

Effect of Cucumber Extract on Soybean Lipoxygenase Activity and the Functional Properties of Soybean Protein Concentrate

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Abstract

The inhibition effect on the soybean lipoxygenase of cucumber extracted by hexane, ethyl acetate, butyl alcohol and methyl alcohol was investigated, in order to determine the inhibitory activity of soybean protein concentrate. The hexane-soluble fraction exhibited superior inhibitory activity with inhibition percentage of 80.24% at concentration of 2 mg/ml. The soybean protein concentrate (HSPC) was prepared by washing the low-temperature defatted soybean flour (DSF) with the mixture of 65% ethanol (v/v) and various amount of hexane-soluble fraction. Then the residual activity of HSPC was measured and the minimum residual activity of HSPC (3.77 ± 0.69 U) was observed at the ratio of 0.3mg cucumber hexane-soluble fraction to 5g DSF. It was closed to that of commercial soybean protein concentrate (SPC). Solubility, emulsifying, foaming and oil absorption properties of HSPC were investigated. It was observed that when pH was less than 3 and more than 5, HSPC showed superior solubility compared with commercial SPC. Emulsifying properties of HSPC exhibited significant higher values of emulsion stability index and emulsifying activity index than those of commercial SPC. Foaming characteristics of HSPC exhibited similar foaming capacity and excellent foaming stability compared with commercial SPC. Oil absorption capacity of HSPC was higher than commercial SPC significantly.

Keywords: Lipoxygenase, Soybean protein concentrate, Functional properties

1. Introduction

Soy protein presents extraordinary nutritional and functional properties as compared to other animal and vegetal proteins. In particular, it has been demonstrated that this protein has certain medical applications (Emmert and Baker 1995), therefore the use of soy protein as functional ingredients in food products is gaining interest and demand (Tiziani and Vodovotz 2005). However, the soybean and green flavors of soybean formed during processing and cooking have hindered its wide utilization (Yukawa *et al.* 1992). The major contributors to these flavors are the volatile carbonyl compounds which are enzymatically derived from the hydroperoxides of unsaturated fatty acids by soybean lipoxygenase (Rackis *et al.* 1979). Lipoxygenase (linoleate: oxygen reductase; EC 1.13.11.12) is an enzyme that catalyzes the oxidation of linoleic acid and other polyunsaturated fatty acids containing a *cis,cis*-1,4-pentadiene moiety to hydroperoxy fatty acids by hydrogen abstraction from the methylene carbon and antarafacial insertion of molecular oxygen (Ivanov *et al.* 2010). These fatty acid hydroperoxides (HPODs) considered to be flavor precursors (Anderson 1992; Andreou and Feussner 2009), are subsequently converted by enzymatic cleavage into flavor compounds, including ketones, aldehydes and alcohols (Kermasha *et al.* 2002).

Heat treatment is the most widely utilized method for inactivating lipoxygenase. However, heat treatment produces a negative impact on foundation properties and induces dissociation, denaturation and aggregation of soy protein (Anderson 1992; Sorgentini *et al.* 1995).

Nonadienal and other volatile C9 compounds are characteristic compounds of cucumber fruits and seedlings. Their biosynthesis is proposed to take place by cleavage of 9-hydroperoxy-octadecadienoic acid which results from the action of lipoxygenase on linoleate (Feußner and Kindl 1992). But when we eat cucumber, could not feel the similar smell like the odour of soybean. Therefore, cucumber maybe contain inhibitory compound on lipoxygenase activity.

The objective of this work was to investigate possibility of the cucumber extract being a substitute for thermal treatment to inhibit lipoxygenase activity. Currently, the functional properties of HSPC were determined.

2. Materials and Methods

2.1 Materials

Soybean was purchased from market. SPC was provided from manufacturer of China. Hexane, ethyl acetate, butyl alcohol, methyl alcohol, ethyl alcohol, linoleic acid and tween 20 were purchased from Wako Pure Chemical, Osaka. Hexane, ethyl acetate, butyl alcohol, methyl alcohol, ethyl alcohol were of guaranteed reagent. All others reagents and chemicals were of analytical reagent.

2.2 Preparation of cucumber extracts

Dried cucumber flour was macerated with MeOH (1:10, w/v) at room temperature for 12 h with constant stirring. The process was repeated twice with fresh extractant. The resulting extracts were combined, filtered, concentrated in vacuo to get the crude extract. This extract was suspended in deionized water and successively partitioned with hexane, EtOAc, and BuOH to afford a hexane-soluble fraction, an EtOAc-soluble fraction and a BuOH-soluble fraction. The remaining aqueous phase was regarded as MeOH-soluble fraction. The

resulting extracts were combined, filtered, concentrated and lyophilized and stored at low temperature ($4 \pm 2^\circ\text{C}$) in dark for further use.

2.3 Preparation of low-temperature defatted soybean flour (DSF)

Preparation of low-temperature defatted soybean flour was performed according to a previously described procedure with some modifications (Kong *et al.* 2008). Soybean was cleaned and dried in an electric oven at 40°C for 12 h to the moisture content below 8%. The dried soybean was dehulled, ground to 60 mesh and defatted with hexane at room temperature and allowed to dry in a fume hood to prepare low-temperature defatted soybean flour.

2.4 Soybean lipoxygenase inhibition assay

In order to determine which fraction could effectively inhibit the lipoxygenase activity, the following experiments were performed.

2.4.1 Preparation of linoleic acid substrate

Linoleic acid substrate was prepared according to Yen *et al.* (Yen *et al.* 2003) with some modifications. Linoleic acid of 0.2 g and tween 20 of 0.2 g as emulsifier and 10 ml sodium borate buffer (0.2M, pH 9.0) were mixed. The mixture was homogenized by magnetic stirrer, added sufficient 1M NaOH to yield a clear solution, adjusting pH to 9.0 by 1M HCl and added sodium borate buffer (0.2M, pH 9.0) to 100ml.

2.4.2 Preparation of the crude soybean lipoxygenase

The crude soybean lipoxygenase was prepared according to the modified method of Hanqing (Hanqing 1996). DSF was blended with 200 volumes (w/v) of sodium phosphate buffer (0.01M, pH 7.0) in a beaker and homogenized by magnetic stirrer for 1h at room temperature. The mixture was centrifuged at 10000 g for 30 min. The supernatant was crude enzyme extract and diluted to one-third with sodium phosphate buffer (0.01M, pH 7.0) before use.

2.4.3 Preparation of samples of cucumber extracts

Sufficient extracts were dissolved by using 0.2% tween 20 sodium phosphate buffer (0.01M, pH 7.0) solution to 2 mg/ml, respectively. The extract solutions were then diluted to 0.67, 0.8, 1, 1.33, 2 mg/ml using 0.2% tween 20 sodium phosphate buffer solution, respectively.

2.5 *In vitro* lipoxygenase inhibition assay

Lipoxygenase activity was measured using a continuous spectrophotometric rate method based on the enzymatic oxidation of linoleic acid to the hydroperoxide of linoleic acid, following a modified procedure described by Indrawati *et al.* (Indrawati *et al.* 2000), with some modifications. 0.5 ml of fresh crude soybean lipoxygenase was incubated with 0.5 ml extract solutions of different concentrations (0.67, 0.8, 1, 1.33, 2 mg/ml) for 30 min at 25°C , respectively. Then 2.5 ml boric acid buffer (0.2 M, pH 9.0) and 0.3ml linoleic acid solution were added into a 10mm path length quartz cuvette. The reaction was initiated by the addition of 0.2 ml incubated crude soybean lipoxygenase and the conversion of linoleic acid to 13-hydroperoxylinoleic acid was measured immediately at 234 nm for 5 min at 25°C by a spectrophotometer (UV-3100PC, shimadzu). The control was prepared with 2.6 ml boric acid buffer, 0.3ml linoleic acid solution and 0.1 ml crude soybean lipoxygenase without incubating. The control was recalibrated before the extract was changed. The activity was determined by the slope of the linear portion of the absorbency curve. The inhibition ratio and IC_{50} of extracts were calculated. The IC_{50} is the concentration of extract which inhibited 50% activity of soybean lipoxygenase. The inhibition ratio of lipoxygenase activity by extracts was calculated as:

$$\text{inhibition}(\%) = \left[\frac{S_c - S_s}{S_s} \right] \quad (1)$$

where S_c and S_s were the slope of the reaction curve of the control and the sample, respectively. All results were an average of at least triplicate measurements.

2.6 Preparation of HSPC

2.6.1 Residual activity of soybean lipoxygenase of HSPC treated by various amount of hexane –soluble fraction

In order to obtain the minimum residual activity of soybean lipoxygenase, the relation between hexane –soluble fraction amount and residual activity of soybean lipoxygenase of HSPC was determined.

HSPC was prepared as follows: Low-temperature defatted soybean flour (5 g) was soaked in the mixture containing 65% aqueous alcohol (1:10, w/v) and hexane-soluble fraction (0.2, 0.3, 0.5, 0.8, 1.2 mg), respectively, for 1h at room temperature to dissolve non-protein components. The slurry was filtered and the resultant cake was washed for 10 min by 75% aqueous alcohol (1:5, w/v) and repeated twice. The cake was evaporated by forced air at room temperature in shallow pans under fume hood for 2h to pre-remove solvent, and then dried overnight in a forced air oven at 40°C. The dried HSPC was ground to pass 200 mesh.

The residual activities of soybean lipoxygenase of HSPC, commercial SPC and DSF were measured according to the method of above. The crude soybean lipoxygenase was extracted from HSPC, SPC and DSF. The reaction mixture contained 2.6 ml boric acid buffer (0.01M, pH 9.0), 0.3ml linoleic acid solution and 0.1 ml crude soybean lipoxygenase. In this experiment, the commercial SPC and DSF were used as controls to verify the inactivation effect of hexane-soluble fraction. One unit of lipoxygenase activity was defined as a change of 0.001 units of absorbance per minute per millilitre of enzyme extract. All measurements were an average of at least triplicate measurements.

2.6.2 Preparation of HSPC

HSPC used for experiments of functional properties was prepared as follow: according to Table 1, low-temperature defatted soybean flour (80g) was soaked in the mixture containing 65% aqueous alcohol (1:10, w/v) and 4.8 mg hexane-soluble fraction for 1h at room temperature to dissolve non-protein components. The slurry was filtered and the resultant cake was repeatedly washed for 10 min by 75% aqueous alcohol (1:5, w/v) and repeated twice. The cake was evaporated by forced air at room temperature in shallow pans under fume hood for 2h to pre-remove solvent, and then dried overnight in a forced-air oven at 40°C. The dried HSPC was ground to pass 200 mesh.

2.7 Functional properties

2.7.1 Protein solubility

The protein content was determined according to the method of Shi et al (Shi *et al.* 2011). SPC and HSPC were mixed with distilled water in the ratio of 1/100 (w/v), and then pH of the mixture was adjusted to 2.0, 3.0, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0 with 1 M NaOH or HCl, respectively. The suspensions were stirred at room temperature for 1 h, and centrifuged at 8000g for 15 min. 6 µl supernatant and 300 µl Coomassie Brilliant Blue (CBB) were added into 96-well plate separately, and then read the optical density (O.D.) of the mixture at 595 nm and the protein content was calculated with BSA solution as the standard. All measurements were an average of at least triplicate measurements. Protein solubility (%) was calculated as:

$$\text{Solubility(\%)} = \frac{\text{protein in the supernatant}}{\text{total protein}} \times 100 \quad (2)$$

2.7.2 Oil absorption capacity (OAC)

OAC was determined according to the method of Zheng et al (Zheng *et al.* 2008). Protein of one gram (W_0) was weighed into pre-weighed 15-ml centrifuge tubes and mixed with 10 ml (V_1) of soybean oil thoroughly using a vortex mixer. Samples were allowed to stand for 30 min. The protein-oil mixture was centrifuged at 3000 g for 20 min. The supernatant was poured into a 10 ml graduated cylinder, and the volume was recorded (V_2). All measurements were an average of at least triplicate measurements. OAC (ml of oil per gram of protein) was calculated as:

$$\text{OAC(ml/g)} = (V_1 - V_2) / W_0 \quad (3)$$

2.7.3 Emulsifying properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) of SPC and HSPC for the protein-stabilised emulsions were determined by the turbidimetric method (Wu and Wang 1998), with minor modifications. For emulsion formation, 6 ml of 0.1% soybean protein dispersion in sodium phosphate buffer (0.01M, pH 7.0) and 2 ml of soybean oil were homogenized by T 10 basic High-Speed Homogenizer (IKA Japan Y.K.) for 1 min at the maximum velocity (15000 rpm). An aliquot (50 µl) of emulsion was taken from the bottom of the homogenized emulsion, immediately (0 min) and 10 min after homogenization, and diluted (1:100, v/v) in 0.1% (w/v) sodium dodecyl sulphate (SDS) solution. After vortex mixing, the absorbance of diluted emulsions was read at 500 nm in the spectrophotometer. All measurements were an average of at least triplicate measurements. EAI and ESI

values were calculated by equation 4 and 5:

$$EAI(m^2/g) = 2T \left[\frac{(A_0 \times \text{dilution factor})}{(C \times \Phi \times 10,000)} \right] \quad (4)$$

where T was 2.303, C was weight of protein per unit volume (g/ml) of the protein aqueous phase before emulsion formation, Φ was oil volume fraction of the emulsion (0.25 in this case) and the dilution factor was 100. A_0 was the absorbance of the diluted emulsions at 0 min.

$$ESI(\text{min}) = \left(\frac{A_0}{\Delta A} \right) t \quad (5)$$

For the equation 5, A_0 was the absorbance of the diluted emulsion immediately after homogenisation. ΔA was the change in absorbance between 0 and 10 min ($A_0 - A_{10}$) and t was the time interval, 10 min in this case.

2.7.4 Foaming properties

Foaming capacity (FC) and foaming stability (FS) were determined according to the method of E.Chove et al (E. Chove *et al.* 2007) with a slight modification. Protein (1.5 g) was whipped with 50 ml (V_1) distilled water in a graduated measuring jug by a T 10 basic High-Speed Homogenizer (IKA Japan Y.K.) for 1 min at the maximum velocity (15000 rpm). Triplicate samples of the dispersions were blended and all measurements were an average of at least triplicate measurements. The volume of foam (V_F) was immediately recorded. FC was calculated using the following equation:

$$FC = V_F / V_1 \quad (6)$$

The remained volume of foam (V_F) was recorded after 2 h at 25°C, FS was calculated using the following equation:

$$FS = V_F / V_1 \quad (7)$$

2.8 Statistical analysis

Experiments were repeated three times. Experimental results were given as means \pm S.D. of three replicate measurements. Analysis of significant difference was performed by Excel of Microsoft.

3. Result and Discussion

3.1 The inhibition of cucumber extracts on lipoxygenase

The inhibition of soybean lipoxygenase activity by cucumber extracts were presented in Figure.1. The hexane-soluble fraction showed the highest inhibition ratio. The inhibition of hexane-soluble fraction exhibited concentration dependence and IC_{50} value of 1.08 ± 0.11 mg/ml was calculated. The inhibition of EtOAc-soluble fraction, BuOH-soluble fraction and MeOH-soluble fractions were exhibited very less inhibition on soybean lipoxygenase activity. Therefore, the hexane-soluble fraction was used as the inhibition the following experiments.

3.2 Residual activity of soybean lipoxygenase of HSPC

The residual activity of HSPC lipoxygenase values were showed in Table 1. The minimum residual activity of lipoxygenase of HSPC (3.77 ± 0.69 U) was observed at the ratio of 0.3 mg cucumber hexane-soluble fraction to 5 g DSF. It was not exhibited concentration dependence. It may be due to the HSPC was prepared by the mixture of alcohol and hexane-soluble fraction, therefore, alcohol induced protein aggregation and then influenced the inhibition of cucumber hexane-soluble fraction on soy lipoxygenase. Meanwhile, lipoxygenase activity of commercial SPC and DSF were determined and they were 1.55 ± 0.19 U and 1965.11 ± 21.74 U, respectively. The residual lipoxygenase activity of HSPC was obviously reduced by alcohol and cucumber hexane-soluble fraction compared with the residual lipoxygenase activity of DSF. Although the minimum residual activity of HSPC (3.77 ± 0.69 U) was higher than SPC (1.55 ± 0.19 U), the characteristic flavors of soybean could not be smelled out at the residual lipoxygenase activity of HSPC with 3.77 ± 0.19 U. Therefore, HSPC used in the following experiments was prepared according to the ratio of 0.3 mg cucumber hexane-soluble fraction to 5 g DSF.

3.3 Functionality of HSPC

3.3.1 Protein solubility

The solubility of HSPC was measured at various pH. Figure 2 showed that at the pH <3 or pH>6, HSPC showed superior solubility compared with commercial SPC and there was a significant difference. Protein solubility were 16.54%, 30.11%, 34.13% and 47.54%, comparison with commercial SPC 4.95%, 8.93%, 13.69% and 20.24% at pH 2, 7, 8 and 9, respectively. At the pH around isoelectric point pH (3.0, 4.0, 4.5, 5.0, 6.0), HSPC and commercial SPC showed similar low solubility. The protein solubility is influenced by the hydrophilicity/hydrophobicity balance, which depends on the amino acid composition, particularly at the protein surface. Higher solubility is related with the presence of a low number of hydrophobic residues, the elevated charge and the electrostatic repulsion and ionic hydration occurring at pH above and below the isoelectric pH. Moreover, the denaturation can alter the hydrophobicity/hydrophilicity ratio of the surface to increase hydrophobic surface was reported in Kilara and Moure (Kilara *et al.* 1986; Moure *et al.* 2006). Therefore the increase of protein solubility may be by the reason that the raw material of HSPC (low-temperature defatted soybean flour) weakened the influence of denaturation on protein solubility.

3.3.2 Oil absorption capacity (OAC)

Oil absorption values of HSPC and SPC were showed in Table 2. The oil absorption of HSPC exhibited significant difference at 1.18 ml/g compared with commercial SPC at 0.88 ml/g. Fat absorption capacity is the binding of fat by nonpolar side chains of proteins (Deshpande *et al.* 1982). Heat treatment made the protein denaturation tends to unfold the protein molecule to increase its effective hydrophobicity. Therefore it is considered that the SPC should show the better oil absorption capacity than HSPC. On the contrary, the HSPC exhibited superior oil absorption capacity compared with commercial SPC. It may be for the reason that cucumber hexane-soluble fraction could influence the structure of protein molecule to expose more hydrophobic group or could combine with protein molecules to increase the amount of hydrophobic group of protein molecule surface.

3.3.3 Foaming properties

Foaming properties is reflected by foaming capacity and foaming stability. The foaming characterizations of HSPC and commercial SPC were showed in Table 2. It was observed that HSPC was similar to FC at 86.67% and no significant difference from the commercial SPC at 88%. HSPC exhibited excellent FS at 73.67% and was different from commercial SPC at 47% significantly, after incubated 2 h. Foam capacity is determined by the ability of the protein to reduce the surface tension, the molecular flexibility and physico-chemical properties (hydrophobicity, net charge and charge distribution, hydrodynamic properties) (Moure *et al.* 2006). Heat treatment and alcohol washing can improve protein emulsifying and foaming properties (Morr 1990; Hua *et al.* 2005). Table 2 showed that hexane-soluble fraction could improve protein foaming capacity of HSPC. It may be because of increasing the amount of hydrophobic group of protein molecule surface by cucumber hexane-soluble fraction.

3.3.4 Emulsifying properties

Emulsifying activity index and emulsion stability index values of HSPC and SPC were showed in Table 2. It was observed that HSPC exhibited superior EAI at 102.68 m²/g compared with the commercial SPC at 96.72 m²/g. The HSPC also showed superior ESI at 34.44 per min compared with commercial SPC at 16.47 per min. The emulsifying properties of protein largely depend on a proper balance between hydrophilic and lipophile groups. Heat treatment made the protein denaturation tends to unfold the protein molecule to increase its effective hydrophobicity. On the contrary, HSPC which was not underwent heat treatment exhibited higher EAI and ESI than SPC. Thus it suggested that the hexane-soluble fraction could improve the emulsifying properties. It may be by the reason that cucumber hexane-soluble fraction could influence the structure of protein molecule to expose more hydrophobic group or could combine with protein molecules and thus increase the amount of hydrophobic groups of protein molecule surface.

4. Conclusions

The cucumber hexane-soluble fraction could inhibit the activity of lipoxygenase effectively and exhibited concentration dependence manner. At the ratio of 0.3mg hexane-soluble fraction to 5g DSF, the minimum residual lipoxygenase activity was observed. Comparing with commercial SPC, HSPC showed superior oil absorbent capacity, foaming property, emulsifying property and superior solubility at the pH <3 or pH>6, similar low solubility at the pH around isoelectric point pH. In brief, the addition of cucumber hexane-soluble fraction in the production process of SPC using low-temperature defatted soybean flour could improve

functional properties of SPC. The cucumber extract could be a substitute for normal thermal treatment to inhibit lipoxygenase activity. In the future, it will be focused on component analysis aiming at cucumber hexane-soluble fraction and the relationship between protein molecular structure and hexane-soluble fraction.

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Table 1. The residual activity of soybean lipoxygenase of HSPC

Sample	amount (mg)				
	0.2	0.3	0.5	0.8	1.2
HSPC	10.44 ± 0.19	3.77 ± 0.69	6.33 ± 0.33	18.00 ± 0.33	20.33 ± 0.88

One unit of lipoxygenase activity was defined as a change of 0.001 units of absorbance per minute per millilitre of enzyme extract.

SPC and DSF were controls.

Standard error of the mean of three assays.

Table 2. Compare of functional properties of HSPC and commercial SPC

Functional properties	HSPC	SPC
Solubility at pH 7.0 (%)	30.11±2.67 ^a	8.93±0.23 ^a
Oil absorption capacity (ml/g)	1.18±0.05 ^a	0.88±0.03 ^a
Emulsifying activity index (m ² /g)	102.68±2.2 ^a	96.72±0.59 ^a
Emulsifying stability index (min)	32.44±1.79 ^a	16.47±0.11 ^a
Foaming capacity (%)	86.67±3.06 ^c	88.00±2.00 ^b
Foaming stability (%)	73.67±3.79 ^a	47.33±2.31 ^a

Data was expressed as means (SD) of triplicate experiments.

a: p<0.01, b: p<0.05, c: no significant difference.

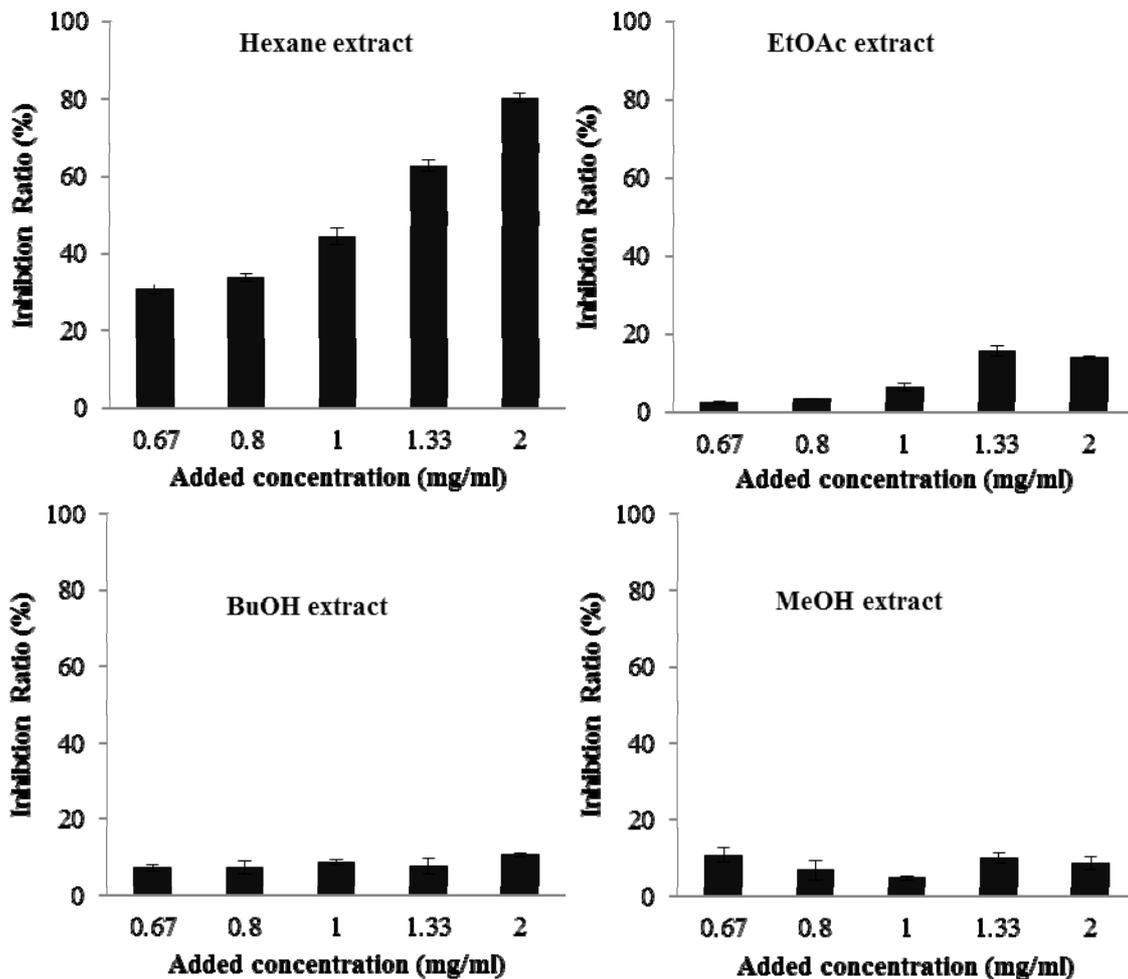


Figure 1. Inhibition of soybean lipoxygenase activity by hexane, EtOAc, BuOH, MeOH soluble fraction at various concentration (0.67, 0.8, 1.0, 1.33, 2.0 mg/ml).(mean ± SD, n = 3)

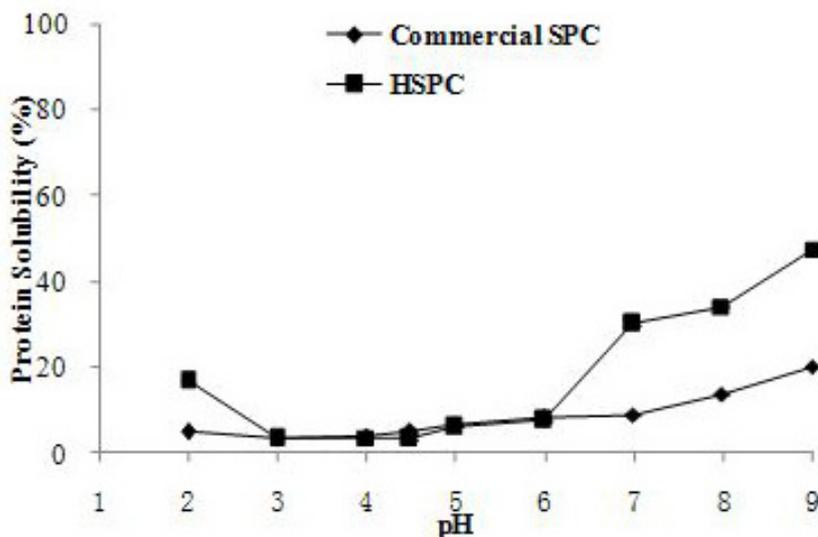


Figure 2. Solubility of HSPC and commercial SPC at different pH. Standard error of the mean of three assays