Ultrasonic-assisted extraction of protein from rapeseed (Brassica napus L.) meal: Optimization of extraction conditions and structural characteristics of the protein

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Abstract

Ultrasonic extraction efficiency to maximize yield of rapeseed meal protein (RSP) was tested by response surface methodology. Protein isolates were obtained by acid precipitation at pH 5.8 and 3.6, pooled and the pH adjusted to 7. Protein isolate structure was screened by measuring free sulfhydryl (SHF) and disulfide bond (SS) contents, hydrophobicity (S0), fluorescence intensity, and Fourier transform infrared (FTIR). A high correlation of quadratic polynomial mathematical model was obtained. The optimal extraction conditions of RSP were: pH 11.71, time 41.48 min, and ultrasound power 40%. Power intensity of 0.228 W/cm² was optimum for high yield of RSP. The yield of protein extracted increased by 43.3% and nitrogen re-solubility by 18.13% over the conventional extraction (control). Changes in amino acids after sonication were noticed. Compared with the control, sonication increased SS and decreased SHF, hydrophobicity, and fluorescence intensity, and changes in FTIR spectra were also found, suggesting protein unfolding and aggregation. Sonication of RSP improves yield and change conformation, providing a future functional protein in food and pharmaceutical systems.

Keywords

Sonication
Extraction efficiency
Rapeseed meal protein
Protein conformation
Nitrogen solubility

Introduction

As the world’s population increases rapidly and against the constraints of limiting land, water and food resources, particularly proteins. Efforts have been directed to develop low-cost protein foods of plant origin. Rapeseed (Brassica napus L.) is an oilseed (~ 40% oil) crop, ranking third with respect to oil production after palm and soybean (Rosillo-Calle et al., 2009). In recent years the annual output of rapeseed meal has exceeded 10 million tons in China, which is one of the largest producers of this crop (Canola Council of Canada 2009). The rapeseed meal, a byproduct of oil industry, is used for livestock feed and aquaculture industries (Seberry et al., 2008). It contains high protein, and well-balanced amino acids characterized by high lysine and sulfur-containing amino acids together with good functional properties (Pastuszewska et al., 2000). The protein content of defatted rapeseed meal is high (35 - 45%). The nutritive and functional properties of the rapeseed are characterized by two main protein families; cruciferin (12S globulin) and napin (2S albumin) (Bos et al., 2007). It is, thus, a kind of ideal and high quality protein resource, with good utilization value. Therefore, efforts have been made to develop efficient methods to prepare acceptable products from rapeseed meal for human benefit (Pan et al., 2011). Protein isolates, however, play role in improving food functional properties and their hydrolysates have pharmaceutical role as well. Alkaline extraction of rapeseed meal is the most commonly used procedure for preparation of protein isolates (Pastuszewska et al., 2000). Attention on plant protein isolates has been focused mainly on cotton seed, peanut, rapeseed, soy protein and sunflower seed and in some areas, commercial preparations are available (Yoshio-Stark et al., 2008).

Ultrasound-assisted technology is widely applied to improve and upgrade the functional and nutritional properties of food proteins. Ultrasonic disintegration, however, depends mainly on the input energy. However, sonication causes cavitation, which creates extremely high local temperatures, high sheer forces, and generation of free radicals. Hence, facilitates disintegration of particles, and destabilizes protein
conformation (Stathopulos et al., 2004; Khanal et al., 2007), which accelerates the chemical reactions and industrial processes.

Alkaline extraction of rapeseed meal, in order to get protein isolate, is the most commonly used procedure for protein extraction. In recent years, ultrafiltration is employed to obtain protein isolates from rapeseed meal (Dong et al., 2011). Sonication, however, improves the extraction efficiency of substances by increasing yield, and reducing processing time and solvent consumption. Moreover, ultrasonic-assisted extraction is done at a low temperature, preventing thermal damage to the extracts and loss of volatile components (Vilkhu et al., 2008). In order to optimize the extraction conditions, such as concentration of solvent, extraction time and ultrasonic power, response surface methodology (RSM) is widely used (Brachet et al., 1999). Limited information on ultrasound assist extraction of rapeseed proteins was established (Dong et al., 2011). Accordingly, this study aimed to assess the potentiality of sonication in enhancing release of protein from rapeseed meal and to monitor the effect of sonication on the structure of extracted protein.

Materials and Methods

Materials

The rapeseed meal was obtained from an oil factory (Danyang town, Jiangsu, China). The meal was cleaned, powdered, sieved to pass 80-mesh sieve and kept in plastic bags at room temperature, pending analysis. All reagents used in the experiments were of analytical grade.

Ultrasonic reactor

The ultrasonic reactor encompasses an ultrasonic generator (HT DTS211 GB/T17215-98), a hexagonal reaction vessel and a feed tank. The ultrasonic generator works with three operating frequencies (25 kHz, 28 kHz and 40 kHz) and different powers (from 10% to 100%), and supplied with pH and temperature gauges. The frequency of 28 KHz was used, without further study, in all sonication experiments. Solution was fed from the feed tank into the reaction vessel (Length of 11.2 cm, area of 325.89 cm$^2$, and a height of 93.5 cm) through an inlet pipe with aid of a suction pump. It equipped with a stirrer (JSCC automation Co. Ltd., Model 90YT 90G V22) for proper mixing of the solution. The triple frequency was set at three sides of the hexagon hosting multiple transducers (10, 10 and 12 on each side). The reaction vessel has a total capacity of 32 L and the reaction solution flows in a continuous loop mode at a rate of 32 L/min, providing pressure of 1250 Pa. The temperature of the system was uncontrollable. The system provided power intensities ranged from 0.057 to 0.573 W/cm$^2$, power densities ranged from 2 to 19 W/L for 32 L sample volume and energy dose ranged from 4.8 to 45.6 KJ/L (Table 3).

Ultrasound energy dose calculation

The ultrasound energy dose (energy density) was determined by the method of Karki et al. (2010) and calculated as:

$$Q = \frac{P}{V}$$

$$\text{Edensity} = Q \cdot t$$

where Q is the power density (W/mL), P is the ultrasound power (W), V is the volume of sample (mL), t is the sonication time (s), Edensity is the energy density (J/mL).

Ultrasound energy efficiency

Calorimetric methods are used to determine the energy efficiency of the ultrasound reactor (Gogate et al., 2001). The power dissipated in the liquid was calculated from the following equation:

$$\text{Power (W)} = m \cdot C_p \cdot \frac{dT}{dt}$$

where m is the mass of solvent (Kg), $C_p$ is the heat capacity of the solvent (J/kg K), $dT$ is the temperature difference between the initial temperature and the final temperature after a specific reaction time (K), and $dt$ is time (s).

Then energy efficiency was calculated as:

$$\text{Energy efficiency} = \frac{\text{Power dissipated in the liquid}}{\text{Power supplied to the system}}.$$

Ultrasound-assisted extraction (UAE) of rapeseed meal protein (RSP)

Single-factor experiments of the UAE of RSP were done to identify the effective independent variables in terms of maximizing the response product. Accordingly, pH ($X_1$), ultrasonic time ($X_2$, min) and power ($X_3$, %) were selected as the variables to be optimized. Extraction was done by dispersing the rapeseed meal in tap water at a solid-liquid ratio of 1:30 (Dong et al., 2011).

Response surface methodology

Box-Behnken design (Design expert software version 8.0.7.1) was used to optimize the effects of pH (10.5, 11.5 and 12.5), ultrasonic power (120, 240 and 360 W) and time (20, 40 and 60 min) on the UAE
of the RSP. The coded values of -1, 0, and 1 were used for low, medium and high actual values. The design included two blocks each with 17 combinations and five center points. Data were analyzed using least square methodology. The effect of $X_1$, $X_2$ and $X_3$ on response product was fit the second order polynomial model as given below:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j$$

where $Y$ is the predicted response variable and $\beta_0$, $\beta_i$, $\beta_{ij}$ are the regression coefficients of variables for intercept, linear, quadratic and interaction terms, respectively, and $X_i$ and $X_j$ are independent variables.

Rapeseed meal protein isolate (RSPI)

After optimization of the interactive variables, the RSP was extracted using the optimum conditions. The sonicated slurry was centrifuged at 5,000 x g for 10 min. The pH of supernatant was adjusted to 5.8 using HCl and centrifuged as before. The residue was washed 3 times with distilled water. The remained supernatant was subjected to acid precipitation at pH 3.6 and the obtained residue was treated as before. The two residues (RSPI) were pooled, the pH adjusted to 7 and then lyophilized (Tan et al., 2011). Protein isolate from traditional extraction was prepared to serve as a control. The protein isolates were kept in tight plastic bags and stored in a freezer, pending analysis.

Determination of protein content

Protein content of the RSP extract was measured by Bradford (1976) method and the protein content (N x 5.7, Mosse`, 1990) of RSPI by Kjeldahl method (AOAC, 2000). The protein content (g/Kg) and protein extraction efficiency (%) were calculated as:

$$\text{Protein} = \frac{\text{protein content (g) / Sample weight (g)}}{1,000}$$

$$\text{Protein efficiency} = \frac{\text{weight of protein in the supernatant (g) / weight of protein in rapeseed meal (g)}}{100}$$

Determination of amino acids (AAs)

The AAs contents of samples were measured using an automatic amino acids analyzer (S433D, Sykam, Eresing, Germany) after hydrolysis at 110°C for 24 h with 6 M hydrochloric acid in sealed glass tubes which were then filled with N₂. Tryptophan was determined after hydrolysis with 4 M LiOH. The AA/ninhydrin reaction products were detected at wavelengths of 570 nm and 440 nm. The amount of each AA was calculated from the area of standards obtained from the integrator.

Solubility determination

The RSPI powder was dispersed in distilled water (1% w/v) and pH was adjusted to 7.0 with 6 N NaOH. Suspensions were (25°C) centrifuged at 5,000 x g for 15 min. The supernatant was diluted in dissociating buffer (50 mM EDTA, 8 M urea, pH 10) to a ratio of 1:10 (v/v) and then absorbance was read at 280 nm (UV-vis spectrophotometer, 756 MC, Leng Guan). Solubility was obtained from the absorbance ratio of the supernatant and the suspension before centrifugation (Britten et al., 1994).

Measurement of intrinsic fluorescence spectrum

Intrinsic fluorescence spectra of a sample in phosphate buffer (0.01M, pH 7.0) was measured at room temperature (25°C) with fluorescence spectrophotometer (Cary Eclipse, Varian Inc., Palo Alto, USA) at 279 nm (excitation wavelength, slit = 5 nm), 300 - 500 nm (emission wavelength, slit = 5 nm) and 10 nm/s of scanning speed. The spectra were an average of ten scans. The phosphate buffer was used as a blank.

Determination of free sulfhydryl (SHF) and disulphide bond (SS) groups

The SHF groups were determined according to Beveridge et al. (1974). Fifty mg of the RSPI were dissolved in 5 mL of a buffer (0.086 M Tris, 0.09 M glycine, 0.004 M EDTA, and 8 M urea, pH 8.0). Forty microliliters of DTNB (5,5-dithiobis-2-nitrobenzoate, 4 mg/mL in methanol) was added to 1 mL aliquot diluted in 4 mL Tris-glycine buffer. For SS, to 1 mg of protein solution was added 4 mL of Tris-glycine buffer and 0.05 mL of 2-mercaptoethanol and the mixture was incubated for 1 h at 20°C.

After additional 1 h incubation with 10 mL of 12% trichloroacetic acid (TCA), the tubes were centrifuged at 5,000 x g for 10 min. The precipitate was twice resuspended in 5 ml of 12% TCA and centrifuged to remove 2-mercaptoethanol. The precipitate was dissolved in 10 ml of 8 M urea in Tris-glycine and the color was developed with 40 µL of DTNB.

After incubation for 15 min, absorbance at 412 nm was read. A molar extinction coefficient (ε) of 13,600/M cm was used. Protein concentration was determined according to Lowry et al. (1951) using bovine serum albumin as standard. The number of SHF was calculated according to the following equation:

$$\text{SH}_f = 73.53 \left[ \text{DO}_1 - \text{DO}_2 - \text{DO}_3 \right] / C$$
where $SH_0$ is the concentration of $SH_0$ in sample ($\mu$mol/g protein), $DO_1$ is the maximum absorbance at 412 nm of sample + DTNB, $DO_2$ is the absorbance at 412 nm of sample without Ellman’s reagent, $DO_3$ is the absorbance of DTNB at 412 nm in urea buffer at 8 M, and C is the sample protein concentration (mg/mL). The number 73.53 is the coefficient that includes $e (13,600 \text{ M}^{-1} \text{ cm}^{-1})$ and constants to express content of SHF or SS in $\mu$mol/g protein.

**Measurement of surface hydrophobicity ($S_0$)**

The $S_0$ of samples was determined according to Kato and Nakai (1980) with some modifications. RSPI solution (1 mg/mL in 0.01 M phosphate buffer, pH 7.0) was centrifuged at 5,000 x g for 15 min. Protein concentration in the supernatant was determined according to Lowry et al. (1951). The supernatant was serially diluted with the same buffer to obtain protein concentrations ranging from 0.1 to 0.0005 mg/mL. Then 60 µL of 1-anilino-8-naphthalene-sulfonate probe (8.0 mM in 0.01 M phosphate buffer, pH 7.0) was added to 3 mL of sample.

Relative fluorescence intensity (RFI) was measured at wavelengths of 388 nm (excitation) and 520 nm (emission) using fluorescence spectrophotometer (Cary Eclipse, Varian Inc., Palo Alto, USA) with a scanning speed of 500 nm/s and a constant excitation and emission slit band width of 5 nm. The slope of the normalized RFI (RFI sample + probe - RFI sample) at the maximum fluorescence intensity wavelength as a function of the protein concentration was used to calculate the protein surface hydrophobicity. The slope of the initial part of the curve was taken as the surface hydrophobicity index of the protein molecules.

**Fourier transform infrared (FTIR) spectroscopy**

The infrared spectra were recorded in absorbance mode from 4000 to 500 cm$^{-1}$ on a FTIR/Nicolet-AVATAR spectrometer (Thermo-Electron Corporation, Madison WI 5371, USA) equipped with a KBr beam splitter and 370 DTGS detector. Solid samples were prepared by grinding protein powder with KBr powder and pressed into a translucent pellet. The recording conditions for each FTIR spectrum were: 32 scans and a resolution of 4 cm$^{-1}$. Analysis of the infrared spectral data was done using OMNIC software.

**Statistical analysis**

Data were means ± SD. Data were subjected to analysis of variance using SPSS software (version 16) and means were separated by DMR test at p < 0.05.

**Results and Discussion**

**Optimization of UAE of RSP**

The protein content of rapeseed meal was 38.09%, wet basis. High levels of the extracted RSP were obtained at zero points of the design. The values of the coefficients in the second-order polynomial equation are presented in Table 1. The response product (Y) and the independent variables (X) are empirically related by the following second-order polynomial equation.

$$Y = -127.79884 + 22.066 X_1 + 0.010525 X_2 + 0.14003 X_3 - 0.95762 X_1^2 - 0.00160656 X_2^2 - 0.00122844 X_3^2 + 0.011312 X_1 X_2 - 0.0025 X_1 X_3 - 0.0002375 X_2 X_3$$

The plots of experimental values of extracted protein (mg/mL) versus those calculated from the above equation indicated a good fit (Data not shown). The statistical significance of polynomial equation was checked by F-test (Table 1). The R$^2$ value > 0.6219, indicating that the model equation was adequate for predicting the yield of RSP under any combination of the independent variables.

The coefficient of variation (CV) of the model was 6.68%; indicating that experiments were compared with good degree of precision. As a general rule, a model can be considered reasonably reproducible if its CV is less than 10% (Kongo-Dia-Moukala and Zhang, 2011). The model coefficients $X_1$, $X_1^2$, $X_2$, $X_1 X_2$, $X_1^3$, $X_2^3$, and $X_2^3$ with p < 0.05 were significant. The coefficient of multiple determination (R$^2$) of the model was 0.9084, implying that 90.84% of the variation in the RSP extracted was attributed to the independent variables (Table 1). Mirhosseini et al. (2009) reported that a model with R$^2$ value > 0.8 indicates a very high correlation. So far, the model developed in this study could adequately represent the real relationship among the parameters chosen.

**Analysis of the response surface**

The relationship between independent variables and dependent variable is interpreted from 3D response surfaces plots (data not shown). Results in the 3D plots showed that the UAE of RSP increased steadily with increasing pH and time until reaching its maximum level at pH 11.71 and time of 41.48 min. Thereafter the yield decreased with increasing the pH and time. However, the induced cavitation with time may rupture plant cells and reduce particle size,
which facilitate the mass transfer of the target solute (Vinatouru, 2001) and therefore the extraction of RSP increased with time. Similar finding was reported earlier (Karki et al., 2010). Nevertheless, solubility and permeability of solvents could also be limited with time due to release of insoluble and cytosolic substances (Zhao et al., 2007). The solute might be re-adsorbed on the plant particles, which negatively affects UAE efficiency (Dong et al., 2011).

The interaction of ultrasound power and pH followed the same trend as time did. The maximum RSP yield was obtained at the power of 41.08% and time of 41.48 min. The decrease in UAE efficiency of RSP at a power higher than 41.08% may be attributed to the increase in cavitation bubbles with increasing the ultrasound power; resulting in attenuated energy input that finally decrease the protein yield (Grönroos, 2010). Moreover, an increase in solvent temperature during sonication was not related proportionally with extracted protein (data not shown). This agreed with the findings reported in the literature (Vinatouru, 2001).

Optimum conditions and model verification
The optimum UAE conditions of RSP were pH 11.71, time 41.48 min, and power 41.08% (40% was used as optimum power because of the limitations in device settings). Suitability of the model equation to predicting the maximum protein content (4.52 mg/mL) was tested using the recommended optimum conditions. Accordingly, the experimental protein value (4.47 mg/mL) was found reasonably close to the predicted value (Table 1).

Ultrasonic energy requirement
Energy is a key determinant in selecting the ultrasound system. It is essential to correlate the protein release with energy input to achieve maximum recovery with minimum energy input (Karki et al., 2010). Power intensity, power density, and energy dose increased with increasing output power while energy efficiency decreased (Table 2). However, the UAE of RSP (i.e protein yield) is not directly proportional to the input power and energy efficiency as well; coinciding with the view of Karki et al. (2010). Ultrasound power densities of 0.057, 0.285 and 0.573 W/cm² resulted in protein yields of 4.13, 4.17 and 4.20 mg/mL, respectively. The maximum protein yield (4.47 mg/mL) was found at the power intensity of 0.228 W/cm² (Table 2). Accordingly, the optimum power intensity that maximizes the protein yield could be 0.228 W/cm².

The existence of optimum power intensity was explained by Gogate et al. (2001) who proclaimed that large numbers of gas bubbles in the solution exist at higher intensity which scatter the sound waves.
to the walls of the vessel or back to the transducer. Therefore, less energy remains in the liquid due to a very high number of cavities per unit volume or area; there is a likely coalescence of the cavities resulting in formation of a larger cavity that may finally decrease the cavitationial yield of the system.

**Rapeseed meal protein**

The UAE of RSP was 13.41% while the conventional extraction efficiency was 9.36%. Sonication, however, increased protein extraction efficiency by 43.3% over the conventional extraction (control). In spite of that the relatively lower power density and energy dose exerted by the ultrasound reactor (Table 2) could be responsible for the relatively low UAE of RSP. The decrease in the pH of sonicated solution from 11.71 (optimum pH) to pH 8 to 10 (isoelectric pH, Dong et al., 2011) with prolonging time (data not shown), may possibly resulted in partial deposition of the extracted protein. The protein content of the isolates from conventional extraction and ultrasound extraction of rapeseed meal protein (RSPI) were 770.1 and 845.9 g/Kg, respectively (Table 3). Dong et al. (2011) reported a value of 796.3 g/Kg for sonicated RSPI.

**Amino acids (AAs) composition**

In Table 3, Glu was the major amino acid (AA) in the RSPIs. Most of the indispensible AAs of the conventional (control) and sonicated protein isolates are comparable to the FAO reference protein (FAO/WHO/UNU, 2007). Trp was found the most limiting AA in both protein isolates. Except for His, Lys, and Trp, the all indispensible AAs of the sonicated RSPI were significantly (p < 0.05) different from those of the control.

**Nitrogen re-solubility**

The nitrogen re-solubility of control and sonicated RSPI were 30.88 and 36.48%, respectively (Table 3). The Improvement in solubility after sonication may be due to cavitations effects that disrupt protein bonds and reduce particle size. Which increases interaction between protein and water and/or enhances formation of soluble amyloid-ressemble aggregates (Stathopulos et al., 2004; Sahoo et al., 2009; Tang et al., 2009). The higher hydrophobicity (S0) and disulfide bond (SS) groups in the sonicated RSPI (Table 3) suggested formation of protein aggregates.

**Free sulfhydryl group (SHF) and disulfide bond (SS)**

In Table 3, the SHF and SS groups of the control RSPI were 0.83 and 9.88 µmol/g, respectively. Compared to the control, sonication significantly decreased (p < 0.05) the SHF group, and increased the SS group. Cavitation and mechanical forces generated by sonication might cause protein unfolding, and then aggregation (Stathopulos et al., 2004).

**Surface hydrophobicity (S0)**

Sonication of the RSPI reduced significantly (p < 0.05) the S0 by 6% compared to the control (Table 3). The lower S0, in the sonicated RSPI may be attributed to the decrease in number of exposed nonpolar amino acid residues due to changes in protein structure. Formation of protein aggregates in the sonicated RSPI due the increase in SS bond groups (Table 3) might be responsible for the decrease in S0. Reduction in S0 due to protein aggregation was reported in the literature (Tang et al., 2009).

**Fluorescence spectra**

Fluorescence spectroscopy is a technique used...
to follow tertiary structure transition in proteins. The fluorescence emission of the control and sonicated RSPI revealed maximum values at wavelengths of 332 nm and 330 nm (excitation wavelength of 279 nm), respectively. The sonicated RSPI showed significantly lower fluorescence emission intensity (39.54 a.u) compared with the control (171.34 a.u) (Table 3); suggesting aggregation of the unfolded rapeseed meal protein molecules (Li et al., 2007).

The decrease in fluorescence intensity may occur due to changes in polarity of the media in contact with Trp, promotion of turbidity effects by soluble protein aggregates and/or proximity between Trp and quenchers, such as disulphide bonds, hydrogen bonds or cations such as iron (Ma et al., 2011).

**FTIR spectra**

Polypeptides and proteins give rise to nine characteristic infra red (IR) absorption bands; namely amide A, B, I, II, III, IV, V VI and VII (Kong and Yu, 2007). In Fig. 1a and b, the two IR spectra of the sonicated RSPI and the control were similar but with different intensities of absorption. The spectra of control (Figure 1) and the sonicated RSPI (Figure 2) showed peaks at 3270 and 3192 cm$^{-1}$, respectively; corresponding mainly to stretching vibrations of the O-H groups of adsorbed water molecules (Gorinstein et al., 2001; Zhao et al., 2008) and/or to NH stretching of –CONH$_2$ group that may belongs to amide B bands. Another two peaks corresponding to hydrophobic interactions were found at 2927 and 2926 cm$^{-1}$ due to C–H stretching, as well as to absorption attributed to the –CH$_2$ alkyl chain.

The peak at 1654 cm$^{-1}$ found in the sonicated RSPI and the control agreed with amide I band region.

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Table 3. Protein content, nitrogen re-solubility, amino acids content, hydrophobicity ($S_0$), free sulphydryl (SHF) and disulfide bond (SS) groups and fluorescence emission intensity$^*$ of RSP isolate$^1$

<table>
<thead>
<tr>
<th>Protein isolate</th>
<th>Conventional extraction</th>
<th>Ultrasound extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein content (g/Kg)</td>
<td>770.1 ± 6.2</td>
<td>845.9 ± 4.6</td>
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<tr>
<td>N re-solubility (%)</td>
<td>30.88 ± 0.3</td>
<td>36.48 ± 0.8</td>
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<tr>
<td>SHF (μmol/g protein)</td>
<td>6.83 ± 0.05</td>
<td>0.30 ± 0.06</td>
</tr>
<tr>
<td>SS (μmol/g protein)</td>
<td>9.88 ± 0.84</td>
<td>16.13 ± 0.42</td>
</tr>
<tr>
<td>$S_0$</td>
<td>114.60</td>
<td>107.31</td>
</tr>
<tr>
<td>Fluorescence emission intensity (a.u)</td>
<td>171.34</td>
<td>39.54</td>
</tr>
</tbody>
</table>

Amino acid (g/Kg)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Essential</th>
<th></th>
<th>Non-essential</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Thr</td>
<td>41.4±1.3</td>
<td>47.4±2.0</td>
<td>45.4±2.0</td>
<td>11.0±0.5</td>
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<tr>
<td>Val</td>
<td>56.1±1.0</td>
<td>50.7±0.7</td>
<td>33.4±0.4</td>
<td>72.1±2.0</td>
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<tr>
<td>Met + Cys</td>
<td>81.4±0.8</td>
<td>89.5±1.1</td>
<td>45.4±1.3</td>
<td>11.0±0.4</td>
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<tr>
<td>Ile</td>
<td>45.4±0.5</td>
<td>41.4±2.0</td>
<td>32.0±0.6</td>
<td>72.1±2.0</td>
</tr>
<tr>
<td>Leu</td>
<td>86.8±2.1</td>
<td>78.6±1.1</td>
<td>45.4±1.0</td>
<td>11.0±0.4</td>
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<tr>
<td>Phe + Tyr</td>
<td>76.1±0.4</td>
<td>68.1±0.8</td>
<td>45.4±1.0</td>
<td>11.0±0.4</td>
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<tr>
<td>His</td>
<td>33.4±0.4</td>
<td>32.0±0.6</td>
<td>32.0±0.6</td>
<td>72.1±2.0</td>
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<tr>
<td>Lys</td>
<td>45.4±1.3</td>
<td>45.4±1.0</td>
<td>45.4±1.0</td>
<td>11.0±0.4</td>
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<tr>
<td>Arg</td>
<td>80.1±0.7</td>
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<td>Trp</td>
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<td>Ser</td>
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<td>Gln</td>
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<td>241.6±2.0</td>
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<td>56.1±0.3</td>
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<td>73.4±2.0</td>
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<tr>
<td>Ala</td>
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<td>73.4±2.0</td>
</tr>
<tr>
<td>Asp</td>
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<td>69.4±0.4</td>
<td>69.4±0.4</td>
<td>73.4±2.0</td>
</tr>
<tr>
<td>Pro</td>
<td>72.1±0.6</td>
<td>72.1±0.6</td>
<td>72.1±0.6</td>
<td>72.1±0.6</td>
</tr>
</tbody>
</table>

1Mean ± SD, n = 3. Within a column, means not sharing a same letter are significantly different according to DMRT at p < 0.05. Sonication conditions: reaction volume is 32 L, F 28 kHz, pH 11.71, time 41.48 min, power 40% and room temperature (~16°C).
Spectral region is sensitive to the protein secondary structural components, which is due entirely to the C=O stretch vibrations of the peptide linkages. The frequencies of the amide I band components are correlated closely to secondary structural elements of the proteins (Kong and Yu, 2007).

The peaks at 1541 and 1449 cm\(^{-1}\) found in both protein isolates, associated with amide II bands. The amide II band derives mainly from NH bending, the C-N stretching vibration, and weak contributions from C-C stretching and C=O in-plane bending (Kavanagh et al., 2000). Amide I and II vibrational bands are of practical use in the protein conformational studies (Kong and Yu, 2007). However, the differences in intensity of absorption revealed in the IR spectra of control and the sonicated RSPIs, especially in amide I region, suggest differences in the secondary structure of these proteins.

In this study, the developed RSM model adequately represents the real relationship among the parameters chosen and reflected the expected optimization. Hence, Optimum extraction conditions that maximize the yield of RSP were; pH of 11.71, time of 41.48 min, and ultrasound power of 40%.

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