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THE RELEASE CHARACTERISTICS OF EPIGALLOCATECHIN GALLATE FROM THE GEL WERE AFFECTED BY ITS REACTION WITH WHEY PROTEIN ISOLATE

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ABSTRACT

The interaction between epigallocatechin gallate (EGCG) and whey protein isolate (WPI) under various pH and ion concentrations conditions was examined by ultraviolet-visible spectrophotometer and fluorescence spectra. The results showed that EGCG with WPI was linked mainly by electrostatic force, and EGCG caused the fluorescence quenching of WPI through a static quenching. Furthermore, in vitro static release results showed that the release process of EGCG from EGCG-WPI gel in release medium of different pH and ion concentrations follows the Korsmeyer-Peppas model and conform to the non-Fick diffusion law. Finally, the binding constant KA and the constant release Kapp kinetic model were established. The model followed the equation $LnK_A=A-\frac{1}{n}LnK_{app}$.

1.Introduction

Whey protein is a by-product of cheese production, and it is mainly composed of β-lactoglobulin (β-Lg), α-lactalbumin, (α-La), bovine serum albumin (BSA), and immunoglobulin (Ig) (Mehla et al. 2020). The functional properties of whey protein such as solubility, gelation, emulsification, foaming, etc. make it widely in food industry application (Minj and Anand 2020). Furthermore, due to its high nutritional value, it also has become one of bioactive supplements for the human body (Marcelo and Rizvi 2008). However, protein gel can be used as a carrier to disperse some functional components (Qiu and Park 2001). There was found that protein gel can not only protect biological activity but also regulate release rate of bioactive compounds (Chen, Remondetto and Subirade 2006) (Gunasekaran, Ko and Xiao 2007, O'Hagan, Singh and Ulmer 2006). Thus, improve the bioavailability of functional components. Previous studies showed that biological activity of VC was improved by encapsulation with WPI gel by controlling condition factors including pH value, temperature, EGCG loading, and ion concentration (Betoret et al. 2011).

EGCG is one of bioactive compounds, it is prepared mainly from green tea and it is contained 50-60 % catechin (Higdon and Frei 2003). The biological activities of EGCG including anti-oxidation, anti-tumor, anti-inflammation activities, thus, it has critical role in prevention of cardiovascular and cerebrovascular diseases. However, EGCG application in food and medicine industries is limited due to its poor stability (Dai et al. 2020).

In this regard, the stability of EGCG by reaction with WPI in the protein gel has not been evaluated. Therefore, the stability of EGCG in the composite gel of EGCG-WPI was evaluated. Furthermore, interaction between the EGCG and WPI was studied.

2. Materials and methods

2.1. Materials and instruments

Whey protein isolates powder (> 95% protein content) and EGCG (98%) were purchased from Le Sueur Cheese CO., USA and Chengdu Purifa technology development CO., LTD, respectively. Other chemicals were bought from Tianjin Fengchuan chemical reagent technology CO., LTD. pH meter (FE20, Mettler Toledo Instrument Shanghai CO., LTD.), visible spectrophotometer (WFJ7200, Uniko, Shanghai instrument CO., LTD.), fluorescence spectrophotometer (Shanghai Lingguang technology CO., LTD.), high-speed centrifuge (Shanghai Anting Scientific instrument factory), multi-head magnetic stirring heater (Changzhou Guohua electric appliance CO. LTD).

2.2. Methods

2.2.1. *Sample preparation*

Firstly, the stability of EGCG of was examined under several pH value by mixing several volumes of (10-300 μL) of EGCG solution (1.2mg/mL), separately with 3 mL of pH phosphate buffer, and then the pH value was adjusted to 2.0 - 9.0, and the ion concentration was set to 0.05 mol/L. After that, 2 mL of WPI solution (1 mg/ mL) was added. Secondly, the stability of EGCG of was examined under difference ion concentration by mixing several volumes of (10-300 μL) of EGCG solution (1.2mg/mL), separately, with several volumes' ion concentration (0.10, 0.15, 0.20 mol/L), 3 mL of phosphate buffer and 2 mL of WPI solution (1 mg/ mL, were added respectively and pH value of 3.0. To prepare the EGCG-WPI gel: the WPI and of EGCG mixtures with difference pH values or ion concentration were prepared as mentioned above. After stirring for 2 - 3 h, the mixtures were stored at 4 °C for 12 h, and then the mixtures were heated at 85°C for 30 min using water bath. After that, the prepared gel was cooled rapidly in the ice water bath.

2.3. *Ultraviolet absorption and Fluorescence spectra analysis*

The absorbance of WPI in EGCG-WPI solution was determined within the wavelength range of 260- 340nm. For fluorescence spectra analysis, the excitation wavelength of fluorescence spectrum was 280 nm, and the scanning emission spectrum range was 280–340 nm (Zhang et al. 2012).

2.4. *In vitro release experiment*

Briefly, 1 ml of prepared gel in 50 mL phosphate buffer solution with difference pH values and ion concentrations was prepared as mentioned above. During the incubation for 360 min at 25°C, the EGCG concentration in mixtures was determined every 20 min by mixing samples mixture with Folin phenol reagent and sodium carbonate (7.5%, w/v), and then the absorbance was measured at 765 nm after reaction at 25 °C for 1 h.

2.5. *Statistical analysis*

Data were analyzed by one-way ANOVA using 25.0 SPSS Statistics (SPSS, Inc., Chicago, IL, USA) and Microsoft Excel 2010 was used for significance analysis. The significance level was $P < 0.05$, and the results were formulated as follows:$\bar{x} \pm$ s.

3.Results and discussions

3.1. Interaction between EGCG and WPI

3.1.1. *Ultraviolet absorption and Fluorescence spectra*

The UV absorption of WPI with EGCG concentration within 260 ~ 340nm is shown in Figure 1. At pH 7.0, WPI native had the maximum absorption peak at 280nm. The absorption of WPI was increased successively after mixing EGCG (0.15mol/L), as well as blue shift occurred from 280.0nm to 276.4nm Figure 1 (a and d). In addition, UV absorption spectra of WPI with EGCG concentration at pH values or ion concentrations presented the same trend (data not shown). Likely, with the increase of EGCG concentration in the WPI solution, the

absorption peak position of the WPI derivative UV spectrum also shifted Figure 1 (b, c, e and f) at the same pH (7.0) and ion level (0.15mol/L), these results indicated that the interaction between EGCG and WPI was occurred.

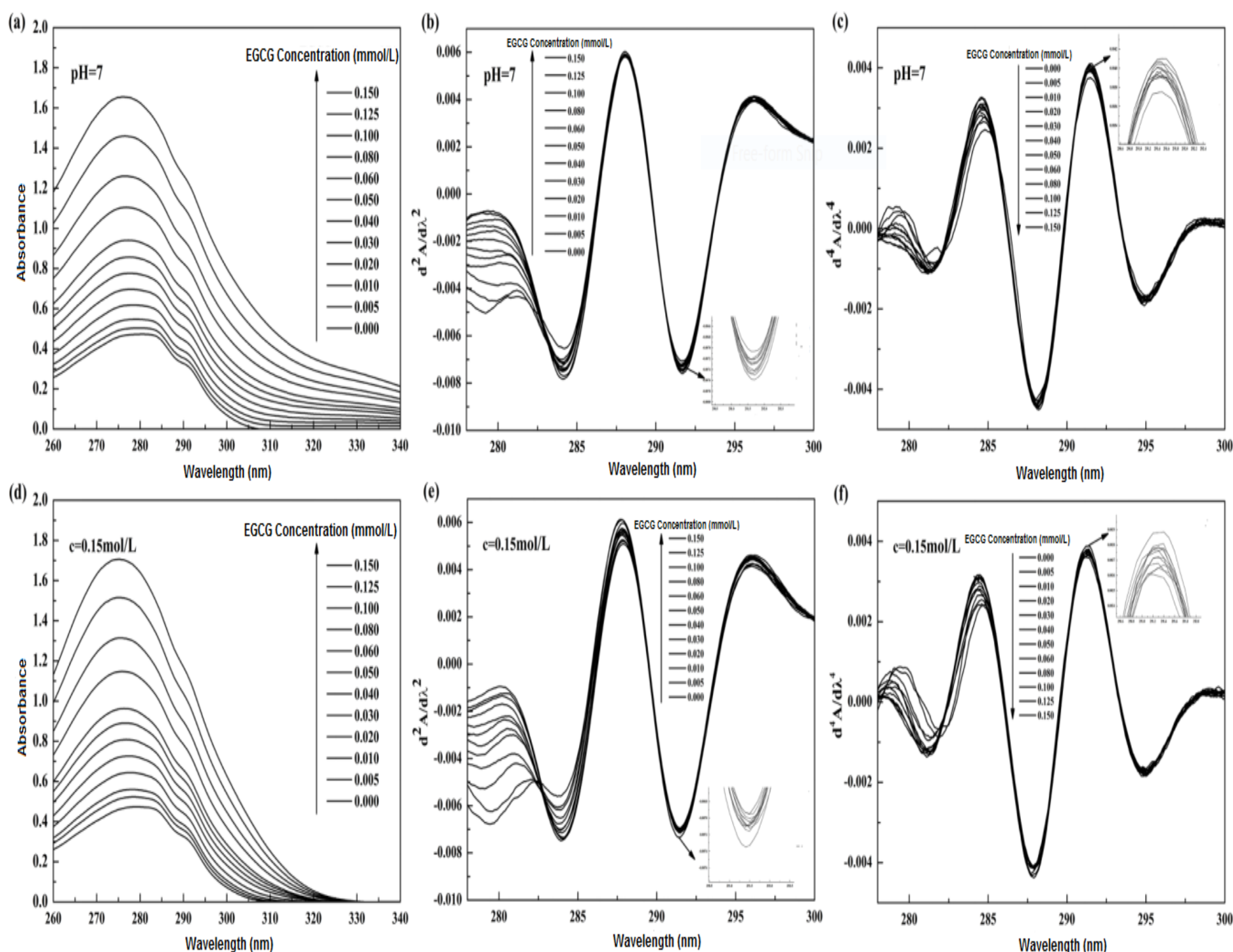

Figure 1. Influence of the concentration of EGCG on the UV absorption spectra of the WPI solution.

3.1.2. *Fluorescence spectrum*

Some amino acid residues include tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) that can emit fluorescence in the protein. Trp and Tyr are usually excited at 280 nm or longer wavelength, while Phe is excited at 295 nm or longer wavelength. Thus, the natural fluorescence and its variation value of this protein can directly reflect the changes of Trp, Tyr, and Phe residues in the protein and their surrounding microenvironment (Hemar et al. 2011).

The fluorescence spectra of WPI with EGCG concentration under experiment condition (pH 7.0, ion level, 0.15mol/L). AS displayed in Figure 2(a and b), at an excitation wavelength of 280 nm, the fluorescence spectrum of WPI solution had the maximum absorption peak near 330 nm. And this fluorescence spectrum did not change with the concentration of WPI, while fluorescence spectrum was gradually increased after added various concentrations of EGCG into WPI solution. Furthermore, the fluorescence intensity of the WPI solution was decreased and slightly redshifted, indicating that EGCG had a quenching effect on the fluorescence of WPI solution. In addition, similar phenomena were observed with other pH

values of 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 9.0 and ion levels of 0.10 and 0.20 mol/L. These results further indicate the interaction between EGCG and WPI was occurred.

Additionally, at the excitation wavelength of 280nm, WPI emitted fluorescence at 330 nm in all pH values (2.0, 3.0, 4.0, 6.0, 7.0, 8.0, 9.0) except for the pH value of 5.0 because the pH environment affects on the solubility of WPI by changing the surface charge distribution. As known, β-Lg is the main component of WPI, and its isoelectric point is around 5.2 (Mantovani, Cavallieri and Cunha 2016).When the ambient pH value is close to the isoelectric point of WPI, the net charge on the surface of WPI molecules is about 0, and the intermolecular repulsion is the minimum. Thus, makes them easy to aggregation (Salminen and Weiss 2014), which is reflected in the fluorescence spectrum and causes the maximum difference in emission wavelength.

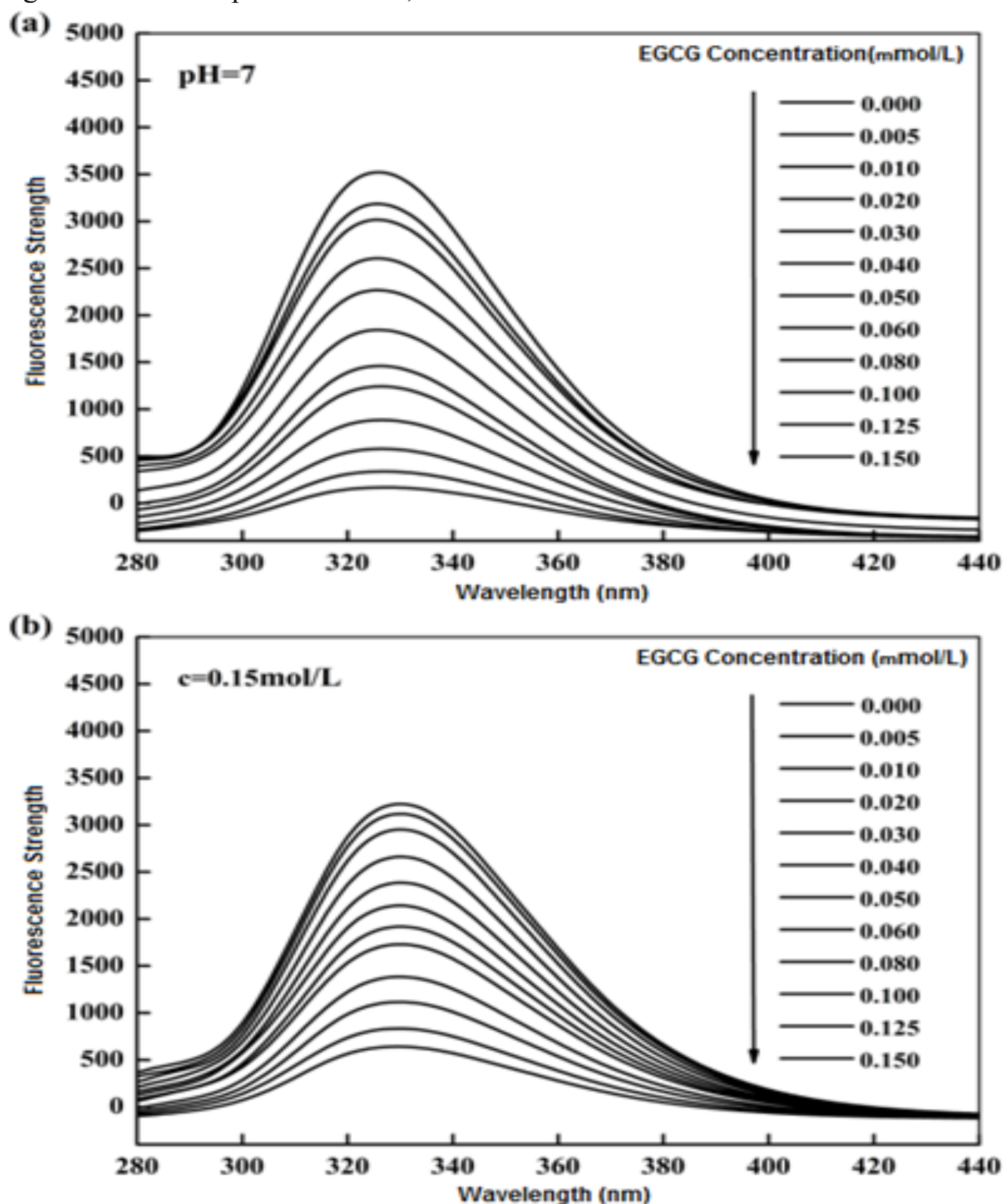

Figure 2. Influence of the concentration of EGCG on fluorescence emission spectra of WPI solution.

3.2.Fluorescence quenching mechanism analysis

The quenching mechanism for the system of biological macromolecules can be determined by comparing the quenching constant with the maximum quenching constant of biological macromolecules(Yang et al. 2018),the fluorescence quenching process caused by the collision of WPI as a fluorescent substance in the solution with the quenching agent EGCG can be analyzed by Stern-Volmer equation (Trnková et al. 2011):

$$F_0/F = 1+K_q\tau_0[Q]=1+K_{sv}[Q] \quad (1)$$

Where, F_0 and F is the fluorescence intensity of WPI solution without and with EGCG, respectively.$[Q]$ is EGCG concentration; K_{sv} is Stern-Volmer quenching constant ($K_{sv}=K_q\times\tau_0$) ; K_q is the rate constant of double molecular quenching;$\tau_0$represents the average fluorescence life of substances without quenching agents, and the average life of biological macromolecules is$1x10^{-8}$s (Yu et al. 2011).

Thus, K_q was much higher than the limiting diffusion rate constant of the biomolecules 2×10^{10} $(mol\cdot L^{-1})^{-1}\cdot s^{-1}$, it indicated that the WPI and EGCG interaction was mainly static quenching. For static quenching, the binding constant (K_s) was calculated using nonlinear least-squares fitting of the experimental data, as shown in Eq. (2) (Hasni et al. 2011):

$$Log\ [F_0 - F/F] = logK_A + m\times log[Q] \quad (2)$$

where m represents the kinetic reaction order (molecularity in Q). M measures the number of Q molecules that interact simultaneously with each site and does not express the number of independent and equivalent binding sites to analyze the reaction mechanism between EGCG and WPI, fluorescence quenching spectral data were combined with Eq. (1) and (2) to calculate the relevant characteristic parameters under different pH values and different concentrations (Table 2).

Table 1. Characteristic parameter in the interaction between EGCG and WPI at different pH values.

pH value	K_{sv}(×10⁵L/mol)	K_q(×10¹³ L/mol·s)	R_1^2	K_A (×10⁵ L/mol)	n	R_2^2
2.0	2.022±0.082	2.022±0.082	0.996	2.383±0.142	1.152±0.079	0.985
3.0	1.751±0.062	1.751±0.062	0.998	2.123±0.193	1.094±0.101	0.975
4.0	1.662±0.066	1.662±0.066	0.998	1.835±0.101	1.044±0.060	0.984
5.0	1.371±0.073	1.371±0.073	0.997	1.527±0.099	0.989±0.064	0.563
6.0	2.025±0.098	2.025±0.098	0.997	1.653±0.359	1.067±0.178	0.973
7.0	2.063±0.123	2.063±0.123	0.998	2.221±0.259	1.034±0.136	0.967
8.0	3.172±0.147	3.172±0.147	0.998	3.423±0.180	1.045±0.094	0.984
9.0	4.133±0.190	4.133±0.190	0.999	5.389±0.103	1.076±0.054	0.985

Table 2 Characteristic parameter in the interaction between EGCG and WPI at different ion concentrations.

Ion concentrations (mol/L)	K_{sv}(×10^5 L/mol)	K_q(×10^{13} L/mol·s)	R_1^2	K_A (×10^5 L/mol)	*n*	R_2^2
0.10	1.884±0.069	1.884±0.069	0.998	2.504±0.053	1.111±0.030	0.996
0.15	1.979±0.076	1.979±0.076	0.985	4.315±0.017	1.355±0.010	0.999
0.20	2.779±0.145	2.779±0.145	0.995	6.882±0.037	1.303±0.022	0.998

In this study, the K_q parameters were used to determine the quenching process, As shown in (Table 1 and 2), under different pH values and ion concentrations. The K_q values found from the reactions were much higher than the limiting diffusion rate constant of the biomolecules2.0×10^{10}L/mol.s, indicating that EGCG could successfully quench the WPI fluorescence via static quenching, which was induced mainly by the formation of WPI-EGCG complex. Besides, the interaction between EGCG and WPI was affected by pH values and ion concentrations, since when the pH value was set as 5.0, and the K_A presented the law of first decreasing and then increasing as well as K_A was increased with the increase of ion concentration (0.10 ~ 0.20 mol/L), indicating the interaction between EGCG and WPI was influenced by pH value via affecting the ionization degree of Trp, Tyr, and Phe in WPI, isoelectric point, charge WPI, and K_A value. However, increasing ion concentration led to protect the electrostatic repulsion, since the K_A value is significantly increased when the ion concentration in the environment is increased. Suggesting the EGCG and WPI were linked mainly by electrostatic force.

3.3.Gel release characteristics of EGCG and WPI

A kinetic model is usually used to describe the release kinetics mechanism to analyze the release process. The ideal model for analysing the release of active ingredients in porous materials is the Korsmeyer-Peppas model (Korsmeyer et al. 1983):

$$X=Kt^n = m_t/m_0 \quad (3)$$

$$\text{where , } m_0=A_{total}\frac{V_{disk}}{V_{total}} \quad (4)$$

Whereas *X* is the cumulative release amount of active ingredients at time *t*; *K* is the kinetic constant; n is the diffusion constant; m_t is the number of active components released at the time of *t*. m_0 is the maximum release amount of active components. A_{total} is the total amount of active components in the gel. V_{disk} is the volume of the gel block used in the release process; V_{total} is the volume of EGCG-WPI solution required for gelation. The kinetic mechanism was determined by *n* at that time $n \leq 0.45$, the release mechanism of active components was consistent with the Fick diffusion mechanism; at that time,$0.45 < n < 0.89$, it was consistent with the non-Fick diffusion mechanism, the coexistence of diffusion and dissolution. At that time, $n \geq 0.89$ followed the mechanism dominated by skeleton corrosion.

EGCG-WPI composite gels prepared under different conditions were respectively placed in phosphate buffer under identical conditions. According to Eq. (3), the EGCG cumulative release concentration changed with time was examined. As shown in (Table 3,6) the correlation coefficients R^2 of dynamic fitting was above of 0.90 in all tested samples, indicating a high degree of model fitting. The release rule of EGCG was similar under different conditions. thus, the release

concentration of EGCG from the gel gradually increased with the increase of release time.

Table 3. Characteristic parameter of EGCG release from WPI-EGCG at different pH values.

Release medium pH	$K(\times10^{-3})$	n	R^2
2.0	2.310±0.238	0.589±0.018	0.989
3.0	2.878±0.544	0.485±0.034	0.945
4.0	1.978±0.321	0.497±0.030	0.961
5.0	1.415±0.217	0.550±0.027	0.973
6.0	1.859±0.311	0.518±0.030	0.963
7.0	1.917±0.282	0.519±0.026	0.971
8.0	2.372±0.347	0.484±0.026	0.966
9.0	2.217±0.298	0.557±0.024	0.980

Table 4. Characteristic parameter of EGCG release from WPI-EGCG gel with drug loading.

EGCG loading (mg/mL)	$K(\times10^{-3})$	n	R^2
6	0.239±0.041	0.656±0.031	0.977
12	0.672±0.112	0.593±0.030	0.973
18	0.942±0.200	0.609±0.038	0.961
24	2.844±0.546	0.487±0.034	0.944

Table 5. Characteristic parameter of release EGCG-WPI composite gel at different pH values.

Gel inside pH	$K(\times10^{-3})$	n	R^2
3.0	2.878±0.545	0.485±0.034	0.945
4.0	2.897±0.306	0.431±0.019	0.976
5.0	1.627±0.190	0.519±0.021	0.981
6.0	1.972±0.245	0.488±0.022	0.975
7.0	2.160±0.198	0.479±0.016	0.986
8.0	2.808±0.359	0.468±0.023	0.971

Table 6. Characteristic parameter of EGCG release from WPI-EGCG gel at different ion concentrations.

Ion concentrations. (mol/L)	K(×10^{-3})	n	R^2
0.05	1.478±0.232	0.568±0.028	0.973
0.10	1.713±0.257	0.552±0.027	0.974
0.15	1.821±0.281	0.550±0.028	0.972
0.20	2.578±0.355	0.494±0.025	0.971

The release fitting curve of EGCG-WPI gel prepared at a pH of 3.0 and an ion concentration of 0.05 mol/L is shown in Figure 3 (a and b). When the pH value is 2-5, the concentration of EGCG released gradually was decreased as the pH value of the medium releasing increases, while in the pH range of 6~9, the EGCG released gradually was increased as the pH of the medium releasing increases.

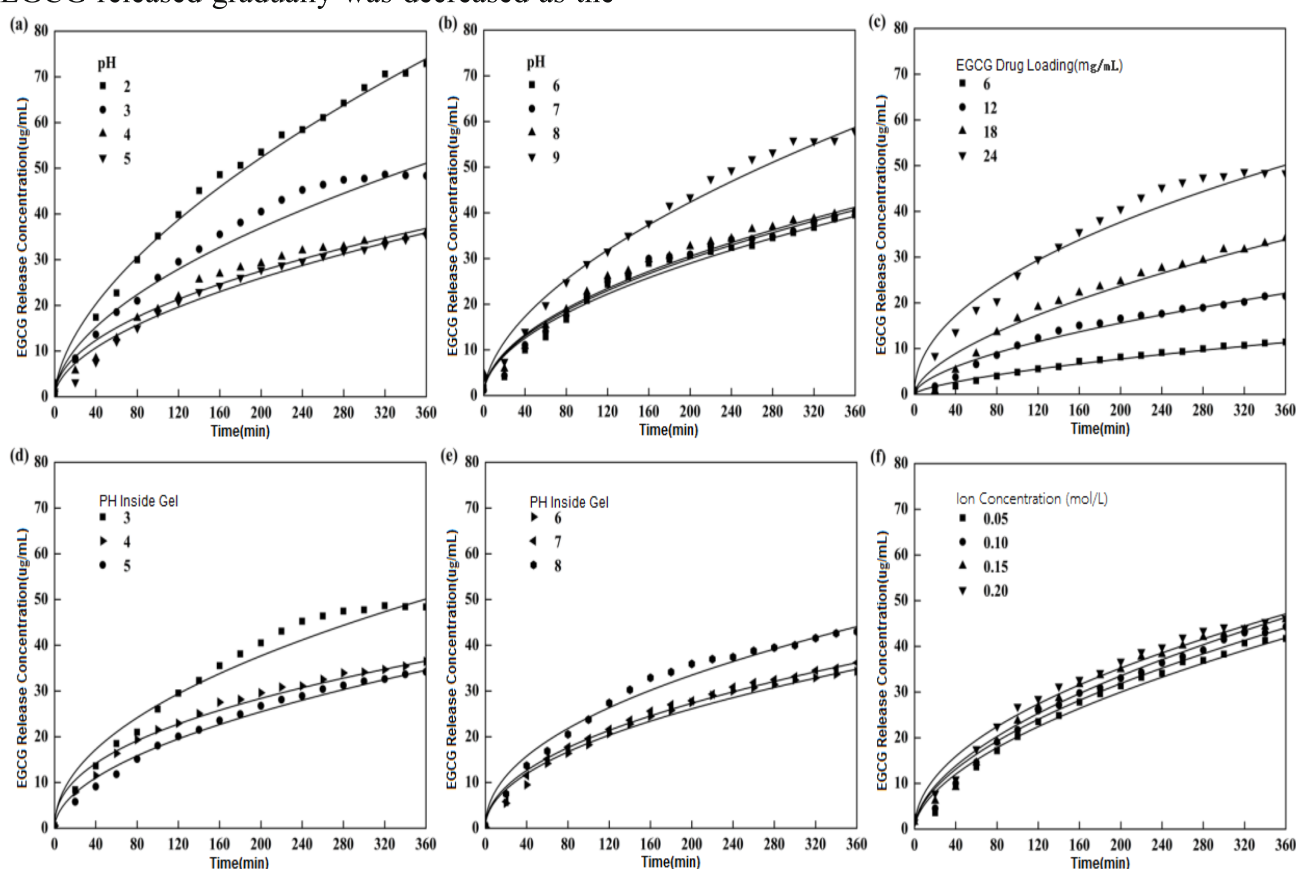

Figure 3. Kinetic fitting of the release of EGCG at different conditions.

Swelling is an important factor due to its effect on release system of the protein gel (Wang et al. 2008). The swelling property of the protein gel depends on the gel density and ionic charge of protein molecules (Caillard, Mateescu and Subirade 2010). In this regards, WPI gel contains a large number of carboxyl and amino groups. With the change of pH value, these groups will ionize, receive, or give protons, resulting in changes in the charge inside and

outside the gel. With high pH value, the ionization of these groups results in the formation of gel macromolecules with positive or negative charges. It makes the positive-positive or negative-negative charges on the WPI gel repel each other, and the gel expands, resulting in the release of EGCG from the gel. As shown in Figure 3C**,** when the gel EGCG level was 6mg/ml, the minimum concentration of EGCG released from the gel was 11.56mg /mL, while the loading increased to 24mg/mL, the release concentration of EGCG was 48.97mg /mL, with the increase of EGCG loading, indicated the positive correlation between the loading EGCG concentration and average releasing. due to gel swelling, the infiltration rate of water molecules into the gel is accelerated, resulting in the smooth release of EGCG (Shin et al. 2016).

At a pH 3.0, and ion concentration of 0.05mol/l, the release concentration of EGCG was the smallest (41.95 µg/mL). while, when the ion concentration increased to 0.20M, the release concentration of EGCG reached the maximum value of 48.09 µg/mL Figure 3f. It might be because the increase of ion concentration in the releasing medium increases osmotic pressure, leading to the rapid dissolution of the gel matrix and the exosmosis of water inside the gel (Jalil and Ferdous 1993), leading to the homeopathic increase of EGCG release concentration.

The relevant fitting parameters in the release process of EGCG from EGCG-WPI composite gel follows the Korsmeyer-Peppas model and conforms to the non-Fick diffusion mechanism under different pH (Table 3) and under different EGCG loading **(**Table 4**)**, release EGCG-WPI composite gel at different pH values (Table 5), and ion concentration release conditions (Table 6).

Kinetic analysis of the influence of EGCG and WPI interaction on the gel release process according to the equation:

$$LnX = LnK + nLnt \qquad (5)$$

The release constant K and release exponent n of EGCG in release medium with different pH values, and ion concentrations can be calculated (Table 7 and 8), respectively.

Table 7. Release constant *K* and release index *n* of EGCG release from WPI-EGCG gel at different pH and ion concentrations.

Release medium pH	***K*(×10⁻⁵)**	***n***	**R^2**
3.0	0.508±0.006	0.658±0.013	0.985
4.0	0.704±0.013	0.540±0.019	0.994
5.0	0.760±0.015	0.501±0.017	0.982
6.0	0.825±0.012	0.490±0.014	0.986
7.0	0.815±0.009	0.500±0.011	0.992
8.0	0.723±0.017	0.557±0.023	0.968

Table 8. Release constant *K* and release index *n* of EGCG release from WPI-EGCG gel at different ion concentrations.

Ion concentrations. (M)	$K(\times 10^{-5})$	n	R^2
0.05	0.411±0.008	0.647±0.020	0.983
0.10	0.453±0.009	0.641±0.023	0.980
0.15	0.371±0.010	0.684±0.027	0.971
0.20	0.466±0.021	0.691±0.038	0.950

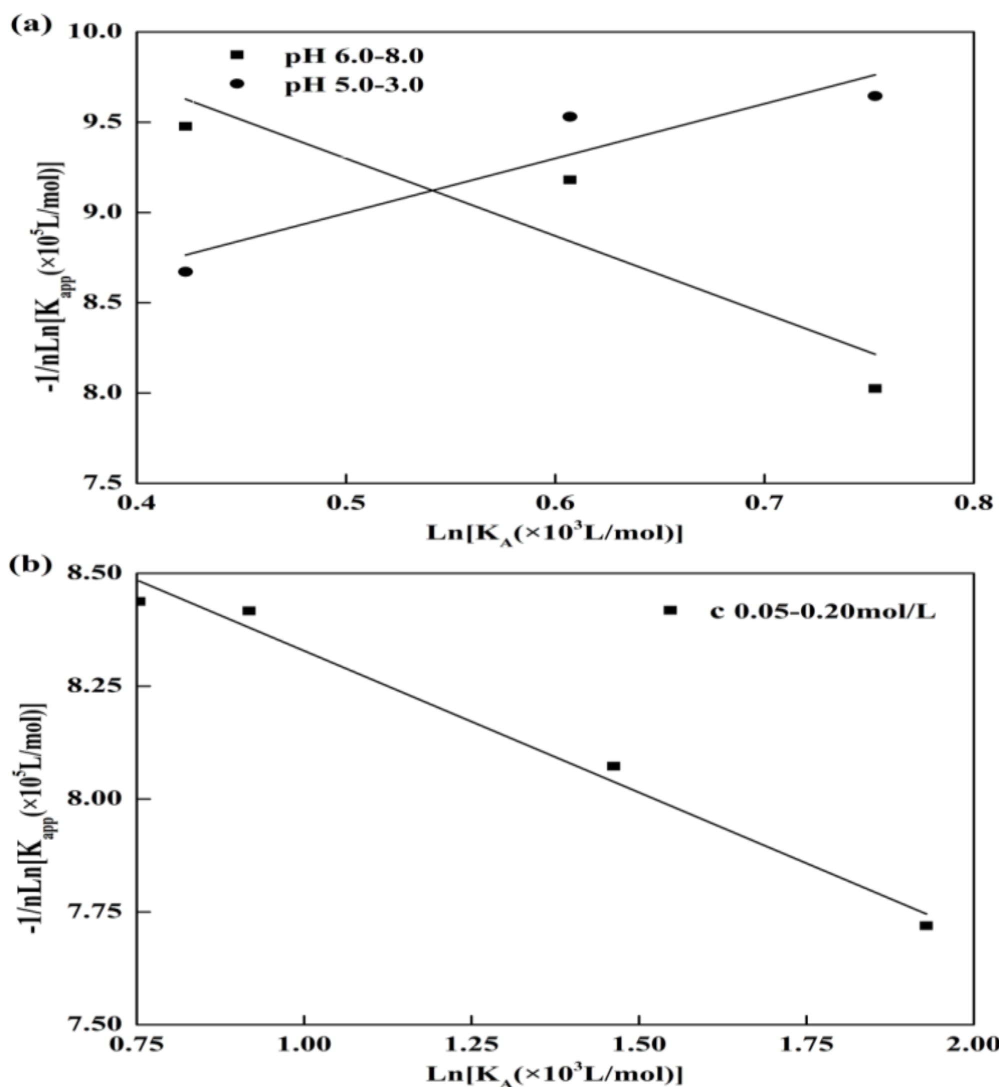

Figure 4. Binding constant K_A dependence of the release constant K_{app} at different conditions.

According to the quenching reaction

$$nQ+B \leftrightarrow Q_nB \quad (6)$$

the instantaneous equilibrium equation can be obtained

$$[Q_nB]=K_A[Q]^n[B_0] \quad (7)$$

According to the mass transfer equation of the existing chemical reaction process, the following equation can be obtained:

$$\frac{\partial C}{\partial t}=D\frac{\partial^2 C}{\partial x^2}-\frac{\partial}{\partial t}\left[Q_nB\right]=D\frac{\partial C^2}{\partial x^2}- K_An[B_0][Q]^{n-1}\frac{\partial}{\partial t}[Q \quad (8)$$

According to equation (3), we get

$$X=K_{app}t^n \quad (9)$$

$$K_{app}=K\left[\frac{D}{1+K_AD}\right]^n \quad (10)$$

Where, K_{app} is the apparent release constant, and D is the mass transfer coefficient, while n≈1,

$$\frac{1}{K_{app}}=\frac{1}{K}\left[\frac{1}{D}+K_A\right] \quad (11)$$

$$\frac{1}{K_{app}} \propto K_A \quad (12)$$

Simultaneous Eq. (5) and (11) that is a dynamic model equation:

$$LnK_A=A-\frac{1}{n}LnK_{app} \quad (13)$$

Formula LnK_A a $-\frac{1}{n}LnK_{app}$ to obtain the relationship between the interaction binding constant K_A and the apparent release constantK_{app} Figure 4a. It can be seen from Figure 4a that in the range of pH value 5.0 ~ 3.0, with the decrease of pH value, LnK_A and $-\frac{1}{n}LnK_{app}$is in direct proportion. In the range of pH value 6.0 ~ 8.0 and ion concentration 0.05 ~ 0.20mol/L, with the increase of pH value and ion concentration, LnK_A and $-\frac{1}{n}LnK_{app}$ represent the inverse proportional relationship Figure 4b.

The binding constant K_A reflects the degree of EGCG binding with protein which has correlation with degree of EGCG binding with protein. When the pH value was 3.0 ~ 5.0, K_A *was* decreased as the pH value increased, also the released EGCG from the gel was increased. Whereas, at pH values 6.0 ~ 8.0, as the pH value increases, K_A *was* increased, and EGCG release from the gel was decreased, due to electric point of WPI, which is close to the mentioned pH value, leading to decrease in surface charge of WPI molecules, and the binding degree between the WPI molecules and EGCG was weakened, thus increase of the released EGCG. On the other hands, K_A was increased with the increase of ion concentration, while the released EGCG was decreased. Increasing ion concentration, resulting in an increase in the binding degree between EGCG and WPI.

4.Conclusion

Briefly, the results of this study showed that reaction between EGCG and WPI was occurred mainly on the electrostatic interaction. The release process of EGCG confirmed Korsmeyer-Peppasmodel from EGCG-WPI gel. Furthermore, a kinetic model of K_A and K_{app} between EGCG and WPI at different pH values and ion concentrations was established, which was consistent with the kinetic equation $LnK_A=A-\frac{1}{n}LnK_{app}$. However, our results provided a further theoretical basis for applying WPI as functional ingredients in the food industry, especially in the development of novel edible gel.

The fitting curve of EGCG-WPI gel prepared at 0.05 mol/L ion concentration and different pH values was released into phosphate buffer release medium under identical conditions, as shown in Figure3(d and e). According to mentioned results in this study, the release concentration of EGCG was the smallest in the release process of gels prepared at pH values of 5.0 and 6.0, respectively 35.15 and 34.89 µg/mL. During the release of gels prepared at pH values of 3.0 and 8.0, EGCG was released at the highest concentrations of 48.97 and 44. 99µg/mL, respectively.

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