# Different forms of whey protein aggregates as curcumin delivery systems: evaluation of free radical scavenging activity and drug release kinetics

**Mehdi Mohammadian[1]** [iD] **, Arasb Dabbagh Moghaddam[1,\*]** [iD] **, Anousheh Sharifan[2]** [iD] **, Parviz Dabaghi[3]** [iD] **, Saeid Hadi [1]** [iD]

[1]Department of Health, Aja University of Medical Sciences, Tehran, Iran
[2]Department of Food Science and Technology, Science and Research Branch, Islamic Azad University, Tehran, Iran
[3]Department of Clinical Psychology, Faculty of Medicine, Aja University of Medical Sciences, Tehran, Iran
*corresponding author e-mail address: **admoghaddam2@gmail.com** | Scopus ID 57195150281

## ABSTRACT

In the present study, different forms of whey protein isolate (WPI) including, native, worm-like aggregates, microgel particles, and nanofibrillar aggregates were employed to encapsulate curcumin as a bioactive ingredient. The results showed that the aggregation improved the capacity of WPI for loading of curcumin and the highest encapsulation efficiency and loading amount were related to the fibrillar aggregates. The curcumin-loaded aggregates also showed a good antioxidant activity as measured by ABTS/DPPH free radical scavenging test. The release of curcumin from aggregates was determined during the simulated gastrointestinal digestion and the results indicated that the capsulation of curcumin in whey protein aggregates can be considered as a hopeful method to control the release of curcumin as well as to site-specific delivery of curcumin. After that, the drug release kinetics and mechanism were evaluated using various kinetic models including zero order, first order, Higuchi, and Korsmeyer-Peppas models. The release modelling results also showed that the release of curcumin from whey protein aggregates was controlled by both diffusing and polymer swelling (i.e. anomalous transport). Generally, this study suggested that the WPI-based aggregates can be used as promising carriers for curcumin delivery.

**Keywords:** *Bioactive delivery; Protein-based carriers; Encapsulation; Antioxidant activity; Release behavior.*

## 1. INTRODUCTION

Curcumin as a bioactive phenolic compound with different health-promoting attributes such as antioxidant, antimicrobial, anticancer, and anti-inflammatory activities has attracted considerable interest in many fields especially food science and pharmaceutical industries [1]. However, the application of curcumin as a functional ingredient was limited due to its poor water solubility, instability towards light and rapid decomposition [2,3]. Therefore, different methods such as encapsulation have been used to improve the solubility and stability of curcumin. In this regard, various biopolymers such as proteins, lipids, and polysaccharides were employed as a carrier for the encapsulation of curcumin [1-3]. Among these biopolymers, food protein-based delivery systems have attracted a lot of interest due to their outstanding properties such as excellent functional and nutritional attributes, amphiphilic nature, biocompatibility, and biodegradability [4]. Accordingly, different food proteins such as soy proteins [5], whey proteins [6], and walnut proteins [7] have been used as a carrier for loading of curcumin.

Whey protein isolate (WPI) as an animal-derived proteins vand a by-product of cheese-making industries has a high nutritional value and multiple bio-functionalities such as good foaming, gelation, βind antioxidant activity which persuaded the researchers to use it in different food products [8]. Whey proteins have the ability to produce different forms of aggregates by applying a heating process with temperature more than the denaturation temperature of whey proteins [9]. The form, size, and shape of the resulting aggregates are mainly dependent on the pH of the protein solution which is going to be heated [10,11].

Prolonged heating of WPI solution at acidic conditions (especially pH value of 2.0) results in the formation of fibrillar aggregates with micrometric length and nanometric diameter [12]. The heating of WPI solution at a pH range of 5.8-6.2 also results in the formation of spherical aggregates or microgel particles with a diameter of 100-600 nm [13]. Heat-denaturation of WPI solution at pH value of 7.5 was also reported that can be resulted in the formation of worm-like aggregates with end-to-end distance below 100 nm and a cross-section of around 6.0 nm [10]. These aggregates are different in terms of functional and biological properties and can be used for diverse purposes [11]. As an important application, whey protein aggregates have been used as carriers for bioactive molecules such as caffeine [14] and gallic acid [8] owing to their superb techno-functional properties and high loading capacity. However, different forms of whey protein aggregates were not compared in the case of their ability to load curcumin as well as their release behavior under simulated gastrointestinal conditions.

An important property for the delivery systems is their drug release process which is influenced by different factors such as the properties of the material matrix (composition, structure, swelling, and degradation), release medium (pH, temperature, ionic strength, and enzymes), and drug (solubility, stability, charges, and possible interactions with matrix) [15]. In fact, the bioavailability of a loaded drug often depends on its gastrointestinal transit rate, therefore the study of drug release kinetics and mechanism of delivery systems as well as the fate of encapsulated drug and bioactive compounds are very important to

establish a suitable sustained release system for drug delivery [16,17]. In this regard, there is no research on the evaluation of curcumin release kinetics form different whey protein aggregates as delivery systems. Therefore, in the present study, different forms of whey protein aggregates were employed as a carrier for curcumin and their ability to load curcumin as a cargo was compared. The antioxidant activity of curcumin-loaded aggregates was also determined. After that, the release of curcumin from aggregates was studied under simulated gastrointestinal conditions and the drug release kinetics and mechanism were evaluated using different models.

## 2. MATERIALS AND METHODS

### 2.1. Materials.

WPI with more than 90% protein content was supplied from Arla Food Ingredients (Viby J, Denmark). Curcumin, pepsin, and pancreatin were obtained from Bio Basic Company (Bio Basic Inc., Canada). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Also, all of the other chemicals used in this research were of analytical grade.

### 2.2. Fabrication of aggregates.

For the fabrication of different whey protein aggregates, at first the WPI solution with a concentration of 40 mg/mL was prepared in distilled water by 2 h stirring at room temperature and then was fully hydrated by storing at 4°C for 12 h. For the formation of worm-like aggregates, the pH of WPI solution was adjusted to 7.5 using NaOH 2.0 M and then was heated for 30 min at 85°C under mild condition of stirring [10]. To produce whey protein microgels, the pH of WPI solution was adjusted to 5.85 using HCl 2.0 M and this was followed by 30 min heating in a water bath at 85°C with stirring [13]. For the preparation of whey protein nanofibrils, also the pH of WPI solution was adjusted to 2.0 using HCl 8.0 M and the protein solution was heated for 5.0 h at 85°C with mild stirring [12]. In all of fabrication methods, the solutions were cooled to room temperature after the heating process and then were kept at 4°C for the subsequent uses.

### 2.3. Loading of curcumin.

After the preparation of different samples including native WPI, worm-like aggregates, microgel particles, and nanofibrils, they were loaded by curcumin. In this regard, the curcumin which was dissolved in ethanol was added to the above sample solutions under stirring condition with a final concentration of 1.0 mg/mL (curcumin to protein ratio of 1:40). After that, the resulting mixtures were stirred in a dark place for 5 h. It should be noted that the final concentration of ethanol in the samples was very low (0.2% v/v) which has no significate effect on the protein structure. The resulting curcumin-loaded samples were addressed as curcumin-loaded native whey protein (C-WPI), curcumin-loaded whey protein worm-like aggregates (C-WLA), curcumin-loaded whey protein microgels (C-WPM), and curcumin-loaded whey protein nanofibrils (C-WPN) throughout the paper.

### 2.4. Encapsulation parameters.

The encapsulation parameters including encapsulation efficiency (EE) and loading amount (LA) were evaluated according to Moghadam et al. [7] with slight modifications. Accordingly, the curcumin-loaded samples were centrifuged for 10 min at 1500 g to eliminate the free or unloaded curcumin. After that, the resulting supernatants were mixed with ethanol to extract the encapsulated curcumin. Then, the absorbance of these mixtures was measured at 420 nm using a UV/Vis spectrophotometer. The curcumin concentration was calculated using the ethanolic standard curve of curcumin ($R^2$= 99.89%).

Finally, the EE and LA were determined using the following equations:

$$EE\ (\%) = \frac{\text{amount of encapsulated curcumin}}{\text{total amount of curcumin}} \times 100$$

$$LA\ \left(\frac{\mu g}{mg}\right) = \frac{\text{loaded amount of curcumin}}{\text{total amount of protein}}$$

### 2.5. Antioxidant activity of samples.

The antioxidant activity of different curcumin-loaded samples was measured by free radical (ABTS and DPPH) scavenging activity. At first, the DPPH ethanolic solution with concentration of 0.1 mM was prepared by dissolving an appropriate amount of DPPH powder in ethanol [5]. The $ABTS^+$ was also produced by reacting 7.4 mM ABTS in phosphate buffered saline (pH 7.4) with 2.6 mM potassium persulfate and storing for 18 h at room temperature and then the $ABTS^+$ was diluted with distilled water to an absorbance of 0.7 at 734 nm [18]. Before the measurements, the samples were also diluted with distilled water with the same pH to a protein concentration of 4 mg/mL and curcumin concentration of 0.1 mg/mL. After that, 100 μL of diluted samples or distilled water as control was mixed with 1.0 mL of ABTS or DPPH solutions. After that, the resulting mixtures were kept in dark for 30 min. Then, for ABTS radical scavenging activity, the absorbance of samples was read at 734 nm and for DPPH radical scavenging activity, the absorbance was measured at 517 nm using a UV-Vis spectrophotometer. Finally, the ABTS/DPPH radical scavenging activity was calculated using the following equation.

$$\text{Free radical scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

### 2.5. *In vitro* release behavior.

The *in vitro* release of curcumin from different forms of whey proteins including native, worm-like aggregates, microgel particles, and nanofibrils was investigated in simulated gastric fluid (SGF) with pH 1.2 and simulated intestinal fluid (SIF) with pH 7.5 using the dialysis bag method. The SGF containing pepsin enzyme and SIF with pancreatin enzyme were prepared according to Maltais et al. [19]. Then, 2.5 mL of curcumin-loaded samples were placed in a dialysis bag (MW: 12 kDa) and charged with 2.5 mL of SGF, and the bag was submerged in 150 mL of enzyme-free SGF as the release medium for 2 h at 37°C and stirring of 100 rpm. After that, the pH of solutions in the bag was adjusted to 7.5 for the inactivation of pepsin and 5 mL of SIF was added to the dialysis sac. After that, the bag was transported to a beaker containing 150 mL of enzyme-free SIF as the release medium and kept for 4 h at 37°C. It should be noted that both outer release media contained 50% (v/v) ethanol to provide good solubility for curcumin as well as maintain the sink condition. At different predetermined time intervals (0, 1, 2, 3, 4, 5, and 6 h) aliquots of the samples were withdrawn from outer release media and the

amount of released curcumin was determined spectrophotometrically at 420 nm to estimate the percentage of the drug released [2,6].

## 2.6. Release kinetics and mechanism.

To understand the release kinetics and mechanism of curcumin from different forms of whey protein aggregates, the experimental release data were applied to different kinetic models including: zero order ($M_t/M_\infty = k_0 t$), first order ($M_t/M_\infty = 1-\exp(-k_1 t)$), Higuchi ($M_t/M_\infty = k_H t^{1/2}$), and Korsmeyer-Peppas ($M_t/M_\infty = kt^n$). In these models, $M_t/M_\infty$ is the fraction of drug released at time t. Moreover, the $k_0$, $k_1$, $k_H$, and $k$ are the rate constants of zero order, first order, Higuchi, and Krosmeyer-Peppas models, respectively. In the Korsemeyer-Peppas model, n also is the release exponent which is used to study the mechanism of drug release [20, 21].

## 2.7. Statistical analysis.

The measurements were repeated three times and resulting data were presented as mean ± standard deviation. The results were analyzed by one-way analysis of variance (ANOVA) and Duncan test ($p < 0.05$) using the SPSS software version 16.

# 3. RESULTS

## 3.1. Encapsulation parameters.

In the present study, different forms of aggregates formed by heating of WPI solution at various pH values including 2.0 (nanofibrillar aggregates), 5.85 (microgel particles), and 7.5 (worm-like aggregates) were compared with native WPI in the term of their ability to load curcumin as a bioactive cargo. The resulting sample solutions before and after loading of curcumin are shown in Figure 1.

**Table 1.** Curcumin encapsulation efficiency (EE) and loading amount (LA) in different forms of whey protein isolate (WPI)-based aggregates. Means with different superscripts in the same column differ significantly ($p < 0.05$).

| Forms of WPI | EE (%) | LA (µg/mg) |
|---|---|---|
| Native | 6.91 ± 0.88[d] | 1.72 ± 0.22[d] |
| Worm-like aggregates | 12.94 ± 2.04[c] | 3.23 ± 0.51[c] |
| Microgel particles | 46.91 ± 2.03[b] | 11.72 ± 0.50[b] |
| Nanofibrillar aggregates | 70.56 ± 1.10[a] | 17.64 ± 0.25[a] |

Determination of encapsulation parameters especially EE and LA for carriers is of utmost importance for evaluating the capacity of a system for loading of a specific drug as well as the therapeutic efficacy of the drug delivery system [2]. Therefore, the curcumin EE and LA for different whey protein aggregates were determined and the results are shown in Table 1. The results showed that the aggregates had a higher EE and LA than native WPI suggesting that the aggregated forms of WPI are suitable carriers for the encapsulation of curcumin. This can be due to the higher surface hydrophobicity of aggregates compared to the native WPI which makes them more appropriate carriers for the curcumin which is a hydrophobic bioactive molecule [21]. Between the aggregates also the highest EE and LA were related to the fibrillar aggregates. This was followed by microgel particles and worm-like aggregates that had higher EE and LA compared to the native WPI, but their capacity for loading of curcumin was lower than the fibrillar aggregates which formed by heating of WPI solution at pH value of 2.0. The higher EE and LA of fibrillar aggregates compared to the other forms of whey protein aggregates can be due to their fibrillar morphology which can provide more hydrophobic binding sites for curcumin. Therefore, curcumin can form soluble complexes with fibrillar aggregates through the formation of hydrophobic interactions. In accordance with our findings, the complex coacervates made of whey protein nanofibrils and gum Arabic also showed a high EE for curcumin which was about 99% [12]. Generally, the determination of encapsulation parameters suggested that the whey protein aggregates can be considered as promising carriers for improving the water solubility of curcumin which can help to expand its applications in the food and drug formulations.

## 3.2. Antioxidant properties.

The antioxidant activity of different curcumin-loaded samples was determined using ABTS and DPPH free radical scavenging tests and the results are presented in Figure 2. The results indicated that in both of the tests, the highest antioxidant activity was related to the curcumin-loaded whey protein nanofibrils and the lowest antioxidant activity was also found with curcumin-loaded native WPI. In fact, there was a direct relationship between encapsulation efficiency and antioxidant activity; samples with higher encapsulation efficiency and loading amount showed a higher antioxidant activity. Therefore, these results suggested that different forms of whey protein aggregates can be suitably used to improve the antioxidant properties of curcumin. In fact, free curcumin has a poor solubility in distilled water and forms large aggregates in distilled water which in turn can restrict the amount of available curcumin for interacting with free radicals of ABTS and DPPH [22]. Therefore, encapsulation of curcumin in whey protein aggregates especially nanofibrils and microgel particles improved its antioxidant activity by increasing its aqueous solubility and increasing the available amount of curcumin for scavenging of free radicals. In consistent with our findings, other studies also reported that the encapsulation of curcumin in different carriers such as soy protein isolate [5], rice bran albumin nanoparticles [23], myofibrillar proteins [24], and whey protein-gum Arabic complex coacervates [12] significantly improved its ability to scavenge the free radicals. Therefore, these findings, suggested that the curcumin-loaded whey protein aggregates can be considered as antioxidant biopolymeric ingredients in the food formulations especially those with high susceptibility to oxidation.

## 3.3. Release behavior.

Determination of drug release behavior for a drug delivery system under simulated physiological conditions is very important because it is closely related to the effectiveness of as-obtained carriers for human health [2]. Therefore, the release of curcumin which was defined as the percentage of curcumin transferred from aggregates to release medium was determined under simulated gastrointestinal conditions and the resulting profiles are shown in Figure 3. After 2 h digestion in SGF, the cumulative curcumin release from native WPI, worm-like aggregates, microgel particles, and fibrillar aggregates was 16.35, 11.57, 9.90, and 7.64%, respectively. After 6 h of release experiment (2 h in SGF and 4 in SIF) the curcumin release from native WPI, worm-like aggregates, microgel particles, and fibrillar aggregates reached to

44.91, 30.08, 25.17, and 21.73%, respectively. Therefore, these results showed that the highest curcumin release during the release test under simulated gastrointestinal conditions was related to the native WPI. Moreover, it was found that the release of curcumin from aggregated forms of WPI was lower than the native WPI and the lowest release was found with nanofibrillar aggregates which were formed by the heating of WPI solution at the acidic condition. This observation suggested that the loading of curcumin in whey protein aggregates can be considered as a promising method to control the release of curcumin. It seems that the lower curcumin release from aggregates can be due to their higher resistance against digestive enzymes (i.e. pepsin in SGF and pancreatin in SIF) compared to the native WPI [11] which makes them more apocopate carriers for protecting of curcumin as a bioactive molecule. In accordance with our findings, a lower release was observed for whey protein aggregates formed by citric acid-mediated cross-linking compared to the non-aggregated WPI attributing to the high resistance of citric acid-induced cross-linked whey proteins against digestion, degradation, and erosion [21].
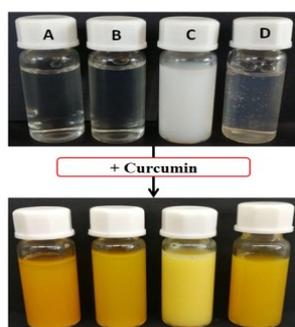


**Figure 1.** Different forms of WPI (A: native, B: worm-like aggregates, C: microgel particles, and D: nanofibrillar aggregates) before and after loading with curcumin.
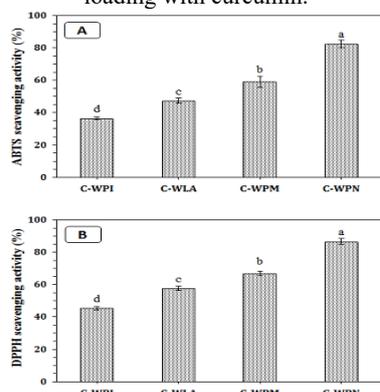


**Figure 2.** ABTS (A) and DPPH (B) radical scavenging activity of curcumin-loaded WPI-based aggregates including native WPI (C-WPI), worm-like aggregates (C-WLA), microgels (C-WPM), and nanofibrils (C-WPN).
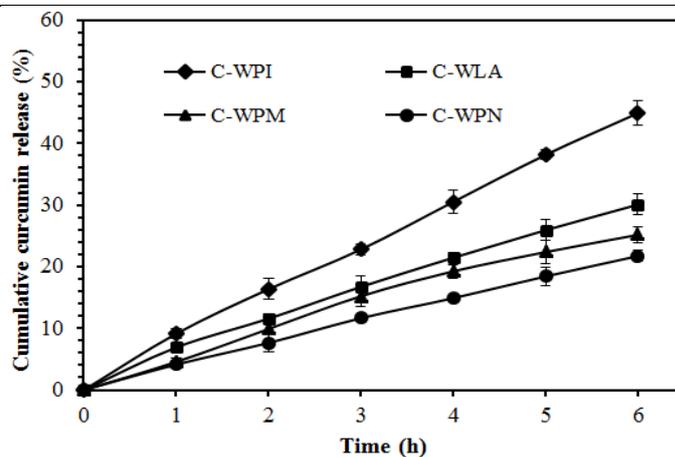


**Figure 3.** Curcumin release profiles for curcumin-loaded WPI-based aggregates including native WPI (C-WPI), worm-like aggregates (C-WLA), microgels (C-WPM), and nanofibrils (C-WPN).

### 3.4. Release kinetics and mechanism.

To investigate the release model that best described the curcumin release, the experimental date from the release test was substituted in equations of zero order, first order, Higuchi, and Korsmeyer-Peppas models and the correlation coefficients of the release profiles at different kinetic models are presented in Table 2. The results showed that for all of the curcumin-loaded samples, the Korsmeyer-Peppas was best fitted with release kinetic data of curcumin. Moreover, high $R^2$ values were also observed for zero order and first order models suggesting that the curcumin release from whey protein aggregates dos not follow only one model or mechanism. Similar results were reported for curcumin-loaded whey protein aggregates which were fabricated by citric acid-mediated cross-linking [21]. The release exponent or n values from Korsmeyer-Peppas as shown in Table 2 was used to determine the mechanism of curcumin release from the whey protein aggregates as: n=0.5 for Fickian diffusion, 0.5 < n < 1 for non-Fickian diffusion or anomalous transport, and n > 1 for case II transport [20]. Our results showed that the release exponent for all of the samples was lower than 1.0 and higher than 0.5. Therefore, the release modelling data showed that the model of curcumin release from whey protein aggregates was non-Fickian or anomalous transport, and the mechanism of drug release is governed by diffusion and swelling. Therefore, the controlling mechanism for curcumin release from both the native and aggregated WPI could be bio-polymer swelling and drug diffusion. This observation indicated that the release of curcumin from the WPI-based carriers is generally controlled by more than one process. In accordance, it was reported that the release of curcumin from nanoparticles of chitosan and gum Arabic was controlled by both diffusing and particle swelling (i.e. anomalous transport) [25].

**Table 2.** Model parameters of curcumin release from curcumin-loaded WPI-based aggregates including native WPI (C-WPI), worm-like aggregates (C-WLA), microgels (C-WPM), and nanofibrils (C-WPN). $R^2$ is the correlation coefficients and $n$ is the release exponent.

| Sample | Zero order $R^2$ | First order $R^2$ | Higuchi $R^2$ | Korsmeyer-Peppas $R^2$ | $n$ |
|---|---|---|---|---|---|
| C-WPI | 0.9973 | 0.9947 | 0.9032 | 0.9991 | 0.928 |
| C-WLA | 0.9895 | 0.9981 | 0.9284 | 0.9997 | 0.846 |
| C-WPM | 0.9835 | 0.9926 | 0.9192 | 0.9936 | 0.849 |
| C-WPN | 0.9979 | 0.9987 | 0.9019 | 0.9997 | 0.930 |

## 4. CONCLUSIONS

Curcumin was encapsulated in different forms of whey protein isolate including native, worm-like aggregates, microgel particles, and nanofibrillar aggregates formed by the heating of WPI solution at different pH values. The highest encapsulation efficiency and loading amount were related to the fibrillar aggregates which can be due to their high surface hydrophobicity. The curcumin-loaded whey protein aggregates showed a high activity to scavenge the free radicals of ABTS and DPPH. Encapsulation of curcumin in whey protein aggregates delayed its release in simulated gastrointestinal conditions. The release modelling results also showed that the mechanism of drug release from whey protein aggregates was a non-Fickian or anomalous transport which is controlled by diffusing and polymer swelling. Generally, the results of this study suggested that the whey protein aggregates can be considered as promising carriers for curcumin delivery in different fields such as food science and drug delivery applications.

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# 6. ACKNOWLEDGEMENTS