. While at any pH value, the calcium-releasing percentage of WPH-Ca chelate was obviously higher than that of $CaCl_2$. The calcium-releasing amount in WPH-Ca chelate was relatively stable in the pH ranged from 2.0 to 8.0 maintaining above 96 %, whereas that of $CaCl_2$ decreased significantly from 94 % at pH 2.0 to 78 % at pH 8.0. The result implied that the calcium in WPH-Ca chelate had good solubility whether at pH 2.0 or pH 8.0. It is well known that the pH value of the human intestinal tract is higher than 7.0, approximately pH 7.2, WPH-Ca chelate would remain relatively high calcium-releasing percentage and keep dissolved in the basic environment of the gastrointestinal tract, which could prevent calcium ion from forming precipitate so that it could be effectively absorbed by intestinal epithelial cells.

The result suggests that the solubility of calcium supplements in the human gastrointestinal tract is of great importance, and calcium nutritional supplements with high solubility probably have prominent bioavailability as reported by Wang *et al.*[12] It's essential to discuss the dissolved status of the peptide-calcium chelate in human gastrointestinal tract because calcium ion probably reacted with phytic acid or oxalic acid to become insoluble in stomach and form $Ca(OH)_2$ in intestinal tract. The finding of our study suggested that WPH-Ca chelate contributed to improve the solubility of calcium under gastrointestinal tract pH values and effectively absorbed by intestinal epithelial cells. The result suggests that it is feasible to produce natural calcium-chelating peptide as

functional food additives.

Effect of WPH–calcium chelate on celluar uptake of calcium

In order to verify whether the WPH-calcium chelate did not inactivate the cell in some way, cell viability was determined. For this purpose, MTT assay was used. This is a rapid colorimetric method based on the cleavage of a yellow tetrazolium salt to purple formazan crystals by mitochondrial enzymes of metabolically active (but not dead) cells. The intensity of the purple color is a measure of cell viability.[22] As the result shown in Fig. 9, the cells were not affected by the WPH-calcium chelate at a concentration of 0 to 10 mmol/L, while the action concentration of chelate and $CaCl_2$ increased to 15 mmol L^{-1}, the cell viability dropped to 86.97 % and 80.07 %, respectively. However, the cell morphology was still maintained normal under a microscope (data not shown). Therefore, the celluar uptake experiment was performed at concentration range between 0 and 15 mmol L^{-1}.

For the uptake studies, Caco-2 cells were pre-incubated with WPH-calcium chelate with different concentrations. Calcium gluconate and $CaCl_2$ were used as control. According to results in Fig. 10, the calcium absorption increased in a dose-dependent manner with the action of WPH-calcium chelate. In comparison with the control, the calcium absorption caused by WPH-calcium chelate was significantly higher when the concentration reached 10 mmol L^{-1}. Results indicated that WPH-calcium chelate could improve calcium absorption at the intestinal level. Similarly, soybean protein hydrolysates bind with calcium could promote calcium uptake in a *in vitro* Caco-2 cell model and the impact was significantly different in the peptides with different MW.[8] It has also been

demonstrated that caseinphosphopeptides (CPPs) can bind a great amount of calcium and prevent it from being precipitated by certain substances and thus, improve calcium uptake.[23,24] This result could support the idea that some proteins/peptides could be considered as mineral carriers because of their ability to bind and solubilize calcium with the possible role in increasing calcium transport across intestinal epithelial cells.[25]

Conclusions

In summary, whey protein hydrolysate possessing the Ca-binding capacity was obtained through enzymatic hydrolysis, and nano-composites of whey protein hydrolysate-calcium chelate was prepared under the optimum process parameters. The structure of WPH-calcium chelate was fully characterized through UV scanning spectrum, fluorescent spectrum, FTIR measurements, dynamic light scattering, and atomic force microscope. Moreover, WPH-Ca chelate demonstrated both stability and absorbability under either acidic or basic conditions. The results suggest that whey protein hydrolysate possibly has the potential to be used as the raw material to produce the calcium-binding dietary supplements in improving the bone health of human body.

Acknowledgments

This work was supported by the National Marine Public Welfare Projects (No. 201305022), Natural Science Foundation of China (No. 31471623), Fujian Natural Science Foundation, China (No. 2013J01132), the open funding of Fujian Provincial Key Laboratory of Photocatalysis–State Key Laboratory Breeding Base, China (No. 038018).

References

1 L. Guénguen and A. Pointillart. *J. Am. Coll. Nutr.*, 2000, **19**, 119–136.

2 A. Cilla, M. J. Lagarda, A. Alegría, B. D. Ancos, M. P. Cano, C. Sánchez-Moren, L. Plaza and R. Barberá. *Food. Res. Int.*, 2011, **44**, 3030–3038.

3 R. S. Adluri, L. Zhan, M. Bagchi, N. Maulik and G. Maulik. *Mol. Cell. Biochem.*, 2010, **340**, 73–80.

4 K. Moseley and S. de Beur. London (UK): Elsevier Academic Press (Chapter 59), 2011, pp. 716–736.

5 S. H. Lee and K. B. Song. *J. Korean Soc. Appl. Bi.*, 2009, **52**, 290–294.

6 F. Bronner and D. Pansu. *J. Nutr.*, 1999, **129**, 9–12.

7 B. M. Donida, E. Mrak, C. Gravaghi, I. Villa, S. Cosentino, E. Zacchi, S. Perego, A. Rubinacci, A. Fiorillia, G. Tettamanti and A. Ferrarettoa. *Peptides*, 2009, **30**, 2233–2241.

8 Y. Lv, H. Liu, J. H. Ren, X. Li and S. T. Guo. *Food Funct.*, 2013,**4**,1245－1251.

9 L. N. Zhao, S. Y. Wang and Y. F. Huang. *Int. J. Food Sci. Tech.*, 2014, 1–8.

10 H. J. Gitelman. *Anal. Biochem.*, 1967, **18**, 521–531.

11 D. Chen, Z. Liu, W. Huang, Y. Zhao, S. Dong and M. Zeng. *J. Funct. Foods*, 2013, 5, 689–697.

12 X. Wang, J. Zhou, P. Tong and X. Mao. *J. Dairy Sci.*, 2011, **94**, 2731–2740.

13 J. V. Adelantado, V. P. Martinez, A. P. Garcia and F. B. Reig. *Talanta*, 1991, **38**, 959–963.

14 M. R. Guo, P. F. Fox, A. Flynn and P. S. Kindstedt. *J. Dairy Sci.*, 1995, **78**, 2336–2344.

15 K. Ou, Y. Liu, L. Zhang, X. Yang, Z. Huang, M. J. Nout and J. Liang. *J. Agric. Food Chem.*, 2010, **58**, 4894–4900.

16 C. A. Blindauer, I. Harvey, K. E. Bunyan, A. J. Stewart, D. Sleep, D. J. Harrison, S. Berezenko and

P. J. Sadler. *J. Biol. Chem.*, 2009, **284**, 23116–23124.

17 R. P. Houser, M. P. Fitzsimons and J. K. Barton. *Inorg. Chem.*, 1999, **38**, 1368–1370.

18 A. Armas, V. Sonois, E. Mothes, H. Zazarguil and P. Faller. *J. Inorg. Biochem.*, 2006, **100**, 1672–1678.

19 S. Y. Wang, B. B. Zhu, D. Z. Li, X. Z. Fu and L. Shi. *Mater. Lett.*, 2012, **83**, 42–45.

20 H. H. Wu, M. Y. Liu, Y. H. Zhao and M. Y. Zeng. *Food. Res. Int.*, 2012, **48**, 435–441.

21 R. Uppal, K. V. Lakshmi and A. M. Valentine. *J. Biol. Inorg. Chem.*, 2008, **13**, 873–885.

22 A. Faria, N. Mateus, V. de Freitas and C. Calhaua. *FEBS Lett.*, 2006, **580**, 155–160.

23 S. Cosentinoa, C. Gravaghia, E. Donettic, B. M. Donidaa, G. Lombardid, M. Bedonic, A. Fiorilli, G. Tettamanti and A. Ferraretto. *J. Nutr. Biochem.*, 2010, **21**, 247–254.

24 H. Naito. A review. *Nippon Eiyo, Shukuryo Gakkaishi*, 1986, **39**, 433–439.

25 W. Daengprok, W. Garnjanagoonchorn, O. Naivikul, P. Pornsinlpatip, K. Issigonis and Y. Mine. *J. Agric. Food Chem.*, 2003, **51**, 6056–6061.

Figure captions

Fig. 1

Time courses of calcium binding capacity and degree of hydrolysis of whey protein hydrolysates obtained with protamex and flavorzyme at different hydrolysis times.

Fig. 2

Effects of chelating reaction time (A), mass ratio of whey protein hydrolysate to calcium (B) on the calcium-binding capacity of whey protein hydrolysate-Ca chelate. All measurements were expressed as means ± SD of 3 independent experiments.

Fig. 3

UV spectra of WPH and WPH-calcium chelate over the wavelength range from 190 to

400 nm.

Fig. 4

Fluorescence spectra of WPH with different $CaCl_2$ concentration.

Fig. 5

Fourier transform infrared (FTIR) spectra of WPH and WPH-calcium chelate in the regions from 4,000 to 400 cm^{-1}.

Fig. 6

Particle size distributions of WPH and WPH-calcium chelate.

Fig. 7

AFM topographic images of WPH **(A)** and WPH-calcium chelate complex **(B)**.

Fig. 8

Calcium releasing percentage of WPH-calcium chelate and $CaCl_2$ at pH values of 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0, respectively.

Fig. 9

Effects of different concentration of WPH-calcium chelate and $CaCl_2$ on Caco-2 cells survival rate.

Fig. 10

Effect of different concentration of reagents on the calcium absorption in Caco-2 cells.

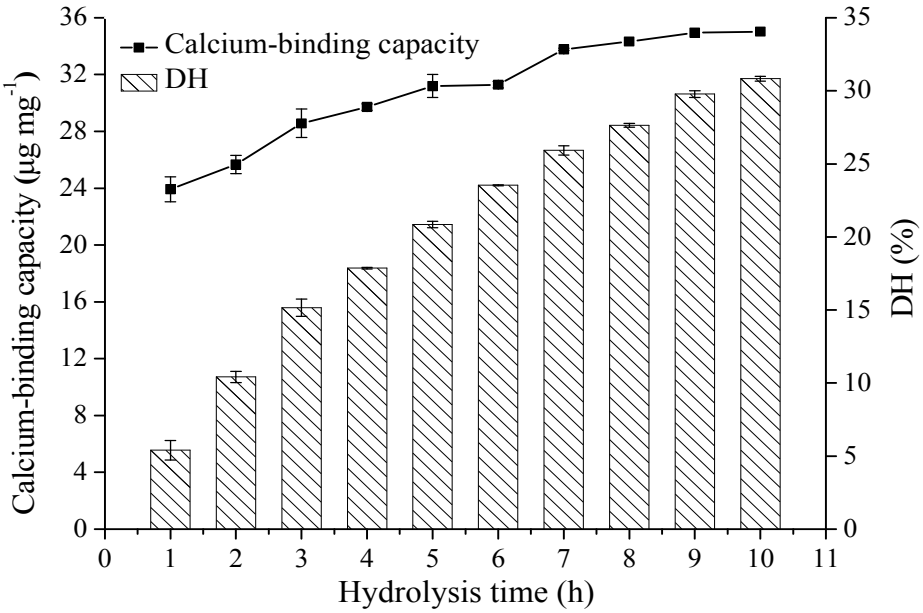


Fig. 1

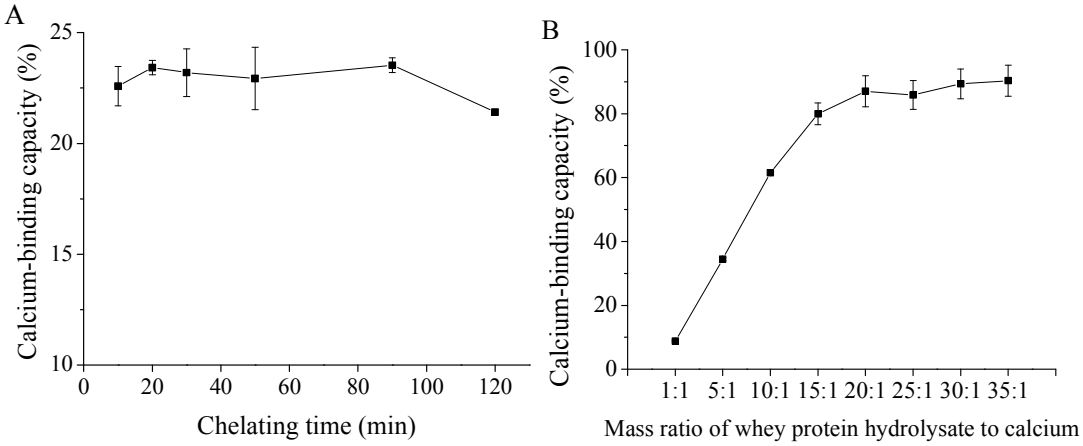


Fig. 2

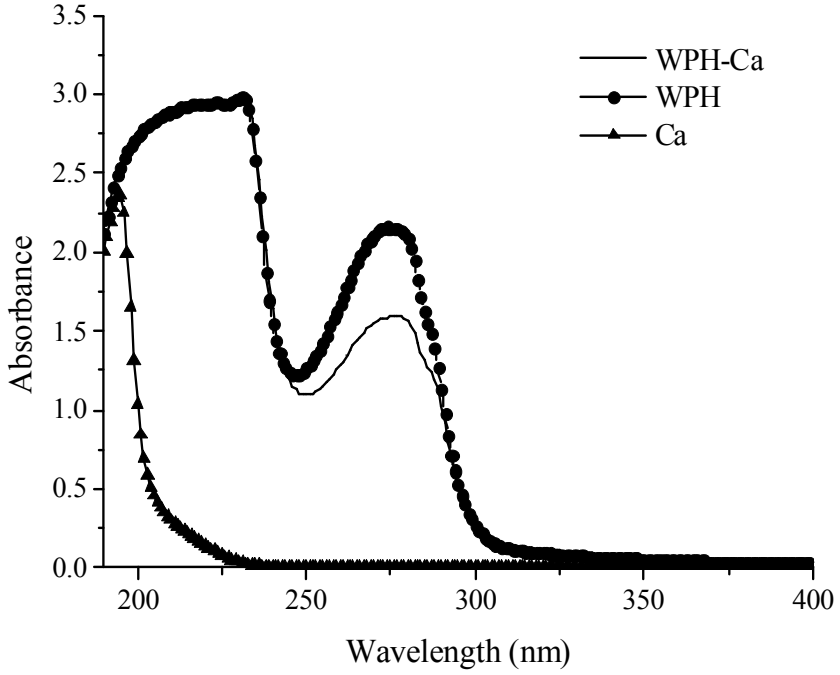


Fig. 3

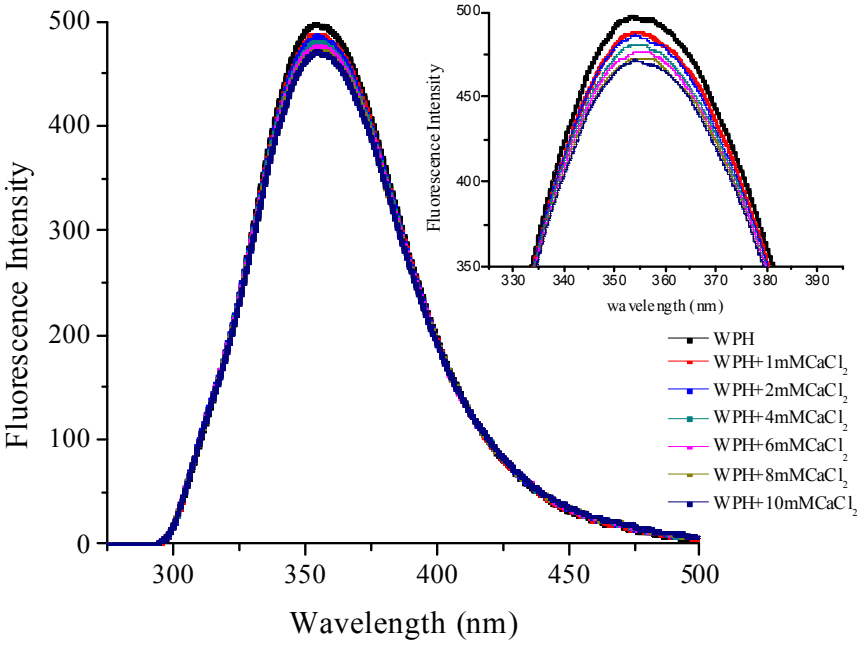


Fig. 4

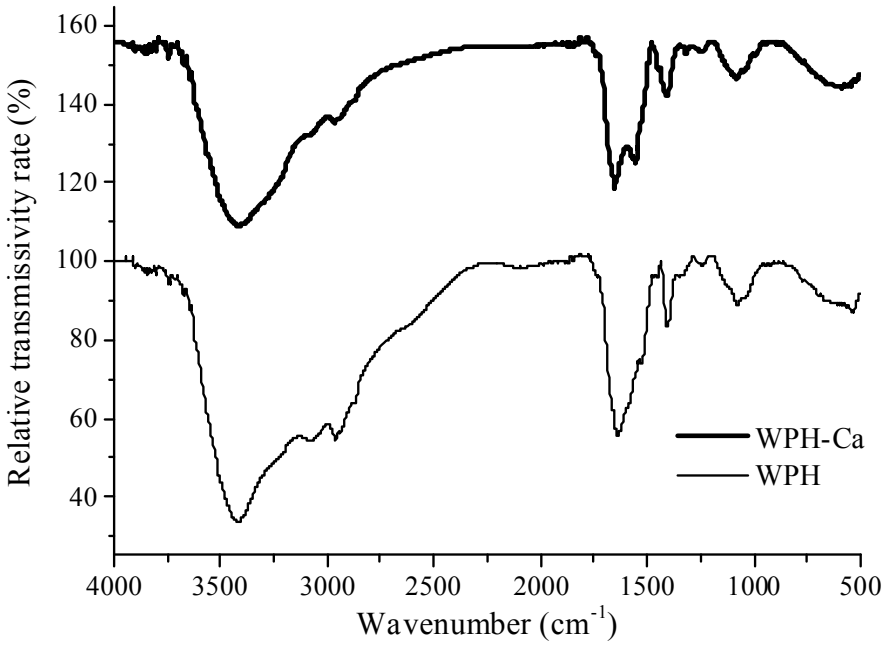


Fig. 5

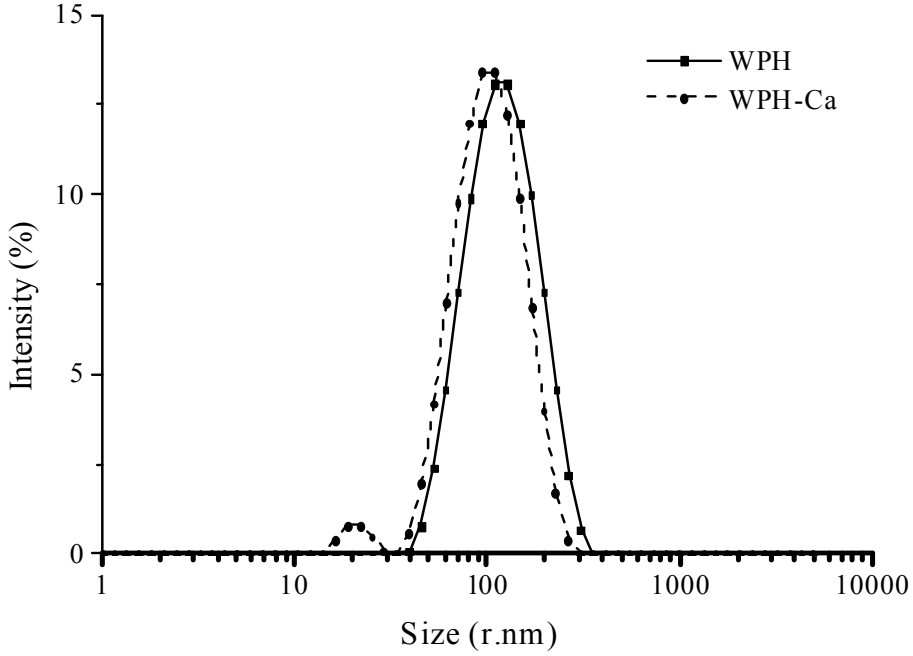


Fig. 6

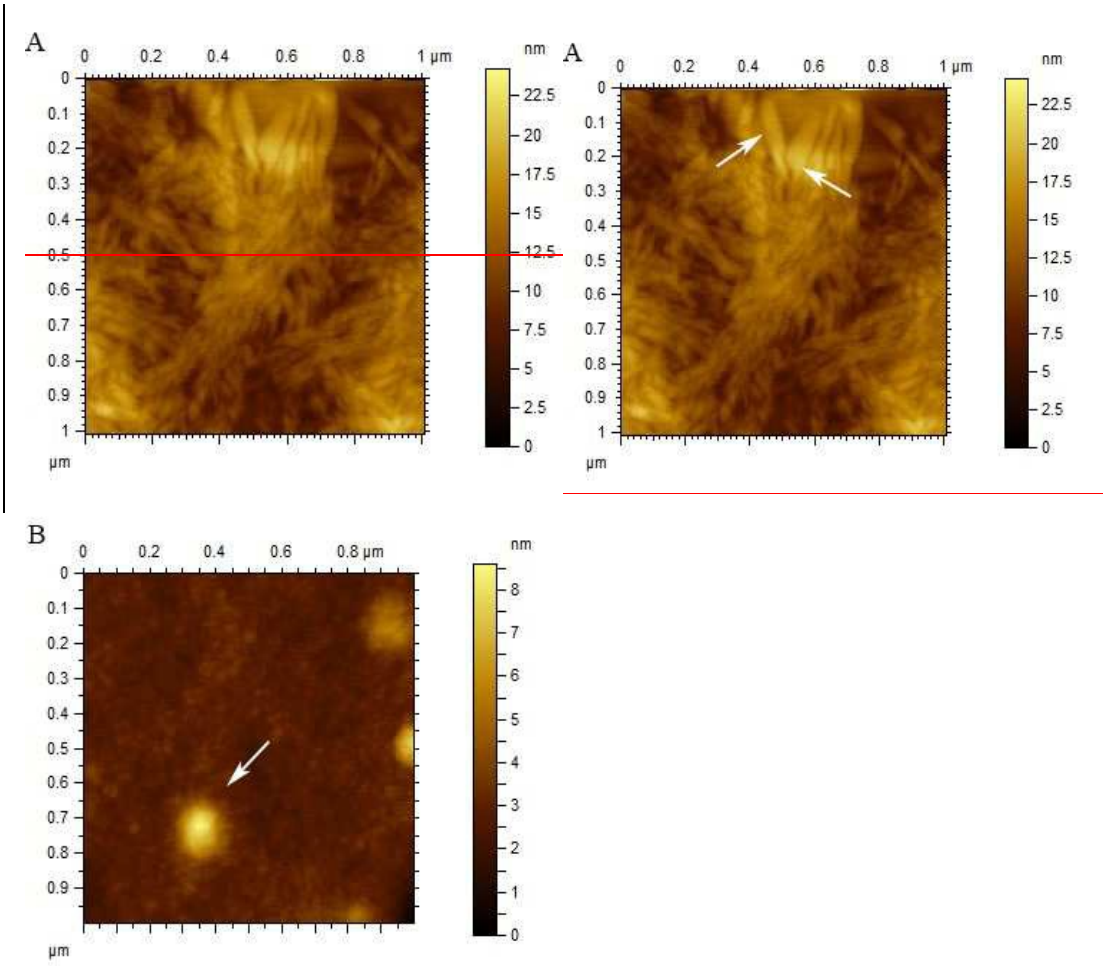


Fig. 7

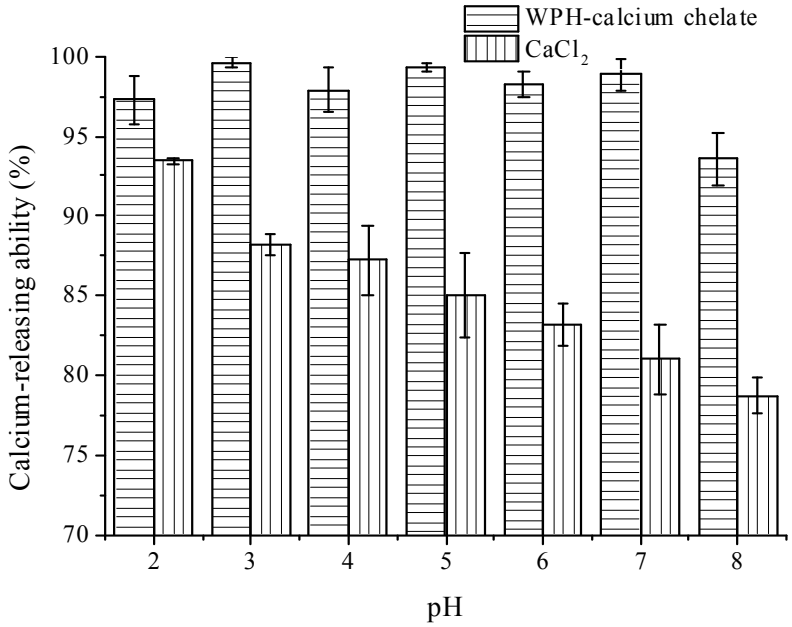


Fig. 8

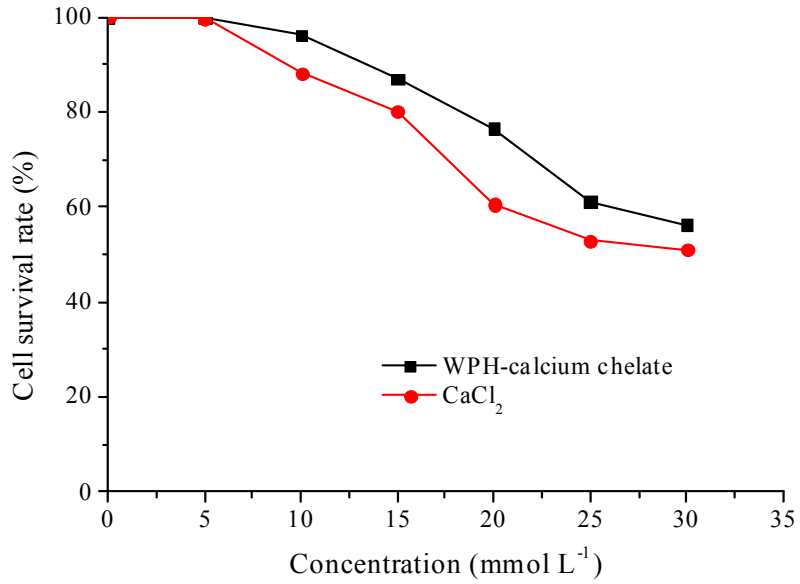


Fig. 9

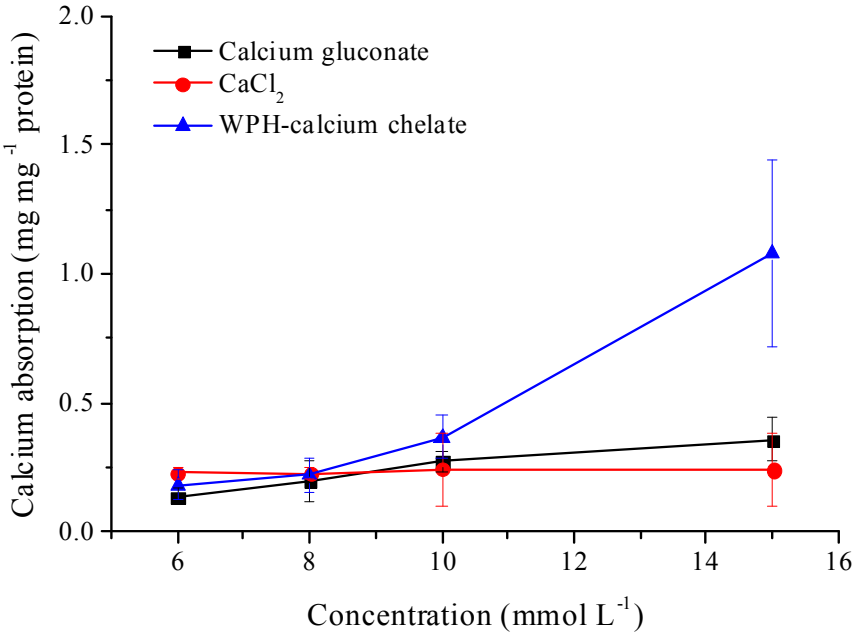


Fig.10

Fabrication and characterization of the nano-composite of whey protein hydrolysate chelated with calcium

Cai Xixi, Zhao Lina, Wang Shaoyun *, Rao Pingfan

Graphical Abstract

The chelation of calcium ions to WPH caused the molecular folding and aggregating leading to the formation of WPH-calcium chelate in nanoparticle size, and the principal sites of calcium-binding corresponded to the carboxyl groups and carbonyl groups of WPH. WPH-calcium chelate exerted the excellent stability and absorbability in both acidic and basic conditions and could significantly improve calcium absorption at the intestinal level.