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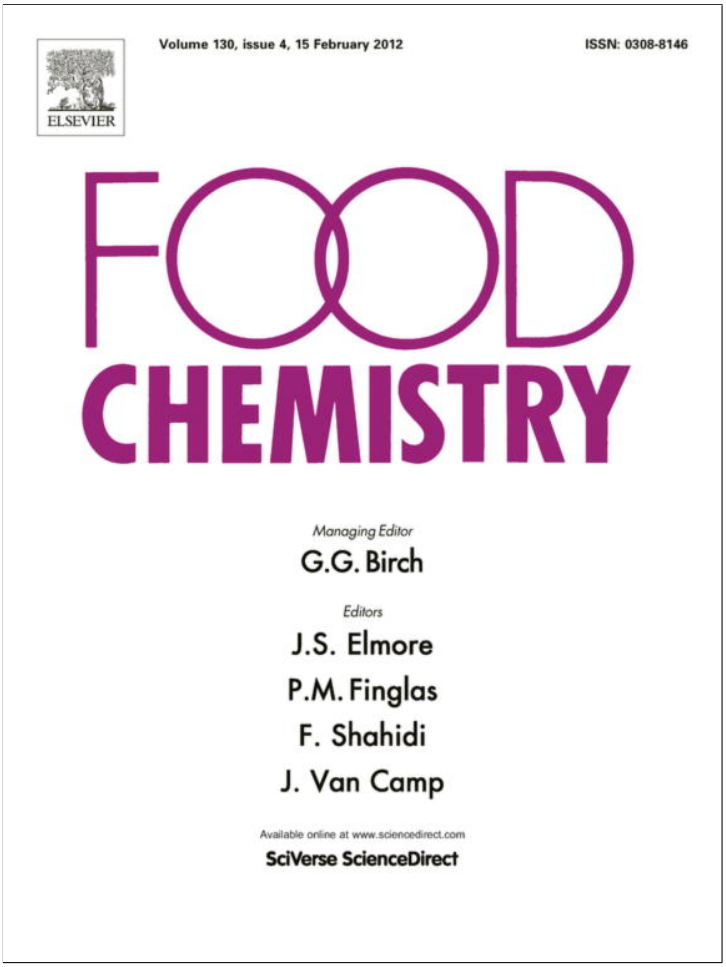

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Complexation of curcumin with soy protein isolate and its implications on solubility and stability of curcumin

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ABSTRACT

Curcumin, a natural polyphenolic food colourant, suffers a low bioavailability because of its low solubility and instability in aqueous solutions. Our study demonstrates that the food derived soy protein isolate (SPI) can form a complex with the curcumin. Fluorescence spectroscopy of the SPI–curcumin complex revealed that the complex is formed through hydrophobic interactions. Moreover, curcumin molecules quench the intrinsic fluorescence of SPI upon binding. Upon complexation, curcumin showed increased water solubility. Stability studies by UV spectroscopy showed that >80% of the curcumin was stable in the SPI–curcumin complex when dissolved in water, simulated gastric and intestinal fluids for 12 h, which would provide sufficient time for intestinal absorption. SPI–curcumin complex exhibits enhanced antioxidant activity and is capable of forming foam and emulsion, indicating its possible utilisation in food product formulation. This study suggests that SPI, being an edible protein, could be used as a material to encapsulate water-insoluble bioactive compounds in functional foods.

1. Introduction

Bioactive food components also known as neutraceuticals can be incorporated into food systems for the development of health promoting functional foods. The effectiveness of a functional food depends on preserving the activity of the bioactive molecule. Moreover, the activity depends on its solubility, stability, absorption and bioavailability. Interest in delivery systems for these neutraceutical products in so-called functional foods has been growing recently. The type of material used to encapsulate the active ingredient and the physicochemical properties of this material are the most critical determinants of the functionality of these food systems. The effectiveness of nutraceutical products in preventing diseases depends on preserving the bioavailability of the active ingredients. This represents a formidable challenge, given that only a small proportion of these remains bioavailable following oral administration, because of their low solubility as well as instability under conditions encountered during food processing or in the gut, which hence limit the activity and potential health benefits of nutraceutical molecules (Bell, 2001).

Curcumin (bis-α,β-unsaturated β-diketone), commonly called as diferuloylmethane, is a low-molecular-weight, natural polyphenolic compound found in the rhizome of turmeric (*Curcuma longa*). Curcumin is primarily used as a food colouring agent because of its strong yellowish colour. It has a wide range of pharmacological activities including anti-inflammatory, antioxidant, antiproliferative and antiangiogenic (Anand, Kunnumakkara, Newman, & Aggarwal, 2007) properties. The antirheumatic activity of curcumin has also been established in patients who showed significant improvement of symptoms after administration of curcumin (Deodhar, Sethi, & Srimal, 1980). The major problem with the limitation of applicability of curcumin as a health promoting agent is its extremely low solubility in aqueous solution and its poor bioavailability (Anand et al., 2007). Curcumin's low systemic bioavailability following oral dosing seems to limit the tissues that it can reach at efficacious concentrations to exert beneficial effects, the attainment of such levels in the gastrointestinal tract, particularly the colon and rectum, has been demonstrated in both animals and humans (Sharma, Gescher, & Steward, 2005). The absorption, biodistribution, metabolism and elimination studies of curcumin have unfortunately shown only poor absorption, rapid metabolism and elimination of curcumin as major reasons for poor bioavailability (Anand et al., 2007). To improve bioavailability of curcumin, numerous approaches have been used. Nanoparticle-, liposome-, micelle-, and phospholipid-complexes are the promising novel formulations, which appear to provide longer circulation, better permeability, and resistance to metabolic processes (Anand et al., 2007). Protection of the bioactive compounds against conditions encountered in food processing and in the gastrointestinal tract (pH, presence of enzymes and other nutrients) is of paramount importance (Bell, 2001). By encapsulation, a bioactive compound can be protected from environmental destructive factors, solubilised and delivered in a controlled manner. Many approaches like

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copolymer micelle, liposome, polymeric nanoparticle, lipid-based nanoparticle, and hydrogel based encapsulation have been made to increase aqueous solubility of curcumin (Abhishek, Naresh, Pranab, & Utpal, 2011; Bisht et al., 2007; Li, Ahmed, Mehta, & Kurzrock, 2007; Sou, Inenaga, Takeoka, & Tsuchida, 2008; Vemula, Li, & John, 2006). Vivek et al. (2010) demonstrated enhanced solubility of curcumin by complexation with cyclodextrin, exhibiting enhanced attributes compared to free curcumin for cellular uptake, as well as for anti-proliferative and anti-inflammatory activities.

In searching food-grade materials to form complexes with curcumin, we focused on soy protein isolate (SPI). SPI is a highly refined and concentrated protein fraction produced from soybean, an abundant, inexpensive, and renewable resource. Using ultracentrifugation, soy proteins have been classified into four fractions, namely 2S, 7S, 11S and 15S globulins. SPI is composed almost exclusively of two globular protein fractions called 7S (β-conglycinin) and 11S (glycinin). Soy proteins are used extensively in food manufacturing, because of their functional properties, low cost, availability and high nutritional value. Their ability to form cold set hydrogels has been widely studied as nutraceutical delivery devices (Maltais, Remondetto, & Subirade, 2009, 2010).

The aim of this study was to investigate the potential of SPI as a carrier for curcumin. An approach was made to enhance the water solubility and stability of curcumin by complexing it with SPI. The potential of SPI as a carrier for curcumin was investigated through interaction studies between SPI and curcumin by fluorescence spectroscopic techniques. The ability of SPI to enhance solubility and stability of curcumin justify the hypothesis that it is a good choice as a material for encapsulation of water insoluble curcumin.

2. Materials and methods

2.1. Materials

Curcumin was purchased from ICN biomedicals, Inc. (Aurora, Ohio). 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was from Sigma Aldrich Chemicals Co. (St. Louis, MO). All other chemicals were of analytical grade. All the solutions were freshly prepared. Curcumin stock solution was prepared in methanol. Absorbance measurements were made on a Shimadzu 1601 double beam spectrophotometer, using a 10 mm path length quartz cell.

2.2. Preparation of soy protein isolate

Soy protein isolate (SPI), was prepared at a pilot plant scale. All preparation procedures were performed at room temperature. Defatted soybean flour was dispersed in 10-fold its weight of distilled water, and the pH value of the slurry was adjusted to 8 with 2 M NaOH. This slurry was stirred for 1hr and then centrifuged to remove the insoluble material. The resulting supernatant was adjusted to pH 4.5 with 2 M HCl, and the insoluble fraction (acid-precipitated soy protein curd) was collected by centrifugation (8000 rpm, 20 min). The precipitate collected by centrifugation was dispersed into four times its weight of distilled water and neutralised to pH 7 with 2 M NaOH before freeze-drying (Lyodryer-LT5S, lyophilisation systems Inc., USA).

2.3. Complexation of curcumin with SPI

An excessive amount of curcumin was added to 5% (w/v) SPI solution in water and homogenised using a homogeniser for 10 min to disperse curcumin into the solution. This was then kept overnight on a magnetic stirrer at room temperature. The free curcumin was removed by centrifugation (8000 rpm, 20 min) and the supernatant was spray-dried to get the dry SPI–curcumin complex powder.

2.4. Stability measurements

To study the stability, SPI–curcumin complex was dissolved in water, simulated gastric and intestinal fluids. The stability of curcumin was calculated by measuring the absorbance at 425 nm at different time intervals. Simulated gastric and intestinal fluid was prepared without enzyme as followed by Maltais et al. (2009).

2.5. Fluorescence measurement

The binding of curcumin with SPI was quantified by fluorescence spectrophotometry. Steady state fluorescence measurements were carried out in a Shimadzu RF 5000 spectrofluorimeter. The fluorescence of curcumin was measured by fixing its concentration at 5 μM in a Tris–HCl buffer pH 7.4 and by varying the concentration of SPI from 0 to 5 mg/ml. The emission spectra were recorded from 450 to 700 nm with an excitation wavelength of 420 nm. The excitation and emission slit widths used were 2.5 and 5 nm respectively. SPI solutions without curcumin were used as controls for the fluorescence measurements. Protein intrinsic fluorescence was measured at a constant SPI concentration (0.5 mg/ml) in the presence of 0 to 10 μM curcumin concentration. Emission spectra were individually recorded from 300 to 450 nm at an excitation wavelength of 280 nm. In this case, free curcumin samples without SPI were used as controls, and fluorescence was similarly recorded. In both cases, the fluorescence spectra of the controls were subtracted from the respective spectra of the samples.

2.6. Foaming capacity

Foaming capacity of samples was determined according to the method of Sathe and Salunkhe (1981). Samples were whipped using a homogeniser (MICCRA, D-8, Milian Labware, Gahanna, OH, USA) for 1 min and the foam volume was measured immediately using a measuring cylinder.

2.7. Emulsion capacity

The emulsion capacity of samples was determined according to the method of Pearce and Kinsella (1978). Briefly, 3 ml protein solutions (1% w/v) in 50 mM Tris–HCl buffer (pH 8) were vortexed with 1 ml of refined groundnut oil for 1 min. Samples of 50 μl aliquots were withdrawn and dissolved in 5 ml of 0.1% (w/v) SDS. The absorbance measured immediately at 500 nm was used as an estimate of the emulsion capacity.

2.8. The antioxidant activity of the samples were measured by the following methods

2.8.1. DPPH radical scavenging activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was measured using the method of Yen and Wu (1999). Samples were dissolved in water to obtain a concentration of 0–10 mg/ml, before 0.5 ml of the sample solution was taken and added to 1 ml 0.2 mM DPPH, then mixed vigorously. After incubation for 30 min, the resulting solution was centrifuged at 8000 rpm for 10 min and the absorbance was measured at 517 nm using a UV-spectrophometer. The solution without any sample was considered as the control.

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.8.2. Reducing power

Reducing power was determined by the method of Oyaizu (1986). The sample solution (0.1 ml, 0–10 mg/ml) was mixed with 0.4 ml 0.2 M phosphate buffer (pH 6.6) and 0.5 ml 1% (w/v) potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. An aliquot (0.5 ml) of 10% (w/v) TCA was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer of solution (0.5 ml) was mixed with 0.5 ml of distilled water and 0.1 ml 0.1% (w/v) ferric chloride, before the absorbance was read at 700 nm. An increase in absorbance indicates increasing reducing power.

2.9. Statistical analysis

For all the measurements, a minimum of triplicates were taken for data analysis. Using the Origin 6.1 software, all of the values were plotted. Data were expressed as means ± standard deviations. One way analysis of variance (ANOVA) was employed to identify significant differences ($p < 0.05$) between data sets using software Origin 6.1.

3. Results and discussion

3.1. Complexation of curcumin with SPI

Formulation development for more efficient delivery of lipophilic compounds is often complicated by its solubility limitation. Solubility and stability are the two important aspects for curcumin formulation development. Curcumin is practically insoluble in water. The solubility of curcumin in aqueous solution in presence of various concentrations of SPI was studied (data not shown). The solubility of curcumin increases significantly with the increase in concentration of SPI which could be because of the soluble complex formation with the SPI.

SPI solution was prepared in water to encapsulate curcumin. After removal of free curcumin, SPI–curcumin solution was clear and yellowish, and was spray-dried for further analysis. The spray–drying process has been used for decades to encapsulate food ingredients such as flavours, lipids, and carotenoids during which the evaporation of solvent, that is most often water, is rapid and the entrapment of the compound of interest occurs quasi-instantaneously (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). The formation of microparticles of SPI–curcumin complex after spray drying was confirmed by SEM observation. The outer topography of the microparticles of SPI–curcumin complex is presented in Fig. 1A. The microparticles are spherical in shape with few dents on the outer surface. According to the scale bar of the SEM images, the particle size ranges between 2 and 10 μm.

The SPI–curcumin complex was extracted with methanol to quantify the amount of curcumin complexed. The concentration of curcumin was calculated using the calibration curve of curcumin methanol solution. It was estimated that the absolute concentration of curcumin was 4.46 μg/5 mg spray dried powder. The spray-dried powder was reconstituted with water which dissolved back to clear solution very quickly and easily, with no noticeable curcumin precipitates (Fig. 1B(a)). One percentage (w/v) SPI–curcumin complex in water would give 8.9 μg/ml solubilised curcumin, compared to solubility of 11 ng/ml (Hailong & Qingrong, 2010) of curcumin in water, thereby showing increased solubility of curcumin by 812-fold through the SPI–curcumin complex. The amount of curcumin solubilised would be directly proportional to the concentration of SPI–curcumin complex dissolved in water. Furthermore, the SPI–curcumin complex powder was completely reconstitutable in simulated gastric and intestinal fluids (Fig. 1B(b and c)). Our findings on successful curcumin complexation corroborate those from other authors. Complexing with various solubilising agents like curcumin–phospholipid complex, (Maiti, Mukherjee, Gantait, Saha, & Mukherjee, 2007) and curcumin–cyclodextrin complex (Tonnesen, Masson, & Loftsson, 2002) have been made to solubilise curcumin. Hailong and Qingrong (2010) reported enhanced 1670-fold water solubility of curcumin after encapsulation in hydophobically modified starch.

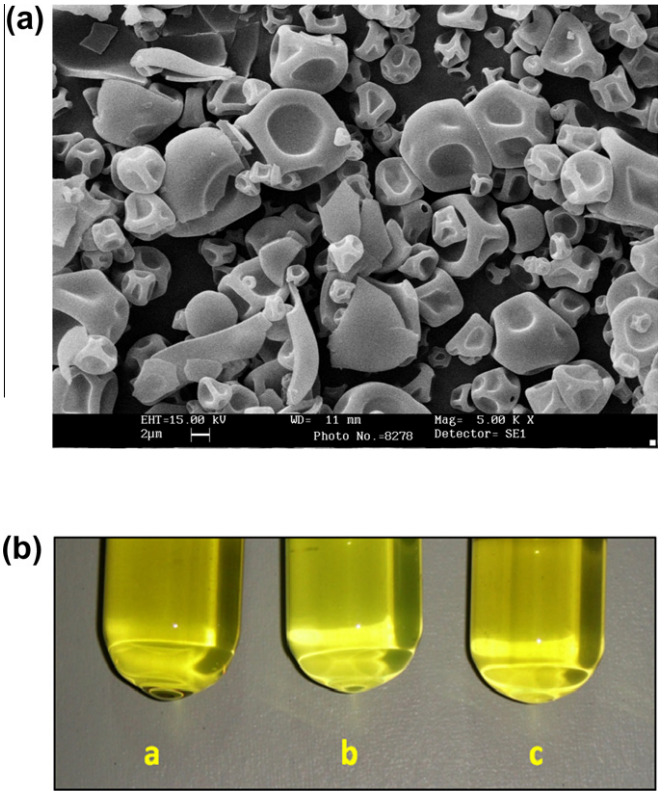

Fig. 1. (a) Scanning electron microscopic image of microparticles of the SPI–curcumin complex. The spray–dried SPI–curcumin complex was coated with gold before viewing under the scanning electron microscope. (b) Image of reconstituted SPI–curcumin powder in (a) water, (b) simulated gastric fluid and (c) simulated intestinal fluid without enzymes.

In addition to insolubility, the instability of curcumin at physiological pH poses another challenge. Wang et al. (1997) reported that curcumin was unstable at neutral and basic pH values and degraded by 90% within 30 min. In the present study, more than 80% of the curcumin was stable in the SPI–curcumin complex in water, simulated gastric and intestinal fluids after 12 h (Fig. 2). This stability would provide sufficient time for intestinal absorption or systemic circulation. Binding of curcumin to proteins may help in improving solubilisation and arresting the degradation. α_{s1}-Casein (Athahalli, Sridevi, & Appurao, 2009), β-lactoglobulin (Athahalli et al., 2010), bovine serum albumin (Barik, Priyadarsni, & Mohan, 2003) and human serum albumin (Leung & Kee, 2009) have also been reported to enhance the stability of curcumin in solution. The stability of curcumin after undergoing the spray–drying process and the property of complete reconstitution in aqueous solution has tremendous advantages for not only long-term storage but also for its applicability in oral administration. Chuan, Hung, Hsu, Ming, and Mei (2009) reported that encapsulation of curcumin in phospholipid based microemulsion prevented the degradation process of curcumin and also increased the concentration of curcumin in aqueous solution. Recently, Efstathia, Spyros, and Vaios (2011) demonstrated that *Saccharomyces cerevisiae*, β-cyclodextrin and modified starch can enhance curcumin solubility in simulated gastric fluid and limit its alkaline degradation in simulated pancreatic fluid. The solubility and stability demonstrated by the SPI-sol-

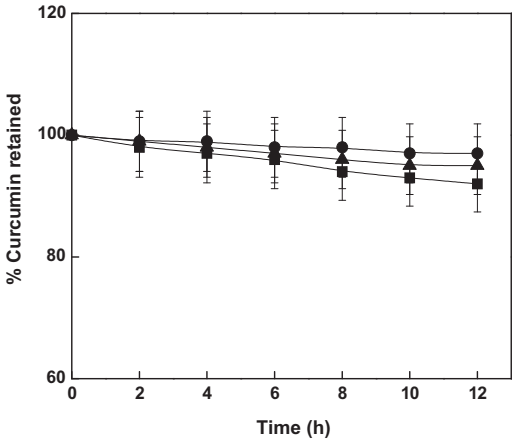

Fig. 2. Stability profile of SPI–curcumin complex as a function of time. The % curcumin retained was monitored by measuring the absorbance at 425 nm at different time intervals. The SPI–curcumin at concentration of 5 mg/ml was dissolved in water (▲), simulated gastric fluid (●) and simulated intestinal fluid (■) without enzymes. Values were expressed as the mean values ± SD and the error bars were the standard deviations of these values.

ubilised curcumin formulation, and the fact that both are food ingredients, would remain advantageous even if low intestinal absorption were to cause low systemic bioavailability.

3.2. Interaction between curcumin and SPI

To investigate the interaction between SPI and curcumin, fluorescence spectra were measured. Curcumin fluorescence in aqueous solution is very low. In our study, we found that when free curcumin was excited at 420 nm in the absence of SPI it showed a low-intensity broad fluorescence peak at around 540 nm in aqueous solution (Fig. 3A). Addition of small increments of SPI results in a sharper fluorescence peak with increased intensity and a blue shift in the emission maximum of curcumin. The fluorescence of curcumin is sensitive to the polarity of its surrounding environment. A shift in the emission maximum from longer to shorter wavelength and increased fluorescence intensity indicated the movement of curcumin from a polar to a less polar environment. This observation is in good agreement with the previous reports on binding of curcumin with proteins such as α_{S1}-casein, casein micelles and human serum albumin (Abhishek, Naresh, & Utpal, 2008; Athahalli et al., 2009; Leung & Kee, 2009). The authors suggest this blue shifting of the peak is due to the binding of the curcumin to the hydrophobic domain of the protein molecules. It is well documented in the literature that the SPI possess a high degree of hydrophobicity. Our observations suggest that curcumin molecules bind within the non-polar regions of SPI through hydrophobic interactions. Binding that is due to charge interactions can be ruled out as curcumin will be in its neutral form at pH 7.4.

The intrinsic fluorescence of proteins has been widely used to investigate the binding of drug molecules to proteins in solutions. At the excitation wavelength of 280 nm, both tryptophan and tyrosine residues have fluorescence emission. SPI shows strong fluorescence emission with a peak at around 345 nm upon excitation at 280 nm. Fig. 3B shows the fluorescence emission spectra of SPI suspension in the presence of different concentrations of curcumin. The intensity of the fluorescence emission of SPI at around 345 nm gradually decreased with an increase in the curcumin concentration. This indicates that it is therefore likely that the curcumin molecules can bind any or all of the Trp or Tyr residues. It is difficult to predict the exact position of binding of curcumin molecules as SPI consists of a mixture of proteins.

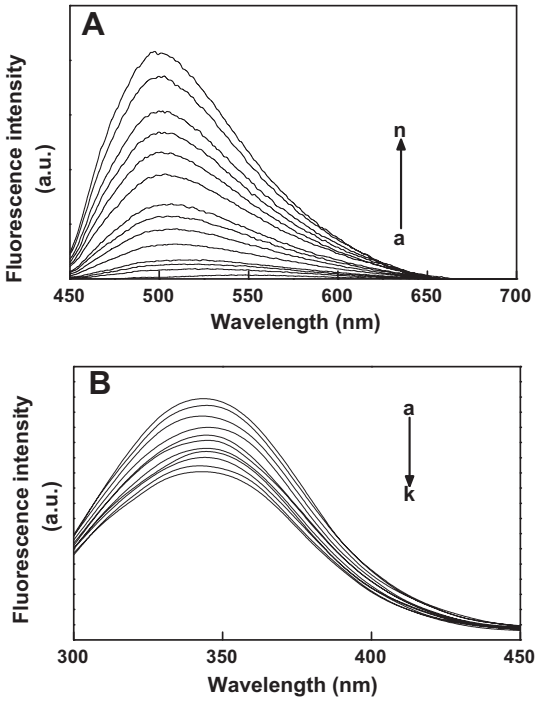

Fig. 3. (A) Fluorescence emission spectra of 5 μM curcumin in Tris–HCl buffer solution (pH 7.4) in the presence of SPI at different concentrations (a) 0, (b) 0.1, (c) 0.15, (d) 0.2, (e) 0.4, (f) 0.6, (g) 0.8, (h) 1, (i) 1.5, (j) 2, (k) 2.5, (l) 3, (m) 4, (n) 5 mg/ml. (B) Quenching of SPI intrinsic fluorescence by curcumin. Fluorescence emission spectra of the SPI suspension at an excitation wavelength of 280 nm in the presence of curcumin at different concentrations (a) 0, (b) 1, (c) 2, (d) 3, (e) 4, (f) 5, (g) 6, (h) 7, (i) 8, (j) 9, (k) 10 μM. Curcumin stock (1 mg/ml) was prepared in methanol.

The UV-absorbance maximum of curcumin is at 425 nm. To get the absorbance spectra of curcumin form the SPI–curcumin complex, we generated a difference spectrum by subtracting the SPI suspension spectrum from the SPI–curcumin complex spectrum. The difference spectrum revealed that the curcumin bound to SPI shows absorbance maxima at 426 nm (inset) (Fig. 4A). Fig. 4B shows the fluorescence emission spectrum of the SPI–curcumin complex and curcumin in water. Thus, an increase in the solubility of curcumin can be due to hydrophobic interactions with SPI. Therefore, curcumin which is poorly soluble in water, can be formulated by the use of SPI.

3.3. Antioxidant activity and functional property

The reaction between an antioxidant and DPPH radicals indicates the ability of the antioxidant to donate its hydrogen atom to O- and N-centred radicals, respectively. Fig. 5A outlines the percentage of DPPH radicals scavenged by the SPI–curcumin complex. When DPPH encounters a proton-donating substance, such as an antioxidant, the radical is scavenged and the absorbance is reduced (Shimada, Fujikawa, Yahara, & Nakamura, 1992). The reducing assay is used to identify the ability of the antioxidant to donate electron. The presence of antioxidants in the test sample will reduce the ferricyanide to its ferrous form. Fig. 5B shows the reducing power of the samples. The reducing power and the DPPH radical scavenging activities increase with an increase in concentration of the samples. The SPI, as such, exhibits antioxidant activity. The antioxidant activity is high in SPI–curcumin complex when compared to SPI. This increase in antioxidant activities can be attributed due to the curcumin present in the SPI–curcumin complex. Curcumin is a phenolic antioxidant. Its effectiveness as an antioxidant is mainly contributed by the phenolic hydroxyl group, which scavenges radicals by donating its H atom to radicals (Jian & Zai, 2009).

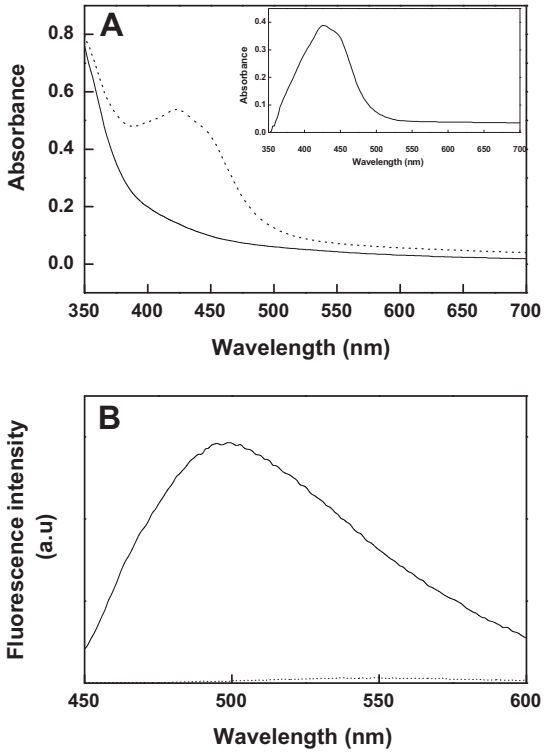

Fig. 4. (A) Absorption spectra of the SPI (––) suspension and the SPI–curcumin complex (···). The difference spectra (inset) revealed that the curcumin bound to SPI shows absorption maxima at 426 nm. (B) Comparison of the fluorescence emission spectra of curcumin (···) and the SPI–curcumin complex (––) dissolved in water. Curcumin stock (1 mg/ml) was prepared in methanol. The excitation wavelength was 420 nm, excitation and emission slit widths were 2.5 and 5 nm, respectively.

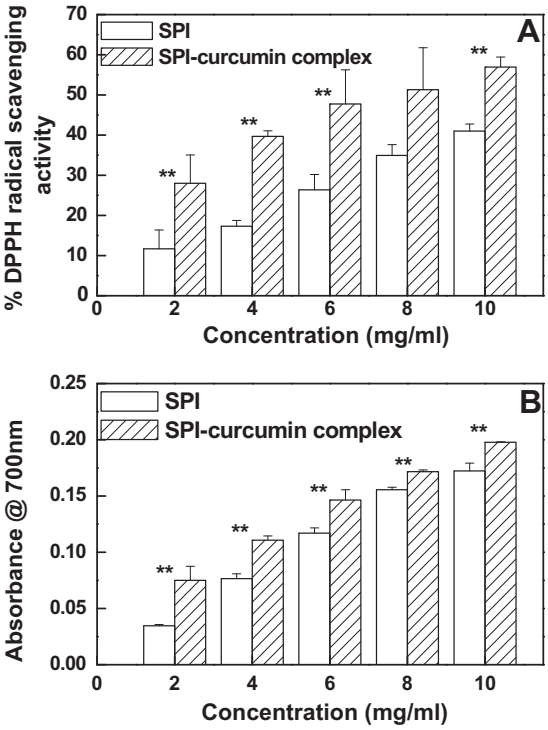

Fig. 5. Antioxidant activity profiles of SPI and SPI–curcumin complex at different concentrations. (A) % DPPH free radical scavenging activity. (B) Reducing power. Values were expressed as the mean values ± SD and the error bars were the standard deviations of these values. **Denotes that the differences are statistically significant.

Table 1
Functional properties of SPI and SPI–curcumin complex.

Concentration of protein (mg/ml)	Foaming capacity (ml)		Emulsion capacity (Absorbance at 500 nm)	
	SPI	SPI–curcumin complex	SPI	SPI–curcumin complex
5	36 ± 2	30 ± 2	0.82 ± 0.02	0.78 ± 0.02
10	38 ± 2	30 ± 2	0.89 ± 0.03	0.87 ± 0.02
15	38 ± 2	32 ± 2	0.97 ± 0.03	0.92 ± 0.02
20	38 ± 2	32 ± 2	1.07 ± 0.05	1.03 ± 0.08

Furthermore considering the effect of complexation of curcumin to SPI the changes in functional property were assayed. The objectives were to determine the emulsifying and foaming properties of SPI and SPI–curcumin complex and to determine the influence of complexation on the performance of the soy protein isolate. Table 1 presents the foaming and emulsion attributes of the samples. There was no major change in the functional properties of SPI after complexation with curcumin. Our results suggest that the SPI–curcumin complex was able to form foam and emulsion, thus indicating its possibility to be used in food as functional ingredients and its application in food product formulations. Soy proteins are used in foods not only as a substitute for animal derived proteins, but also as functional and nutritional ingredients (Rhee, 1994). The effective use of a protein in a food processing system is dependent on the protein's functional properties. Emulsification and foaming are two of the most important functionalities that proteins and other amphoteric molecules contribute to in the development of traditional or novel foods in order to enhance acceptability and increase usage for the formulation of foods. There is therefore a need to provide some basic information on their ability to act as emulsifiers and foaming agents.

4. Conclusion

In the present study, it was shown that SPI can form complexes with curcumin. It was observed that curcumin molecules interact with SPI by binding to the low-polarity regions of SPI. Upon complexation the water solubility of curcumin increased. The SPI–curcumin complex was reconstitutable in water, simulated gastric and intestinal fluids. Furthermore it was stable in all these aqueous solutions. The antioxidant activity of SPI increases after complexation with curcumin. The functional attributes suggests that SPI–curcumin is able to form foam and emulsion which is essential for food product formulations. This study suggests that SPI could be used as a material to encapsulate water-insoluble bioactive compounds in functional food.

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