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THE USE OF WHEY PROTEIN ISOLATE IN MICROENCAPSULATION OF CURCUMIN

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ABSTRACT

Whey protein isolate (WPI) was used as a carrier of curcumin (CCM). Micro particles from WPI-CCM complexes were prepared under different conditions. WPI-CCM complexes characteristics were investigated. Fourier Transform infrared (FT- IR) showed significant interaction between WPI and CCM. The highest interaction occurred at acidic pHs and then decreased at alkali pHs. The micro encapsulation efficiency (EE) of curcumin with WPI was found to more 98.32%. Swelling ratio (SR) reached the maximum when swelling medium pH was to pH 10, while SR reached the minimum when swelling medium pH was close to the pI of the whey protein (~5.4).In vitro study, the release of curcumin from WPI micro particles were checked under acidic conditions of pH 2.0 in the SGF about 0.74% over a period of 12 hrs. About 56% of curcumin was released from the WPI micro particles under neutral pH conditions.Scanning electron microscopy (SEM) micrographs of WPI- CCM micro particles showed spherical particles with wrinkled surface without cracks or disruptions at pH 7, 8 and 9, while at pH 4 the particles resembled structures protein gels with a opaque appearance.

Key words: Whey protein isolates, Curcumin, Microencapsulation, FT-IR, and Microstructure.

INTRODUCTION

There is a growing interest in the use of whey proteins (WP) as safe biopolymers in the delivery of bioactive compounds and drugs (Tavares *et al.*, 2014). The ability of WP to form gels and microcapsules under relatively mild heating conditions and without the need of chemicals makes them attractive materials for controlled delivery of many hydrophobic and hydrophilic compounds for food and medical applications. The formation of hydrogels and nanoparticles depends on their thermal denaturation and gelation under controlled condition of pH and ionic strength. (Edwards and Janseon, 2014).

Curcumin is a fat soluble phenolic compound (diferuloyl methane) obtained from the roots of turmeric (*Curcuma longa L.*). It exhibits several physiological and pharmacological effects such as antioxidants, anti-inflammatory, antiangiogenic, antiamyloid, anticancer, antimicrobial, wound-healing, and hepatoprotective effects (Aggarwal*et al.*, 2007).

However, the potential uses of curcumin as efficient medical cure are limited due to its poor solubility, stability and bioavailability in aqueous media. Thus curcumin undergoes rapid degradation by hydrolysis (even at physiological pH) followed by molecular fragmentation within 30 min (Wang *et al.*, 1997; Liang *et al.*, 2009).Formation of complexes with natural polymers has been proposed to enhance the solubility and stability of curcumin. β-lactoglobulin (β-lg) has been reported to be capable to bind curcumin (Sneharani*et al.*, 2010 ; Li *et al.*, 2013) forming a complex which enhanced the solubility of curcumin 6.7 times in comparison to the free curcumin. The curcumin was found to bind to the central calyx of β-lg by hydrophobic interaction without affecting conformation or the state of association of the protein. (Sneharani*et al.*, 2010).β-lg nanoparticles can encapsulate curcumin and the stability of the entrapped curcumin in β-lg nanoparticles was significantly increased (Aditya*et al.*, 2015). Curcumin was also found to interact strongly with α-lactalbumin (α-la) by two hydrogen bonds (Mohammadi*et al.*, 2015). Due to low intrinsic toxicity of curcumin for healthy (normal) cells, several clinical trials are either underway or have been completed with an aim to develop curcumin into a treatment agent (Sahu*et al.*, 2008; Cheng *et al.*, 2001). Curcumin is extremely safe even at very high doses of 8–12 g/day (Cheng *et al.*, 2001).

The use of whey protein isolate as carrier for curcumin has received much less attention than the individual whey proteins. Whey protein preparations can offer more feasible and economic materials for this purpose. Therefore, the objective of the present study was to prepare micro or nanoparticles from whey protein isolate and to from their complexes with bioactive compounds.

MATERIAL AND METHODS

MATERIALS

BiPro®, a commercial whey protein isolate (WPI) was obtained from Davisco Foods International Inc.,

(Minnesota, USA). Theβ-lactoglobulin (β-lg) and α-lactalbumin (α-la) contents WPI were estimated to be 82% and 16%, respectively according to thesupplier. Curcumin (CCM) from *curcum longa* (≥65% pure), pepsin and pancreatinwerepurchased from Sigma (st. Louis, mo).

2.2.1Preparation of WPI and CCM solutions

WPI was dissolved in sterilized milli-Q water (10 %, w/v) at 4° C under slight agitation (180 rpm)for 16 h.The pH of aliquots of the protein solution were adjusted to pH 4, 5, 6, 7, 8 and 9 respectively using 1N HCl or 1N NaOH. The protein solutions were subsequently heated at 80°C for 30 min under agitation (95 rpm), cooled rapidlyin ice bath and stored at 4°C overnight. Calcium chloride solution (0.2 M) was used as a curing medium.

Freshly prepared CCM solution was made up by dissolving the CCM in ethanol to give 2 mM concentration. The exact concentration of CCM was measured by using a molar absorption coefficient $^{\varepsilon}429$ nm = 55,000 M^{-1} cm^{-1} (Mohammadi*et al.*, 2009). The CCM solution was protected from light throughout the experiments. Complex samples were prepared by mixing WPI and CCM solutions in different ratios to give 1, 3 and 5 mg CCM/ g protein. The ethanol concentration never exceeded 5% (v/v), to avoid any appreciable effect on the protein structure (Liang, *et al.*, 2007).

MICROENCAPSULATION SYSTEM

Monodisperse whey protein micro-beads were prepared using the Inotech IE-50R Encapsulator (Inotech AG, Dottikon, Switzerland),based on laminar jet break-up technology (Serp*et al.*, 2000).The WPI-CCM complex was aseptically extruded through a 150 mm nozzle into 250 mL of tempered (35° C) calcium chloride to induced flocculation of microbeads.The formed micro-beads were curedby standing in calcium chloride solution at room temperature for 20 min. Micro-bead analysis was performed during five independent trials with the mean and standard deviation (SD) being reported in triplicate for respective conditions.

FT-IR SPECTROSCOPIC MEASUREMENT

The freeze dried WPI- CCM complexes were grounded to fine powder, mixed with KBr at the ratio of 25 mg complex: 225 mg KBr (Lim *et al.*, 2014). The infrared spectra of the samples were recorded using FTIR spectrometer (Jasco FT-IR 6100) at wave number 250 – 4000 cm^{-1}.

MICROENCAPSULATION EFFICIENCY

The micro particles were calculated from $CaCl_2$ curing medium solutions then the dispersed in chloroform (1:10). The absorbance of the solutions was measured at 419 nm. Since interaction between curcumin molecules and WPI molecules may change the absorbance of the curcumin solutions, a standard curve of curcumin concentrations in the presence of the exact amount of free WPI molecules in the supernatant was plotted (R2 = 0.9998) according to (Sadeghi et al., 2014). Formula was used to estimate the encapsulation efficiency (EE) of curcumin was calculated from the following equation

Encapsulation efficiency (E. E)% = $\frac{initial\ CCM\ on. - Free\ CCM\ con.}{initial\ CCM\ on.} \times 100$

SWELLING

Triplicates of dried microparticles were weighed and placed in 20 mL of phosphate buffer solutions of pH 1.2, 3, 5.2, 6.8, 8 and 10 (ionic strength = 0.2*M*). Temperature was maintained at 37.5°C ± 0.5°C in an incubator (Model 2005; VWR Scientific Inc., West Chester, PA) (Saglam*et al.*, 2013). Microparticles were removed from the buffer solution at successive intervals of 15, 30, 60, 180 and 360 min., respectively, excess solutions was removed by blotting with tissue paper and then weighed. The swelling ratio (SR) was calculated from the mass measurements of wet gel (mw) and dry gel (md) as follo

$$SR = \frac{m_w - m_d}{m_d}$$

IN- VITRO DIGESTION

Enzymatic digestion of WPI–curcumin complexes was performed according to Puyfoulhoux*et al.*, (2001) with some modifications. Briefly, WPI –curcumin complexes re-suspended in pH 7.4 Dulbecco's Phosphate-Buffered Saline (DPBS) containing 0.9 mM calcium and 0.5 mM magnesium (called Phosphate-Buffered Saline PBS^{2+}) were incubated with porcine pepsin (25 mg mL^{-1} final) in PBS^{2+} brought to pH 2.0 with HCl, or with porcine pancreatin (2 mg mL^{-1} final) in PBS^{2+} at pH 7.4, to simulated gastric fluids (SGF) or simulated intestinal fluids (SIF) digestion, respectively. As negative or positive control for curcumin release, WPI–curcumin complexes were respectively incubated in PBS^{2+}put in dialysis bag . WPI–curcumin complexes were incubated at 37° C under gentle shaking for 12hrs.

After appropriate incubation-time, the released curcumin from dialysis bagin PBS^{2+}Curcumin was extracted from solutions with 5 mL chloroform, Curcumin release was expressed as:

Curcumin releases = 100 × ([curcumin] t_0 – [curcumin]t_i) / [curcumin] t_0

Where [curcumin]t_0 and [curcumin]t_i are curcumin concentration measured in chloroform at 419nm t_0 and after the various incubation times (t_i), respectively. Results werethe means of three independent experiments carried out on different days (Benzaria *et al.*, 2013).

SCANNING ELECTRON MICROSCOPY

The samples were freeze dried and obtained powder were deposited on a brass holder and sputtered with gold and examined using scanning electron microscope (Model JSM-6360, JEOL) at an accelerated voltage of 10 kV.

RESULTS AND DISCUSSIONS

Figure 1:- IR spectroscopy of WPIFigure 1:- IR spectroscopy of WPI (——), CCM (——) and WPI-CCM Complex (-----) at pH 4 (A), pH 5(B), pH 6 (C), pH 7 (D), pH 8(E), pH 9 (F) respectively.

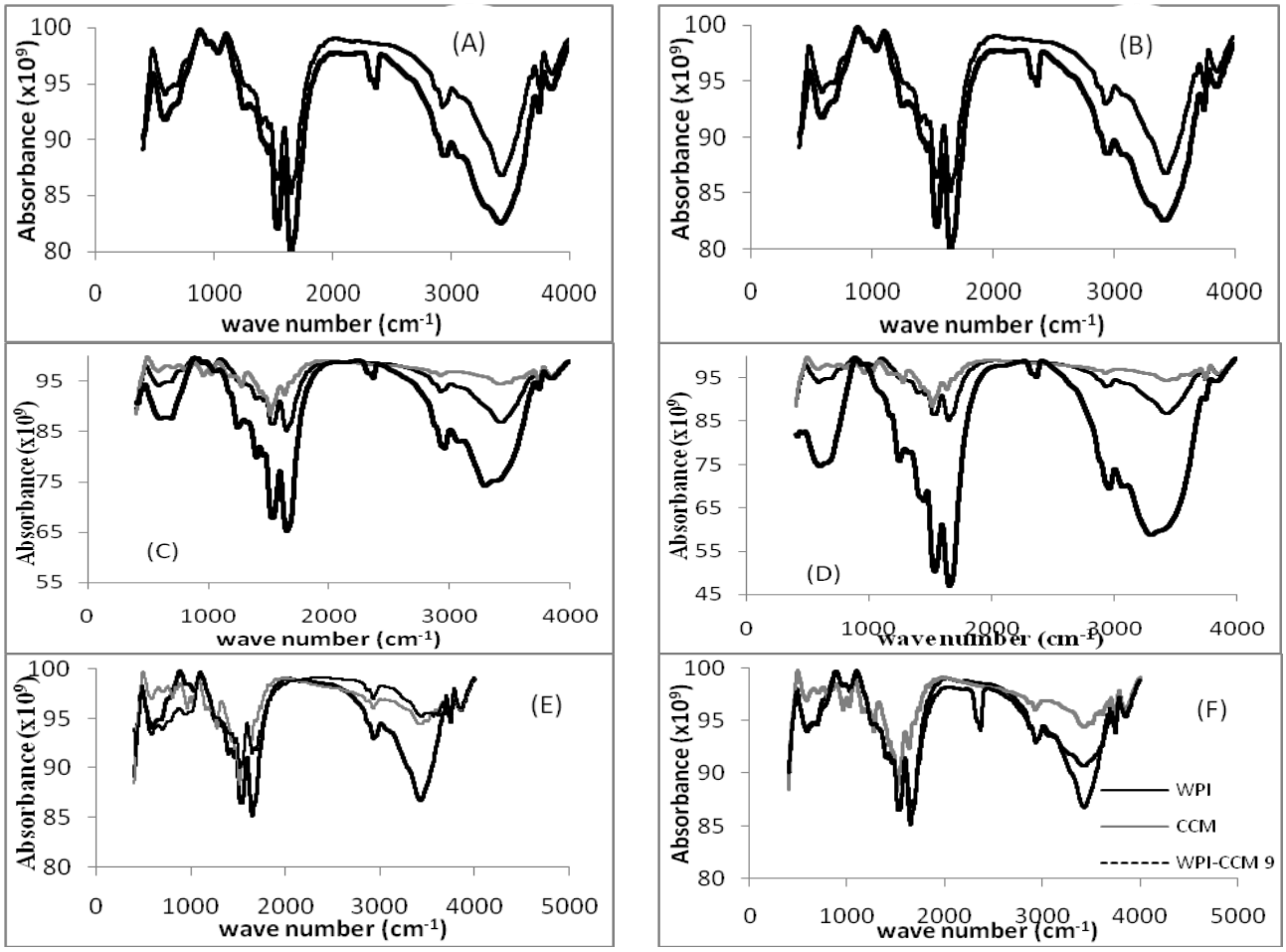


Fig.1 shows the infrared spectra of WPI, CCM and WPI-CCM Complex at pH 4 to 9. The IR spectra were recorded at wave numbers ranged from 4000 - 250 cm^{-1}.However the bands in the region 4000 - 1400 cm^{-1} were only analyzed in details, since they are characterize the OH groups on other hand NH groups were appeared at (3000 - 4000 cm) of various protonic species that undergo hydrogen bonding interactions. Another region of interest was that from 1800 – 1400 cm^{-1}, characteristic of the bending vibrations of the same group. Since the bands in this region are wide and complex.

Curcumin molecule has been placed appropriately near the side chain groups of the peptide. The functional groups OH and COOH of curcumin have been assumed to act as a hydrogen bond donor/acceptor for different side chain groups of amino acid (Madhan*et al.,* 2001). Fourier transform infrared spectroscopy (FTIR) spectrum of pure WPI displayed two characteristic bands, namely amide I (1649 cm^{-1}) bond associated with C=O stretching vibration, and amide Π (1547 cm^{-1}) bond due toN-H bending vibration. The significant in the shiftspeak position of amide I and Π in the IR spectra of WPI-CCM complex bands can be attributed to hydrogen bonding between curcumin and whey protein isolate. This may be due to interaction between CCM and WPI at pH 4, 5, 6, and 7 stretch band at 1631 with C=O , biding with N-H at 1507, -N=C=O stretch at 2327 and –C(triple bond) C–H: C–H stretch functional group alkynes (terminal) at 3327. However pH 8, 9 the terminal alkyne ≡ C-H – possible at 3291due interaction containing amine with curcumin.

The van der Waals interactions, hydrogen bonds, hydrophobic forces and electrostatic interactions are the general intermolecular interacting forces between small molecules and proteins (Mohammadi*et al.,* 2015). The total number of hydrophobic contacts made by curcumin with the protein is 21. The central calyx of β-LG is lined by hydrophobic amino acid residues that are gated by the protonation/deprotonation of Glu89 of the EF loop. The pH dependence shows that binding increases with increasing pH (Spector and Fletcher, 1970; Frapin*et al.,* 1993) in accord with the movement of the EF loop that acts as a lid to the cavity (Qin *et al.,* 1998a, b; Wu *et al.,* 1999; Ragona*et al.,* 2000, 2003). Conversely, the positively charged N, ,Ntrimethyl-dodecylammonium ion appears not to bind but to precipitate β-Lg (Lu et al., 2006) , or at least to bind differently (Magdassi*et al.,* 1996) in keeping with the presence of the positively charged sentinel lysines. (Lovrien and Anderson 1969) found two somewhat different anionic binding sites for N-methyl-2-anilino-6-naphthalenesulphonate at pH 8 but only one at pH 6, probably that for l-anilino-8-naphthalenesulphonate (ANS). (D'Alfonso*et al.,* 1999) found significant pH and ionic strength dependence for ANS with two distinct types of behavior, concluding that the interaction was largely electrostatic, and an electrostatic analysis of the protein structure indicated that there may be more than one binding site for negatively charged ligands (Collini*et al.,* 2000, 2003; Considine*et al.,* 2005) . (Kontopidis*et al.,* 2004) found that vitamin D_2 and curcumin bound independently in the calyx site so that the principal binding

site is in the central calyx which is capable of accommodating quite sizeable molecules.

Encapsulation efficiency

The micro encapsulation efficiency (EE) of curcumin in WPI was shown in table 1. The encapsulation efficiency was between 99.99 and 98.32 %. The main reason for the ratio 5 mg/g encapsulation efficiency was the use pH 8, 9s prescribed by the results of FT-IR.The effect of pH on that, the binding of curcumin and the binding measurements with denatured β-lg indicated the lower binding affinity of curcumin to β-lg with increase in pH, pointing to the restriction in entry and binding of curcumin to the internal cavity of β-lg. Lys60 is in the near vicinity of the methoxy group of curcumin (n 17). Pro38 is in contact with the hydroxyl group of curcumin. (Sneharanie*t al*., 2010). In α-lactalbumin Trp-26 and Trp-60 are the most buried tryptophan residues in bovinelactalbumin (BLa) and Trp-104 is almost buried in the α-la domain.

Table 1 effect of pH on encapsulation efficiency curcumin with whey protein isolate

WPI- CCM	Ratio of CCM		
	1 mg/g	3mg/g	5mg/g
pH 4	99.997±0.26	99.985 ± 0.68	98.978 ± 0.01
pH 7	99.999 ± 0.03	99.977 ± 0.01	99.896 ±0.01
pH 8	99.278 ± 0.06	99.322 ± 0.16	98.799 ± 0.04
pH 9	98.617 ± 0.07	98.543 ± 0.02	98.788 ± 0.01

SWELLING

Figure 3 illustrates the swelling ratio of WPI-CCM micro particles (hydrogel) as affected by pH. The results showed that the lowest swelling ratio of those hydrogels gelled at a pH 3 and 5.2, which increased when pH increased to pH 8 and 10.

This is results promising especially with regard to the use of the whey protein isolate hydrogels as matrices for the pH-controlled release of compounds (Betz *et al.*, 2012).At all swelling medium pHs, there is a general trend that the higher the gelation pH. Because the structure of thermally-denatured protein gels depends on pH of the protein solution. When pH <pI, the extent of increase in swelling ratio (SR) at acidic swelling medium was very small, because there are very few amine groups exist at protein chains so that the positive charges are very limited. SR reached the minimum when swelling medium pH was close to the pI of the whey protein (=5.4). This is because the net charge of whey protein molecules is close to zero at pI, which means almost no electrostatic repulsion between chains in thermally denatured whey protein and minimum SR exists. On the other hand, when pH >pI, there are a lot of negatively-charged groups in the protein chains, so the gels would contain a lot of net charges when the swelling medium of high pH value is used, which results in increased equilibrium SR. The higher the pH, the more surface charges, the higher electrostatic repulsive force, and higher equilibrium SR(Gunasekaran*et al.*, 2007).

According to (Gunasekaran*et al.*, 2007) microparticles swelling is also governed by ionization of negatively-charged groups. When the swelling medium pH = 10, the number of negatively charged groups is the most, so the SR is the highest because of the strong electrostatic repulsion. When the swelling medium pH = 6.8, the protons from the swelling medium neutralizes most of the negatively-charged groups, so the SR is lower due to the

reduced electrostatic repulsion. When the swelling medium pH = 1.2, all negatively-charged groups are neutralized; instead, there would be some positive amine groups. Because the amine groups in the microparticles are fewer than the carboxyl groups, the net charges in this case are few, so that the SR is very low.

IN- VITRO DIGESTION

Table 2 In- Vitro release percent % of curcumin from WPI micro particles

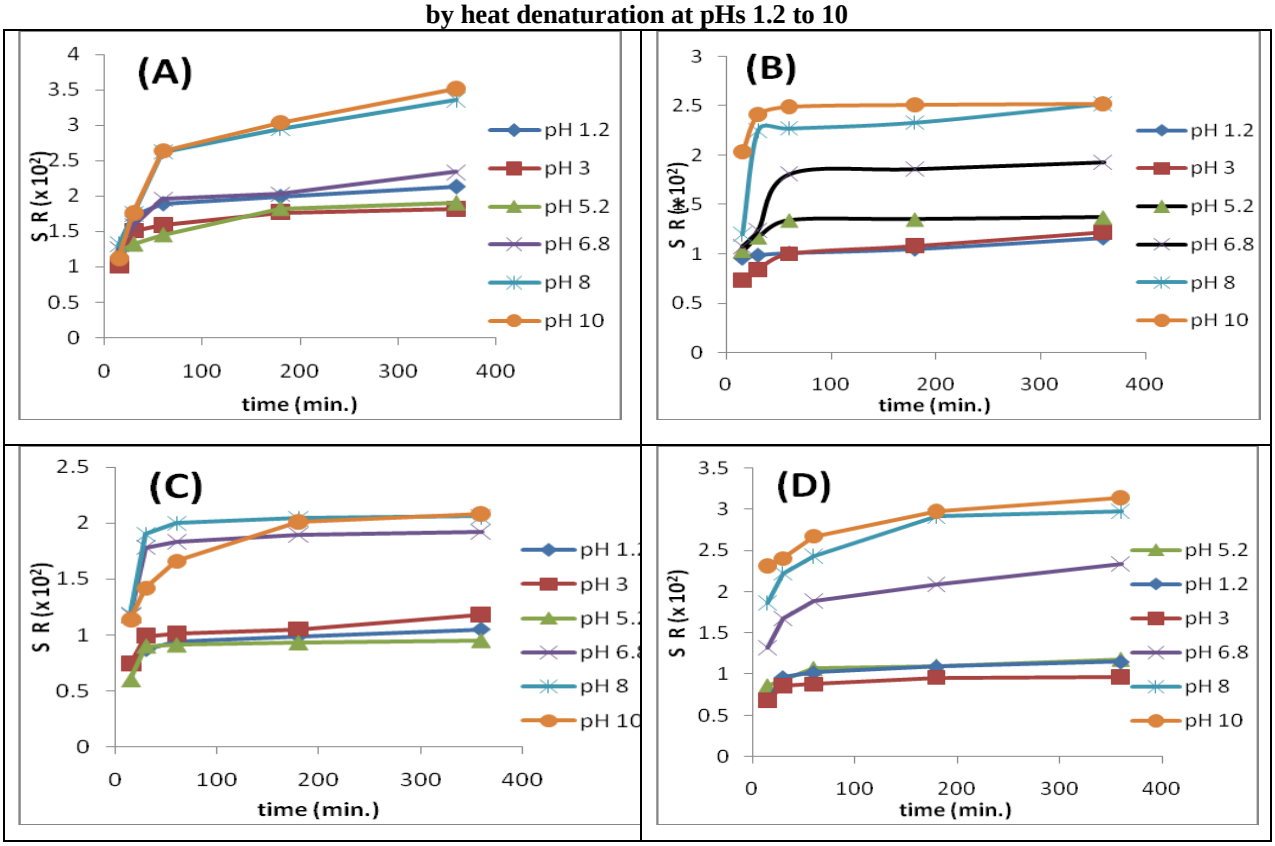
Sample	1 mg/g		3 mg/g		5 mg /g	
	SGF	SIF	SGF	SIF	SGF	SIF
pH4	0.74	49.76	0.81	50.29	0.85	50.39
pH7	0.71	51.90	0.79	53.34	0.80	54.3
pH8	0.68	52.38	0.75	53.95	0.75	55.53
pH9	0.60	53.76	0.65	55.12	0.71	56.72

In –vitro release of curcumin from WPI microparicles were shown in table 2. The results indicated that the release of

curcumin from WPI micro particles was checked under acidic conditions pH 2.0 in the SGF about 0.74% release of curcumin was observed, over a period of 12 h, from the nanoparticles. The concentration of protein and ligand affects the encapsulation efficiency as reported by (Somchue*et al.*, 2009). To prolong the release of curcumin in simulated intestinal conditions, coating with WPI was carried out as there was immediate release of curcumin in

simulated gastric conditions (Somchue*et al.*, 2009). Nearly 56% of curcumin is released from the WPI microparticles under neutral pH conditions. In the SGF after 12 hours only 0.74% of curcumin was released from the particles, which increased up to 50.39% in SIF after 12 hours from WPI-CCM pH4. However the maximum of release for produced micro particles at pH 9 then pH 8 the lowest value at pH4.

Figure 3 Swelling ratio at pH 4 (A), pH 7 (B), pH 8 (C), and pH 9 (D) of WPI-CCM complexes hydrogels prepared by heat denaturation at pHs 1.2 to 10



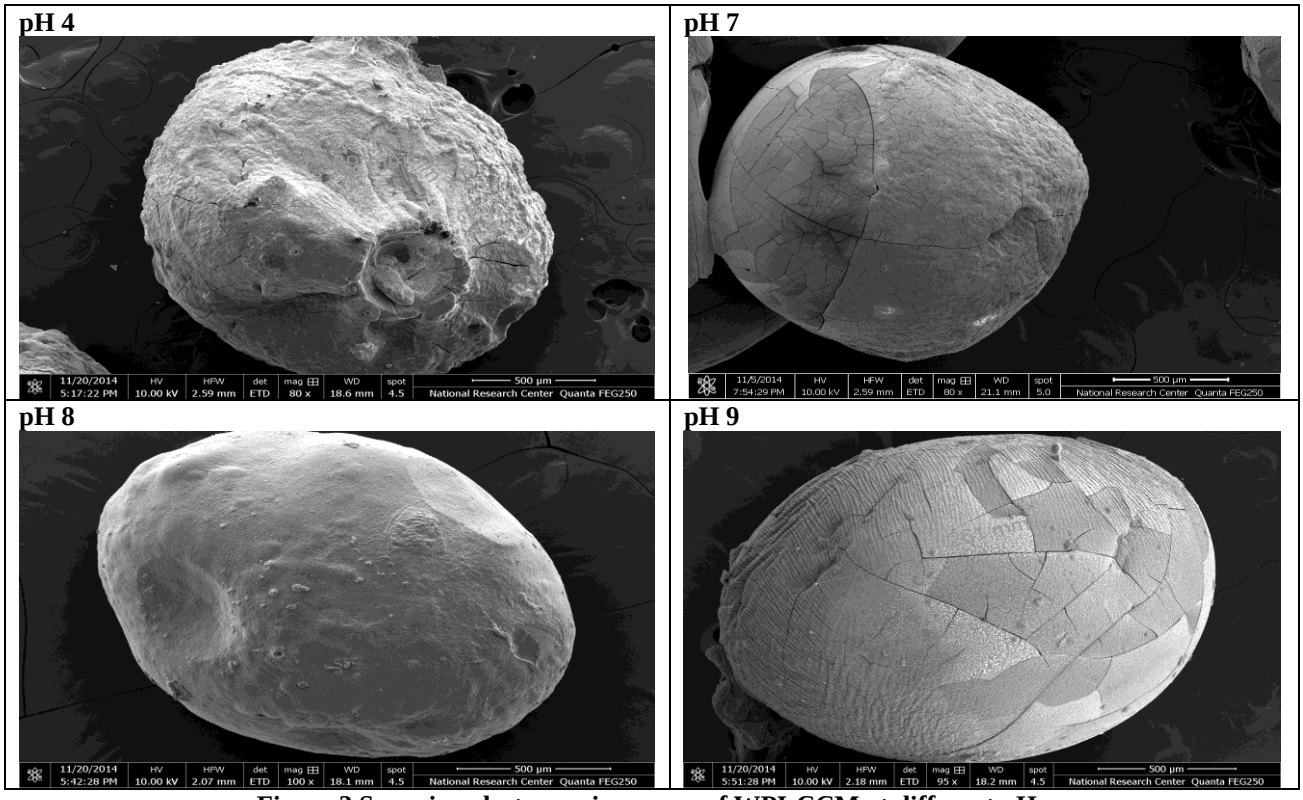


Figure 3 Scanning electron microscope of WPI-CCM at different pHs

WPI is proposed to be utilized for encapsulation as well as controlled release of bioactive compounds by preparing hydrogels or nanoparticles based on its superior gelling property and its ability to be a natural carrier of many biochemically important hydrophobic compounds.

MICROSTRUCTURE AND MORPHOLOGY OF MICROPARTICLES

Scanning electron microscopy micrographs of WPI- CCM micro particles at different pHs at pH 4, 7, 8 and 9 were shown effect of variation of pH on the surface morphology and size of the micro particles in Figure 3.Microparticles of WPI-CCM complex showed a rounded shape and wrinkled surface without fissures, cracks or disruptions at pH 7, 8 and 9. While at pH 4 the particulate structures reported for protein gels with a white opaque appearance (Clark *et al.*, 1981; Doi and Kitabatake, 1989; Stading *et al.*, 1993) the micro particles showed unround shape and surface not spherical may be effect the protein surface charge. Proteins are charged species and many of their functional properties depend on the level of charge and the electrostatic interaction caused as a result of this.The effect, therefore, of the pH on the charge in terms of z-potential of these systems is ofimportance and was investigated.

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