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Original Article

Preparation and *In Vitro* Characterization of Crocin-loaded Casein Hydrogels

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HIGHLIGHTS

- Casein-based hydrogels were developed for delivery of crocin.
- Casein-based hydrogels provided a controlled *in vitro* release profile for crocin.
- Hydrogel with a lower casein ratio exhibited a higher release rate of crocin.

ABSTRACT

Crocin, the main active constituent of saffron, has many important biological activities. Due to its anti-inflammatory properties, crocin can be potentially effective in different pathological conditions including oral ulcers. Novel drug delivery systems such as hydrogels have been used to increase the stability of crocin and provide a controlled release of this compound. Casein is the main protein of milk that possesses suitable properties for the fabrication of hydrogels. In this paper, casein-based hydrogels with different casein to crocin weight ratios were synthesized using the acid-gelation method. The prepared crocin-loaded hydrogels were characterized regarding their rheological behavior, drug content, swelling ratio, surface morphology, thermal stability, and *in vitro* release profile. The structure of casein hydrogels was characterized using Fourier transform infrared and X-ray diffraction. All formulations exhibited a pseudoplastic rheological behavior and there was no statistically significant difference in viscosity among them. Hydrogel with casein to crocin weight ratio of 10:1 had larger pores and demonstrated a higher swelling percentage and suitable thermal stability. All casein-based hydrogels demonstrated a slow release of crocin over 24 hours and the hydrogel with lower casein to crocin weight ratio had an increased release rate. Taken together, casein-based hydrogels were found to be effective carriers to provide a controlled release system for crocin delivery.

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Introduction

Crocin, also known as crocetin digentiobiose ester, is a hydrophilic natural carotenoid compound and one of the main bioactive constituents of the dried stigmas of *Crocus sativus* L. plant (Sung and Kim, 2018; Puglia et al., 2019). Crocin has shown multiple favorable effects such as high anti-oxidant, anti-inflammatory, and anti-tumor activities on different body tissues and organs (Sung and Kim, 2018). According to the pharmacological studies, crocin can be useful in various pathological conditions such as diabetes, bronchitis, depression, and cancer (Esposito et al., 2016; Omidkhoda et al., 2020). Moreover, due to the anti-inflammatory properties of crocin, it can be effective in inflammatory conditions such as oral ulcers (Edgar et al., 2017; Liu et al., 2018). The protective effects of crocin in periodontitis, atopic dermatitis, skin aging and burn wounds have been demonstrated (Alemzadeh and Oryan, 2018; Fagot et al., 2018; Sung and Kim, 2018; Kocaman et al., 2021). Despite the beneficial effects of crocin, the unfavorable chemical and physical properties of this carotenoid have limited its therapeutic applications (Esposito et al., 2016; Puglia et al., 2019). For example, the low chemical stability of crocin has made it sensitive to light, oxygen, and heat (Tsimidou and Biliaderis, 1997). Moreover, using crocin is associated with poor absorption, low bioavailability, and rapid elimination (Puglia et al., 2019). Using the topical route of administration for local delivery of therapeutic substances has multiple advantages such as avoidance of first-pass metabolism, selective drug delivery to specific sites, and patient convenience. The conventional topical delivery systems have several drawbacks including low bioavailability and poor retention. These limitations can be conquered using novel topical drug delivery systems, which can improve the efficacy and safety of a therapeutic agent and reduce its adverse effects (Madhusudhan et al., 2020). To overcome the aforementioned drawbacks associated with using crocin and to obtain a controlled release system for this compound, several studies have developed crocin-loaded novel drug delivery systems that can be used for the topical administration of crocin, including organogels (Esposito et al., 2016), ethosomes (Esposito et al., 2016), nanostructured lipid dispersions (Esposito et al., 2017), and hydrogels (Zeka et al., 2018).

Hydrogels are three-dimensional materials composed of hydrophilic polymeric chains (Sharma et al., 2019; Hanna et al., 2020). Various appreciable properties of hydrogels such as resemblance to the extracellular matrix, suitable biocompatibility, adjustable mechanical strength, and ability to swell by absorbing tremendous volume of water-based fluid while maintaining its structure have made it an ideal candidate for different applications including tissue engineering, subcutaneous inserts, contact lenses, and wound dressing (Rassu et al., 2016; Hafeez et al., 2018; Sharma et al., 2019). Furthermore, hydrogels have been used for the delivery of a wide range of drugs and bioactive molecules because their unique porous nature provides the possibility of delivering drugs in a controlled manner (Hoare and Kohane, 2008; Rassu et al., 2016).

Proteins are biocompatible components consisting of one or multiple amino acid residues (Xu et al., 2018). Using proteins, as natural biopolymers for developing drug delivery systems, has attracted a lot of attention in recent years because of their excellent and splendid features (Hu et al., 2015). Milk proteins are known as a natural vehicle for bioactive molecules (Elzoghby et al., 2011). Casein phosphoproteins are the major components of bovine milk that are obtained by precipitation through acidification of raw milk (Hanna et al., 2020). The beneficial characteristics of casein such as good biocompatibility, high hydrophilicity, low toxicity, and low price have made it an interesting option for developing novel drug delivery systems (Hanna et al., 2020; Simão et al., 2020). In this regard, several casein-based drug delivery systems including cross-linked micelles (Picchio et al., 2018), nanocomposite films (Kajthunyakarn et al., 2018), and hydrogels (Song et al., 2010; Khodaverdi et al., 2018) were successfully developed. Casein-based hydrogels can potentially be used in an extensive range of applications. The high swelling capacities and water absorption of casein hydrogels can provide the entrapment of therapeutic agents and result in a controlled release (Nascimento et al., 2020). Taken together, it can be concluded that using casein is a promising approach to develop biocompatible hydrogels for the controlled release of active substances.

Our study aims to investigate the potential use of casein-based hydrogels for the delivery of crocin in a controlled manner. Casein-based hydrogels with three different casein to crocin weight ratios were generated through acid-induced gelation. The crocin content and *in vitro* release behavior of crocin from these self-linked casein matrices were examined. Moreover, the swelling behavior, surface morphology, rheological properties, thermal stability, and chemical structure of the prepared hydrogels were investigated in detail.

Materials and Methods

Chemicals

Casein and crocin were purchased from Merck KGaA (Germany). All other chemicals and solvents such as HCl and NaOH were of analytical grade and were received from commercial sources.

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Preparation of blank and crocin-loaded casein hydrogels

The synthesis of casein hydrogels was carried out according to the method described by Tan et al. (2019) with slight modifications. Aqueous casein solution was prepared by mixing casein with distilled water to get a final concentration of 8.0 wt%. Appropriate amount of NaOH solution (2.0 M) was slowly added under constant magnetic stirring, until casein was completely dissolved. Then, by adding 0.5 M HCl, the pH value of the solution was adjusted to 1.0-2.0. After 24 hours, the solution was centrifuged for 20 minutes at 2000 g. After replacing the supernatant with distilled water, the process of centrifugation was repeated 4 more times to get near to neutral pH. For the preparation of crocin-loaded hydrogels, different amounts of crocin were added before the centrifugation process to obtain three distinct casein to crocin weight ratios (10:1, 25:1, and 50:1).

Characterization of hydrogels

a. Viscosity measurements

Viscometric measurements of casein hydrogels were performed at room temperature using programmable rheometer (Brookfield DV-III ultra, USA), equipped with a suitable spindle (CPE 42) and rotated at different rpm.

b. Swelling studies

The swelling behavior of freeze-dried hydrogels was investigated. Definite amount of hydrogels were weighted and immersed in simulated saliva solution (pH 6.8) at 37 °C. Hydrogels were taken out at regular time intervals and weighted after removing the excess water on the surface of hydrogels using a filter paper. The swelling percentage of hydrogels was determined using equation 1,

$$\text{Degree of swelling (\%)} = \frac{Ws - Wd}{Wd} \times 100 \qquad \text{Equation 1}$$

where W_d is the original weight of the dry hydrogel sample and W_s is the weight of swollen hydrogel at a specific time.

c. Scanning electron microscopy (SEM)

The surface morphology and the inner pore structures of casein-based hydrogels were analyzed using SEM at various magnifications on MIRA3 (TESCAN, Czech Republic). Prior to analysis, the samples were lyophilized to keep the porous structure and avoid any collapse and changes in morphology. The freeze-dried samples were then sliced into small pieces and fixed on aluminum stubs by double-sided adhesive tape. Sputter coating with gold was used to enhance the electrical conductivity and improve the imaging of hydrogel samples.

d. Determination of crocin content

During the formation of casein-based hydrogels, the supernatants of centrifugation were collected to determine the crocin content of hydrogels and were quantified using UV-vis spectrophotometry (Ultra-3660, Rigol, China) and the standard calibration curve.

e. In vitro drug release from casein hydrogels

The release profiles of crocin from hydrogels were studied using Franz diffusion cells. The acceptor compartment was filled with 25 mL of simulated saliva fluid (pH 6.8) and accurately weighted hydrogel formulations were placed in the donor compartment. A pre-soaked cellulose dialysis membrane with a molecular weight cut-off of 12,000 Da was used to separate the compartments. The temperature of the receptor compartment was adjusted at 37 °C and a magnetic stirring bar was used for constant stirring. Samples were withdrawn from the acceptor phase at predetermined time intervals over 24 hours and were instantly replaced with an equal volume of fresh simulated saliva solution to maintain a constant volume. The samples were analyzed using a UV-vis spectrophotometer and the absorbance was read at the specific absorption peak of crocin (485 nm). The cumulative amount of crocin released at different time intervals was calculated by equation 2 and the standard calibration curve and then was plotted against time,

$$\text{Cumulative \% of drug release} = \frac{Mt}{M} \times 100 \qquad \text{Equation 2}$$

where M is the total amount of crocin loaded in hydrogels and M_t is the cumulative amount of crocin released at time t.

f. Thermogravimetric analysis (TGA)

Thermogravimetric analysis was used to evaluate the thermal stability of the prepared casein hydrogel. Crocin, casein, and the freeze-dried casein hydrogel were heated using TGA-50H analyzer (Shimadzu, Japan) under nitrogen atmosphere with the flow rate of 30 mL/min and the temperature range of 20-400 °C.

g. X-ray diffraction (XRD) studies

XRD patterns of crocin, casein, physical mixture of casein and crocin, and freeze-dried hydrogels with or

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without crocin were collected using an Explorer (Italy, GNR company), with Cu Kα radiation at a voltage of 40 kV and current of 30 mA. The scanning rate of the test was 0.033°/s and the angle of diffraction was ranged from 5° to 80°.

h. Fourier transform infrared (FTIR) spectra analysis

An FTIR spectrophotometer (FTIR-8400S, Shimadzu, Japan) was used to confirm the chemical structure and the functional groups of crocin, casein, and empty and crocin-loaded hydrogels. Applying the potassium bromide (KBr) pellet method, the samples were grounded to obtain a fine powder and then the powders mixed with dry KBr disks were used for recording the spectra. Measurements were acquired within the scanning range of 4000 to 500 cm^{-1} at room temperature and a resolution of 4.0 cm^{-1}.

Statistical analysis

The data were analyzed by using GraphPad Prism (version 8.0.2; Graphpad Software Incorporated) and reported as means ± standard error of the mean (SEM). The results were performed in triplicate (n= 3). The student's t-test or one-way analysis of variance (ANOVA) followed by a Turkey's post-test were used to compare repeating values. When the P-value was below 0.05, the differences among results were considered statistically significant.

Results and Discussion

Preparation of casein hydrogels

The great anti-inflammatory and anti-oxidant properties of crocin, the main active carotenoid of saffron, have made it an ideal potential candidate for the management of inflammatory processes including oral ulcers (Borhan-Mojabi et al., 2014; Esposito et al., 2016). However, the unfavorable properties of crocin such as low physicochemical stability, poor absorption and low bioavailability have limited its application in topical therapy (Esposito et al., 2016). Several studies have developed novel drug delivery systems to overcome these limitations or to provide a controlled release system of crocin. In this regard, Esposito and co-workers (2017) developed nanostructural lipid dispersions containing crocin to evaluate its anti-oxidant and anti-proliferative properties. The resultant topical drug delivery system was effective in controlling the skin diffusion of crocin, protecting it from degradation, and prolonging its anti-oxidant activities, as well as increasing the cytotoxic effects of crocin. In another study, crocin was loaded in polyvinyl pyrrolidone and polyethylene glycol-based hydrogels and its antioxidant effects on mouse fibroblasts were evaluated. The synthesized hydrogels were biocompatible and able to simulate the expansion of fibroblasts (Zeka et al., 2018).

Using food proteins to develop drug delivery systems offers a lot of promise owing to their desirable functional properties. As the major protein of milk, casein is cheap, non-toxic, readily available, biodegradable, and biocompatible. Moreover, casein possesses multiple favorable characteristics that make it a suitable candidate for developing drug delivery systems, particularly hydrogel biomaterials. These characteristics include high hydrophilicity, the presence of reactive sites that are used for chemical modifications, and excellent water-binding and gelation capacity (Elzoghby et al., 2011). Various methods, such as physical or chemical cross-linking, heating, compression, and acid-induced gelation have been applied to improve the gelation process of aqueous casein systems (Tan et al., 2019). For instance, Song et al. (2009) developed novel casein-based hydrogels using genipin as a cross-linker. The prepared hydrogels were able to provide a controlled release of bovine serum albumin. Moreover, the casein-based hydrogels exhibited various drug release and swelling properties depending on their genipin content.

In this study, aqueous 8.0% casein solution was prepared by dissolving casein in an alkaline solution. Acidification of this solution resulted in the gelation of casein. Different concentrations of crocin were used to obtain hydrogels with final casein to crocin weight ratios of 10:1, 25:1, and 50:1. The synthesized casein gels were collected after centrifugation. The synthesis of blank hydrogels was the same as crocin-loaded hydrogels without adding crocin. The preparation process of casein-based hydrogels is shown in Fig. 1.

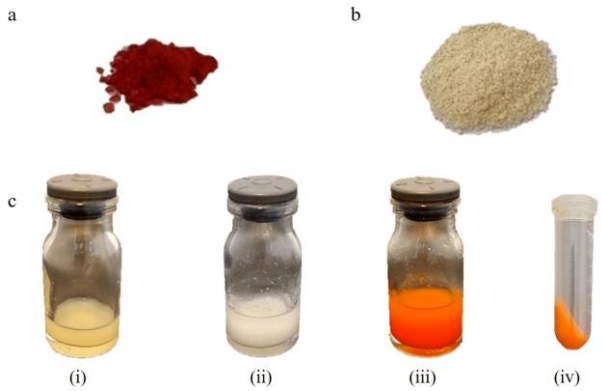

Figure 1. Images of the (a) crocin powder, (b) casein powder, and (c) the process of gelation: (i) casein was dissolved after adjusting pH to 10, (ii) the pH of the solution was adjusted to 1-2, (iii) crocin solution was added after 24 hours and (iv) casein hydrogel collected after centrifugation.

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Characterization of casein hydrogel

Viscosity is an important parameter that can potentially influence the release behavior of active ingredients of hydrogels. The viscosity of prepared casein hydrogels was measured with the rotation speed set between 1 and 15 rpm. The viscosity curves of hydrogels with three different casein to crocin weight ratios (50:1, 25:1, and 10:1) were obtained. As shown in Fig. 2A, there was no statistically significant difference in the viscosity profile of the designed hydrogel formulations.

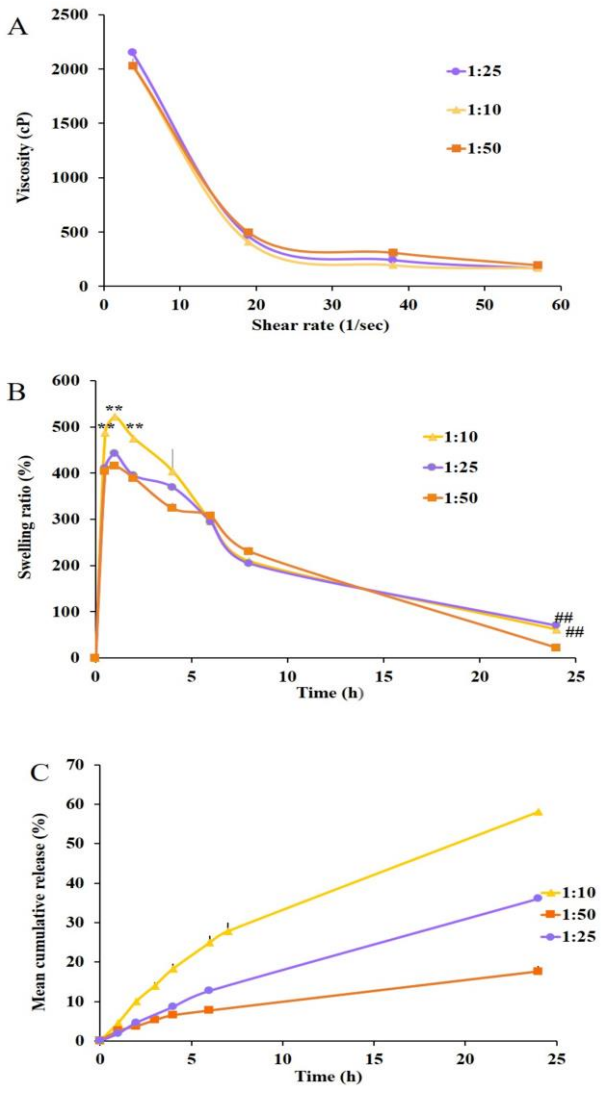

Figure 2. (A) Viscosity curves of hydrogels with three different casein to crocin weight ratios (10:1, 25:1, and 50:1). (B) The swelling ratio of casein hydrogels with three different casein to crocin weight ratios (10:1, 25:1, and 50:1) was evaluated in simulated saliva fluid. (C) *In vitro* cumulative release profile of crocin from casein-based hydrogels with three different casein to crocin weight ratios (10:1, 25:1, and 50:1). ** Indicates statistical significance compared to hydrogels with casein to crocin weight ratio of 50:1 and 25:1 ($P < 0.01$). ## Indicates statistical significance compared to hydrogels with casein to crocin weight ratio of 50:1 ($P < 0.01$).

Moreover, the tested hydrogels showed a non-Newtonian pseudoplastic behavior with shear-thinning properties (Mahmoud et al., 2020). Pseudoplastic behavior is a desirable characteristic for the topical administration of hydrogels because the gel will flow easily at higher shear rates, supporting its spreadability, and it will exhibit a higher consistency in the case of low shear rates, recovering the rheological properties prior to the administration (Ghica et al., 2016).

The ability to swell in aqueous solutions is one of the favorable properties of hydrogels (Ganji et al., 2010). Determination of the rate and the degree of swelling is important, as it is one of the main essential factors that affects the loading capacity and the release profile of a therapeutic agent from a synthesized polymeric matrix (Eyigor et al., 2018). The swelling rate is determined by multiple physicochemical parameters including the degree of porosity (Ganji et al., 2010). The swelling profiles of the casein hydrogels with three different casein to crocin weight ratios (50:1, 25:1, and 10:1) were studied up to 24 hours in simulated saliva fluid (pH 6.8). The swelling of casein hydrogels increased in the first hour and then gradually decreased over time (Fig. 2B). This could be due to the weight loss of hydrogels caused by the degradation of casein or the breakdown of dry casein hydrogels in the presence of the fluid (Johnson et al., 2020). The graph also indicates that the degree of swelling was higher in the first hour in hydrogels with lower casein to crocin weight ratio. This could imply that the formulation with lower casein to crocin weight ratio has a looser polymeric network and higher porosity.

To gain information on the surface morphology of synthesized casein hydrogels, freeze-dried crocin-free and crocin-loaded hydrogels were analyzed using SEM. As observed in Fig. 3, all hydrogels presented a porous structure that resulted from losing water in the freeze-drying process. The morphology of hydrogels with casein to crocin weight ratio of 25:1 and 50:1 exhibited a denser network structure compared to blank hydrogels and the hydrogel with the casein to crocin weight ratio of 10:1, which confirms the finding of the swelling test.

The crocin content of designed hydrogel formulations was determined by quantification of the absorbance of supernatant after centrifugation. The calculated crocin content of hydrogels with the casein to crocin weight ratios of 10:1, 25:1, and 50:1 were 89.88 ± 0.0004, 37.83 ± 0.0006, and 19.10 ± 0.0012 mg/g of hydrogel, respectively. The hydrogel with a lower casein to crocin weight ratio showed a higher crocin content ($p < 0.0001$). This could be due to the higher concentration of loaded crocin in hydrogels with lower casein to crocin weight ratios. Another reason could be the less compact structure of the hydrogel with less casein to crocin weight ratio which could increase the diffusion of crocin in the matrix of gel (Hanna et al., 2020).

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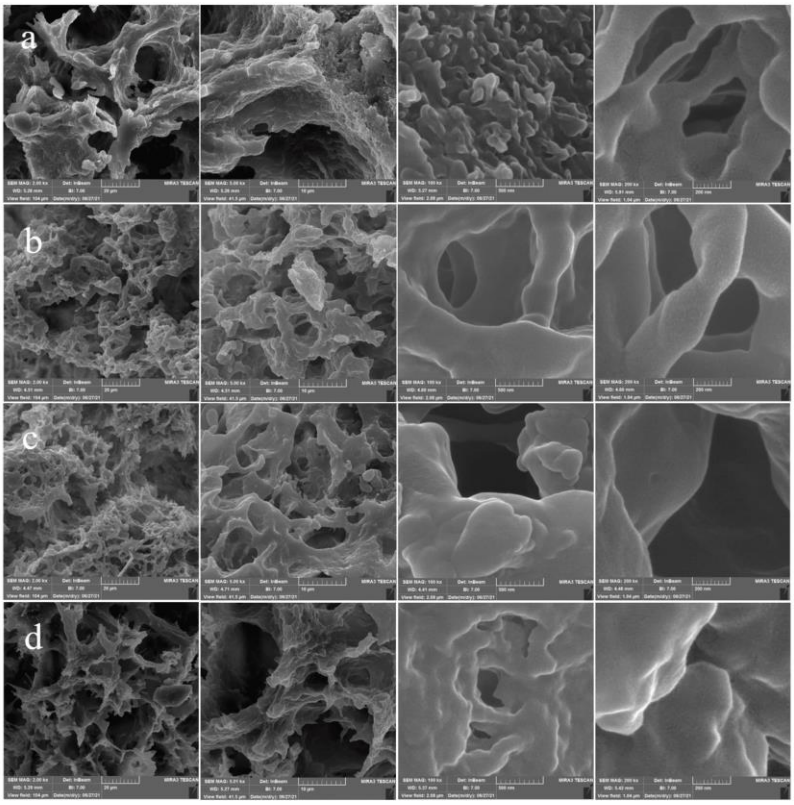

Figure 3. SEM images of (a) unloaded casein hydrogel and loaded casein hydrogels with different casein to crocin weight ratios: (b) 50:1, (c) 25:1 and (d) 10:1.

The mechanism of crocin release from different formulations of synthesized hydrogels was studied in simulated saliva fluid (pH 6.8) at 37 °C. Fig. 2C shows the crocin release behavior from hydrogel samples. All hydrogel samples released the drug in a sustained manner up to 24 hours. The hydrogel with the casein to crocin weight ratio of 10:1 had released larger amounts of crocin compared to the others ($p < 0.05$) with an ultimate cumulative release of 58.07% after 24 hours. This might be due to the fact that hydrogels with higher casein to crocin weight ratio have a denser network structure that increases their drug grasping power (Sharma et al., 2019). While the hydrogel with the casein to crocin weight ratio of 10:1 has larger pore sizes and a subsequent higher swelling, which correlates with SEM and swelling test results, as the maximum swelling ratio was observed in this formulation. Based on these findings, the hydrogel with the casein to crocin weight ratio of 10:1 was selected as the optimum formulation and it was used for further analysis.

The TGA curves of crocin, casein, and freeze-dried casein hydrogel are shown in Fig. 4. Crocin started to lose approximately 2% of its weight at 336 °C. The thermograms of casein powder and casein hydrogel exhibited similar profiles, which indicates that the gelation process does not change the thermal stability of casein. Below 200 °C, the casein powder and the casein hydrogel demonstrated good thermal stability, having a small weight loss due to the removal of the entrapped water of the samples. Both samples started losing weight and decomposing at around 260 °C, which could be attributed to the breakdown of the polymer (Yi et al., 2019).

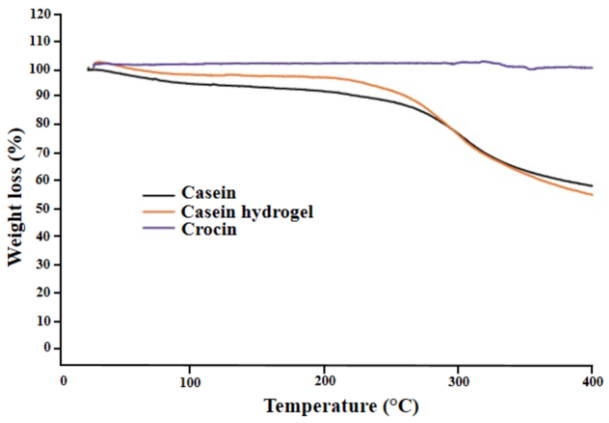

Figure 4. TGA curves of casein, crocin, and casein hydrogel.

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XRD patterns of casein, crocin, the physical mixture of casein and crocin, crocin-free hydrogel, and crocin-loaded hydrogel were analyzed (Fig. 5). As indicated by the XRD pattern of crocin, it has a crystalline nature. Casein has an amorphous appearance and its broad peak appears at the diffraction angle (2θ) value around 20°. The sharp peaks of crocin disappeared after being entrapped in casein hydrogels (Purushothaman et al., 2019).

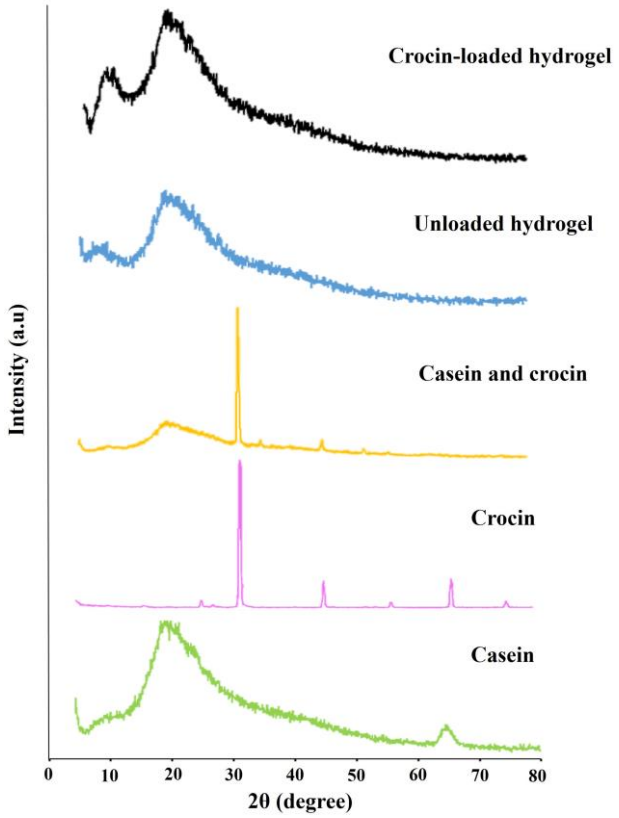

Figure 5. XRD patterns of casein, crocin, the physical mixture of casein and crocin, unloaded hydrogel, and crocin-loaded hydrogel.

FTIR spectroscopy was used to confirm the chemical synthesis of casein hydrogel and to investigate the presence of functional groups of the components that were used in the synthesis of the hydrogel. The FTIR spectra of the prepared blank hydrogels, crocin-loaded hydrogels, and their components are illustrated in Fig. 6. The main absorption frequencies of casein appeared at 3441 cm^{-1} which corresponded to the free amino group, 2929 cm^{-1} and 2855 cm^{-1} due to the symmetric and asymmetric vibrations of the C-H bonds, 1654 cm^{-1} attributed to amide I group, and 1528 cm^{-1} owing to the N-H bending and C=O stretching vibrations (Purushothaman et al., 2019). For crocin, the main peaks were observed at 1635 cm^{-1} for C=O, 1202 cm^{-1} for C-O stretching vibrations, and 3443 cm^{-1} and 1036 cm^{-1} for the O-H and the C-O sugar groups of the glycosyl esters (Rahaiee et al., 2015). The FTIR spectrum of casein hydrogels reveals the same notable peaks at 1000-1800 cm^{-1}, which could imply that the process of gelation does not affect casein at the molecular level (Tan et al., 2019). The reduction in the intensity of crocin peaks after loading in hydrogels states the entrapment of crocin in casein hydrogel (Purushothaman et al., 2019).

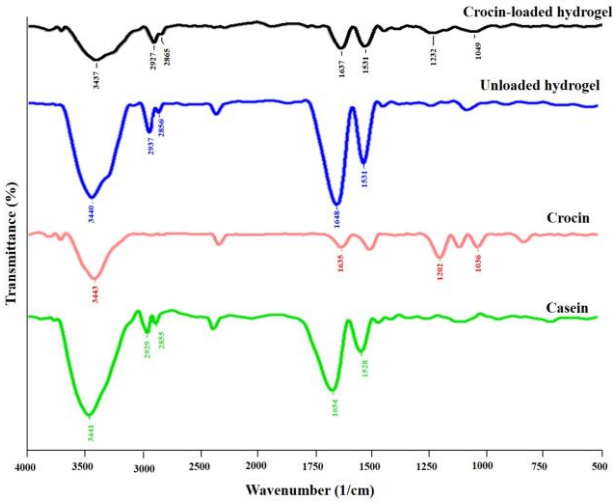

Figure 6. FTIR spectra of casein, crocin, unloaded hydrogel, and crocin-loaded hydrogel.

Conclusion

In this work, hydrogels based on casein from milk protein were successfully prepared by using the acid-induced gelation method to achieve a controlled release of crocin, the main carotenoid of saffron. All the prepared casein hydrogels showed a non-Newtonian pseudoplastic behavior. The casein hydrogel with the lowest casein to crocin weight ratio (10:1) exhibited higher crocin content, maximum swelling in simulated saliva fluid during the first hour, and larger released amount of crocin (58.07% over 24 hours) compared to hydrogels with higher casein to crocin weight ratios (25:1 and 50:1). These characteristics were attributed to the enhanced porosity in the structure of this hydrogel formulation. All formulations were able to provide a slow *in vitro* release of crocin. As a result, this study has provided a potential drug delivery system based on casein proteins as an effective approach to achieve controlled release of crocin. Crocin-loaded casein hydrogels could be proposed as a good candidate for topical administration of this carotenoid. Further *in vitro* and *in vivo* research is required to confirm these findings.

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Ethical Statement

This article does not contain any studies with human and animal subjects performed by any of the authors.

Competing Interests

The authors declare no conflict of interest.

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Authors Contribution

Fatemeh Mehryab and Fatemeh Taghizadeh equally contributed to performing the experiments, data collection and analyses, interpretation of results, and manuscript writing and revisions. Azadeh Haeri contributed to conception and supervision, study design, data collection and analyses, interpretation of results, and manuscript revisions.

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