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Research Article
Preparation and Characterization of Sustained Released Zinc Citrate Encapsulated in Whey Protein Nanoparticles

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Abstract

Background and Objectives: The use of milk proteins for drug delivery is a new trend in functional foods and pharmaceutical. Recently, researchers have focused on the utilization of whey proteins in the preparation of nanoparticle and carrier for drugs and micronutrients. The objectives of this paper were to use whey proteins isolate (WPI) nanoparticles for the encapsulation of zinc citrate micronutrients and characterization of the prepared nanoparticles. **Materials and Methods:** Nanoparticles were prepared from WPI with pH cycling and used for the encapsulation and sustained release of zinc citrate with three ratios (7, 14 and 28 mM) of zinc citrate per gram WPI. The particle size of the prepared nanoparticles was characterization and examined by transmission electron microscopy. The release of Zinc from the prepared nanoparticles was carried out using simulated gastric fluid at pH 1.2 using dialysis membranes, the amount of zinc citrate loaded whey protein (14.36 mg Zinc in 1 g WPI) within range of daily dose of zinc for healthy adults. **Results:** The WPI nanoparticles were able to encapsulate efficiently zinc, with encapsulation efficiency that ranged between 99.79 and 96.31%. Zinc was highly released from the prepared nanoparticles in acidic media (pH 1.2). **Conclusion:** It can be concluded that WPI can be used as an effective vehicle for the protection and sustained release of zinc in food and pharmaceutical preparations.

Key words: Zinc citrate, nanoencapsulation, whey protein isolate, sustained release, encapsulation efficiency

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Zinc (Zn) is the second most common trace mineral in the body after iron, nearly found in 100 enzymes, and is present in every living cell. Zn is the second most common trace mineral in the body after iron, nearly found in 100 enzymes, and is present in every living cell. The human body contains approximately 3 g of Zn, the highest concentrations of which are found in the prostate gland and the eyes[1]. For healthy adults the required daily intake from Zn is around 2-3 mg. The world health organization (WHO) and UNICEF recommended short-term Zn supplementation (10-20 mg of Zn per day) for infants under 6 months, for 10-14 days to treat acute childhood diarrhea. Globally Zn deficiency might be as high as 40% particularly in developing countries possibly due to the low Zn bio-availability and insufficient consumption of Zn-rich foods[2]. Zinc deficiency is linked to the incidence of impaired or severe cognitive function, persistent diarrhea, lower respiratory infections and low birth weight[3].

Whey protein isolate (WPI) has been recognized as one of the most nutritious and valuable milk ingredients that can improve the general health and combat diseases. Nanoparticles (with a diameter ranging from 100-300 nm) can be prepared from WPI by pH cycling of denatured of WPI solution at low concentration[4]. Natural biopolymers including food proteins have many advantages as drug delivery systems[5], because of their compatibility, high nutritional value and excellent functional properties[6]. Nanoparticles delivery systems for nutrients and nutraceutical have been developed and are now in the process of industrial applications in food and pharmaceutical products. In order to reduce the side effects of nanoparticles considering such as digestion *in vitro* testing of these material must be performed release to ensure their efficiency and stability[7]. *In vitro* dissolution testing is an important tool for quality control purpose is one of the areas that need to be explored, as well as the potential side effects of these nanoparticles carriers[8]. Therefore, particle size determination of nanoparticles and their properties are a prerequisite for controlling biological properties and/or stability of nanoparticles[9].

The scope of the present work was to investigate the potential use of whey protein isolate nanoparticles for entrapment and sustained release of zinc. The preparation of zinc loaded whey protein nanoparticles and characterization of these particles was done through these morphology, particle size and stability and loading efficiency of nanoparticles. Also, the release behaviour of the zinc from the encapsulated whey protein nanoparticles was evaluated.

MATERIALS AND METHODS

Bipro whey protein isolate 92.6% protein (w/w) was obtained from Davisco Foods International Inc., Eden Prairie, USA. Calcium chloride and pepsin 1:3000 from Scharlau S.L., Spain, sodium azide (≥98%) from Acros Organics, Belgium, sodium hydroxide (≥98%) from Bio. chem, Egypt. Sodium phosphate dibasic obtained from Fine Chem, Egypt. Trypsin enzyme 2656.7 μ mg^{-1} obtained from FAIZYME laboratories, Egypt, zinc citrate was obtained from El Nasr. Pharmaceutical Chemicals Company. All other chemicals and reagents used were of analytical grade.

Preparation of whey protein soluble polymers: Whey protein isolate (WPI) was dispersed in Milli-Q water to form 8% (w/v) and adjusted with 1N NaOH to pH 7. The dispersion was kept refrigerated at 4°C overnight for complete hydration and then it was warmed at room temperature and degassed for 20 min under vacuum (560 mm Hg). The dispersion was then heated up in a water bath up to 80°C heating rate of 8°C min^{-1} and kept for 15 min at this temperature. Heated suspension was cooled to room temperature in an ice bath and then diluted to 2% (w/v) using Milli-Q water.

Preparation of zinc citrate loaded nanoparticles: The loaded WPI nanoparticles were prepared by the pH-cycling method previously described by Giroux *et al.*[10]. Zinc citrate was added to the prepared WPI soluble polymer at the ratios 7, 14 and 28 mM/ g protein. The mixture (250 mL) was then acidified under stirring using 0.1 N HCl to an aggregation pH of 6.0 and then 1 mL of 62.5 mg mL^{-1} $CaCl_2$ solution was add to give to reach the final Ca concentration 2.25 mM in the final mixture and left formation di-sulphide bonds. The dispersion was then brought to pH 7 using 1N NaOH at room temperature, homogenized, using 2-stage Rannie homogenizer Copenhagen, Denmark at 20 MPa for the first stage and 3.5 MPa for the second stage respectively. In order to obtain nanoparticle of low average size, part of the preparation was kept in solution (50 mL) for particle size, transmission electron microscope and atomic absorption analysis, while the rest of the formula (200 mL) was dried.

Sample drying: About 200 mL from the zinc loaded nanoparticles preparation before homogenization was filtered through 3 μm pore size filter paper. The precipitate was then washed using 20 mL Milli Q-water then the precipitate was collected in a porcelain dish and allowed to dry under vacuum

overnight. The large particles in the dried precipitate was then ground using a mortar and pestle. The particles were used then for encapsulation efficiency analysis and *in vitro* release study.

Particle size analysis: The z-average diameter and size distribution of nano-encapsulated Zn samples were carried out at 25±0.1°C using Nano ZS/ ZEN3600 Zetasizer (Malvern Instruments Ltd., UK) with a He/Ne laser (λ = 633 nm), scattering angle 90° scattering optics and refractive index of 1.35. Samples were diluted and filtered through 0.45 μm membrane (Millipore, USA) to obtain a count rate in the appropriate range 100-450 nm to avoid multiple scattering phenomena due to inter-particle interaction. Immediately, the diluted sample was transferred into the polystyrene cuvette for size determination and then the z-average diameter (Dz) and particle dispersity index (PDI) were recorded by dynamic light scattering (DLS) as described by Giroux *et al.*[10].

Transmission electron microscopy: Samples were prepared for transmission electron microscopy by the negative staining method[11]. Glutaraldehyde was added to WPI and WPI-Zn nanoparticles at the ratio of 1:7 v/v and then dilution (1:100 v/v) with deionized water. A drop of diluted suspension was placed on formvar-coated electron microscopy grid left for 1 min and then a drop of phosphotungstic acid solution (2% at pH 7.2) was added. The grid was air dried and subsequently examined by transmission electron microscopy (TEM) using a JEOL JEM-1400 plus TEM with an accelerating voltage of 100 kV at a magnification of 200,000x.

Preparation of gastric and intestinal fluid simulated: Two solutions were prepared: Gastric fluid simulated (GFS) and Intestinal fluid simulated (IFS) according to USP[12].

***In vitro* Zn release:** The assays were performed in triplicate, in 500 mL beaker, using 450 mL of GFS or IFS with 266 mg of WPI nanoparticles. This was added to 50 mL of the same solution into previously hydrated dialysis membranes (MEMBRA-CEL® dialysis tubing, MWCO 10000, 34 mm, 14×100 CLR). The systems were kept at 37°C under gently magnetic stirring and the solutions (GFS and IFS) and 25 mL aliquots were collected in different time intervals in GFS (0, 5, 10, 15, 20, 25, 30, 60 min and 2 h) the solutions were replenished with the same volume. Aliquots were stored and submitted to atomic absorption analysis AAS to determine the amount of Zn^{+2} released each time in the simulated media.

Zn^{+2} determinations by atomic absorption: The amount of zinc loaded whey protein (7.5 mg zinc in 1 g WPI) was measured by atomic absorption flame spectrophotometer (AnalytikJena, Germany) computed with Aspect CS 2.1.1.0 software. Zinc was measured at its primary wavelength 357.9 nm, the flame was acetylene-air mixture, the lamp used was xenon short arc lamp and CCD detector was used for complete background correction. Automatic optimization of the apparatus was used to obtain the best condition for measurement.

Statistical analysis: The data, reported as mean±standard deviation, are from experiments conducted in triplicate. One-way analysis of variance (ANOVA) was performed using SPSS (ver. 16) software. ANOVA was used to check the assumptions of variance homogeneity and normality and compare the treatment means. Differences among mean values were examined by the least significant difference (LSD) and Duncan's test at Pb 0.05 significance level.

RESULTS AND DISCUSSION

Encapsulation efficiency: The effect of Zn concentration on encapsulation efficiency was presented in Table 1. The encapsulation efficiency was between 99.79 and 96.31%. There were significant differences in encapsulation efficiency with different Zn concentrations 7, 14, 28 mM zinc citrate. From the data in Table 1, it can be noticed that EE was higher than 96.31% for all studied samples indicating a high Zn encapsulation. It could be generally observed from results in Table 1 that WPI have high ability to bind Zn. This results agreement with Tang and Skibsted[13] were showed that Zinc amino acid or peptide complexes improved zinc absorption. This investigated the interaction between zinc ion and whey proteins. Cysteine and histidine exhibited strong zinc binding ability.

Table 1: Mean encapsulation efficiency of Zn in WPI, z-particle size (nm) and zeta potential (mv) and polydispersity index (PDI) before and after loaded

Samples	Encapsulation efficiency (%)	Size (nm)	Zeta potential (mV)	Calculated PDI
WPI nanoparticles	-	94.8±15.69[a]	-94.9	0.346
WPI loaded 7 mM Zn	99.79±0.08[c]	141.9±20.23[b]	-114.0	0.157
WPI loaded 14 mM Zn	98.27±0.02[b]	196.1±11.14[c]	-84.9	0.422
WPI loaded 28 mM Zn	96.31± 0.21[a]	228.0±12.78[d]	-79.0	0.647

[a]Numbers are mean±standard deviation from triplicate samples, Different superscripts indicated differences in the means ($p<0.05$)

The essential trace metal the cation Se^{+3} was formed quickly when dissolving complexes showed significant interactions with BSA[14]. In general, all proteins could interact with metal ions. On the surface of each protein amino acid residues such as thiolat, carboxylate, imidazole, amines and amides, which can bind to existing metal ions[15], which may explained the present finding.

Size distribution of encapsulated Zn: Data in Table 1 summarized the dynamic light scattering results of zinc loaded whey protein nanoparticles compared to the blank particles. The polydispersity index (PdI) for WPI, WPI-Zn were determined to be in an acceptable range (0.0-0.7) implying that the particle size distribution values for all samples was not too high and the resulting quality according to these values were acceptable. However, there was a substantial difference between the particle sizes of whey protein nanoparticles and Zn loaded nanoparticles as shown in Table 1. The size Z-average of whey protein nanoparticles was determined to be 94.8 nm, while the size Z-average of Zn loaded nanoparticles was shown to be 141.9, 196.1 and 228 nm increasing the size add-up ratio of zinc citrate 7, 14 and 28 mg. Giroux *et al.*[10] confirmed that whey protein particles as size Z-average of whey protein nanoparticles prepared at pH 6 and 22 h aging time was 100 nm. Same explanation could be applied to the results of formulated zinc loaded nanoparticles, as some particles may aggregate to shift the Z-average to higher value. This was confirmed by the presence of a small peak at the right side of the Fig. 1.

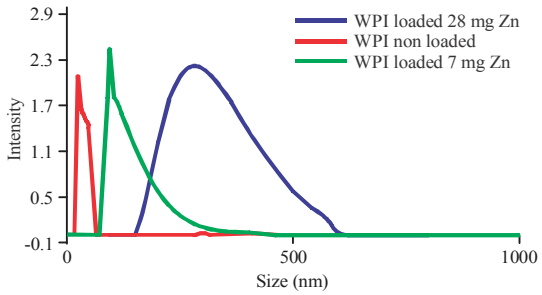

Fig. 1: Size distribution curves of WPI nanoparticles and encapsulated 7 and 28 mM zinc citrate respectively

Electron microscopy of encapsulated: The morphology of the as-prepared sample was investigated by TEM Fig. 2 showed a typical TEM image of the WPI-Zn nanoparticles in which the dark part corresponded to WPI and the pale part to Zn. From this TEM micrograph, it was cleared that current product was a composite of Zn and WPI, i.e., WPI granule with a size of about 100 nm and WPI-Zn nanoparticles a size of about 200 nm. These nanoparticles were uniform and monodisperse with an average diameter of 96 nm, which correlated very well with size scale obtained from DLS data (228 nm). The WPI-Zn particles were stable under the electron beam in the vacuum used for TEM measurements, suggesting that the binding between Zn and WPI was strong.

***In vitro* zinc release:** The Table 2 showed the recoveries of zinc concentration at interval time in comparison with calibration curve experiment was carried out at 5, 10, 15, 20,

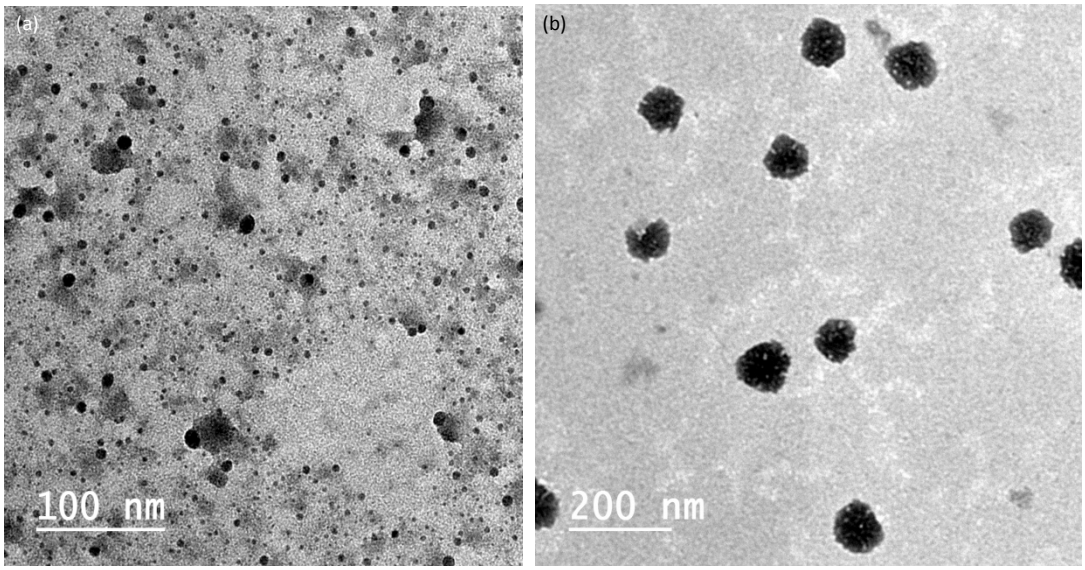

Fig. 2(a-b): Transmission electron micrograph of (a) WPI nanoparticles and (b) Encapsulated Zn nanoparticles

Table 2: Zinc release profile in gastric fluid from zinc loaded nanoparticles for (WPI loaded 14 mM Zn)

Time (min)	Release (%)
5	28.57±1.51[g]
10	51.43±1.14[f]
15	57.14±1.01[e]
20	67.14±1.26[d]
25	80.29±1.34[c]
30	91.43±1.20[b]
60	94.29±1.00[a]

[a-g]Numbers are mean±standard deviation from triplicate samples, different superscripts indicated differences in the means ($p<0.05$)

25, 30, 60 min and after 2 h intervals. The obtained results from triplicate showed that the release rate was quite high after 60 min the release was more than 94% of the analysis and 2 h completely release 100% (equilibrium time). Respectively a high recovery of zinc was obtained after 60 min proved that, the suggested formula of zinc with loaded whey protein was more active for the stability and the duration of zinc release up to two hours. Wegmuller *et al.*[16] found that zinc absorption from zinc citrate was relatively high and was equivalent to that from zinc gluconate. The measured value of 61% zinc absorption from zinc citrate was similar to previously reported 71% zinc absorption from zinc sulfate for adults consuming a 10 mg zinc dose that was measured using the same method. Present result indicated that the WPI nanoparticles were good carrier and could act as a reservoir for zinc which may be added to food formulation and pharmaceutical preparation. This finding confirmed that WPI nanoparticles can be used to encapsulate, protect and enhance the release profile of micronutrients to be used for nutraceuticals. These results were great advantage for zinc absorption through the stomach (i.e., at low PH in the presence of WPI. The obtained data were agreement with Gulseren *et al.*[17] succeeded in incorporating the daily required amount of zinc for adults in whey protein isolate (WPI) nanoparticles by applying the ethanol desolvation method. They reported that the encapsulation capacity of whey protein nanoparticles for zinc retained was high and remained stable for 30 days at 22°C. It was found the increased solubility of zinc and bio-availability. Zinc citrate did not exceed the solubility of 61% as finding of Wegmuller *et al.*[16], while in this study, found when loaded on WPI nearly increased to 94%. The use of pH cycling method was better than the method of desolvation as finding Gulseren *et al.*[18] and Shao *et al.*[18] this research had not used ethanol. Finally, can be accomplished that the WPI to encapsulate sensitive micronutrients of nanoparticles by ph-cycling to enhance solubility and sustained release and apply it in food and pharmaceutical industries.

CONCLUSION

This study concerned that the zinc loading on the whey protein isolate by pH cycling has given high solubility and bio-availability up to almost 94% comparative with Zinc citrate does not exceed the solubility of 61%. The encapsulation efficiency was 99.79% with Zn concentration 7 mM zinc citrate and the average size was 141.9 nm. The polydispersity index (PdI) for WPI-Zn 7 Mm it was 0.157 that indicated for homogeneity and clarity of solution.

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