Microwave-Assisted Phosphorylation of Soybean Protein Isolates and their Physicochemical Properties

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Abstract

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In order to improve the functional properties of soybean protein isolates (SPI), microwave-assisted phosphorylation (MAP) was applied. The result showed that after microwaving at 600 W for 3 min, the phosphorylation level of SPI reached 35.72 mg/g, emulsifying activity and stability were increased 2 times and 1.4 times, respectively, the solubility was increased by 26.0% and the apparent viscosity was decreased by 13.5%. The charge density, content of sulfhydryl groups, and surface hydrophobicity increased significantly. The infra-red spectroscopic analysis indicated PO_4^{3-} primary and lysine residues for phosphoric acid esterification. The change of amide bond I and fluorescence spectrum of variation suggested that the MAP made the secondary and tertiary structures of SPI into a compact conformation. Compared to the regular phosphorylation, the preparation time applied in MAP of SPI was much shorter. These results indicated that MAP can be used as an efficient method to improve the functional properties of SPI.

Keywords: soybean protein; sodium tripoly phosphate; microwave-phosphorylation; emulsifying properties; molecular characteristics

Soybean protein, due to its high nutritional value (Kinsella 1979), good functional properties, and low price, has become an important raw material in food processing industry. Although it has been widely used in the food field, researchers, seek methods to improve the functional properties due to the fact that they cannot satisfy the demands of modern food processing (Qi *et al.* 2006; Luan *et al.* 2007).

Soybean protein modification aims to enhance or improve the functional properties of the protein by means of changing the molecular structure. There are several methods such as physical (Wang *et al.* 2008), chemical (Sabina *et al.* 2010), enzymatic (Gan *et al.* 2008), and biological engineering modification (Herman *et al.* 2003) etc., to modify the functional properties of soybean protein isolates (SPI). Most of these are currently at the research stage, only some of them have been applied to large-scale production. Phosphorylation is an efficient way to improve the emulsifying activity of SPI, however, there are also obvious disadvantages such as the too long modification time, huge energy consumption, pretreatment of raw materials, and low reaction efficiency. Therefore, the main problem is to boost the reaction efficiency between protein and phosphorylation reagents which has become the focal point of international research.

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Based on its high efficiency and energy conservation, microwave technology has been extensively used in the food and chemical industries. As a new branch of science, microwave chemistry studies the stimulative and alterant actions of the microwave field on chemical reactions in chemical systems,which has currently become one of the leading-edge disciplines (Lidstrom *et al.* 2001). This paper aims to modify the SPI using the microwave technique (microwave-assisted phosphorylation, MAP) to improve the emulsifying property of SPI, and the emulsibility and molecular characteristics of both modified and unmodified SPI were studied.

MATERIAL AND METHODS

Material. SPI was prepared from soybean flakes produced by Harbin Hi-tech Soybean Food Co., Ltd. (Heilongjiang, China) through alkaline extraction followed by acidic precipitation following the method of Wagner *et al*. (2000). Corn oil was purchased in the market. Sodium tripoly phosphate (STP), ammonium molybdate (AM), 8-anilino-1-naphthalenesulfonate (ANS), potassium bromide, and 5, 5'-dithio-2-nitrobenzoic acid (DTNB) were purchased from Sigma Chemical Co. (Beijing, China). All the chemicals used in this research were of analytical grade.

Preparation of phosphorylated SPI. For common phosphorylation, SPI solution (6% w/v) was prepared and homogenised at 10 000 rpm for 2 min with a high speed homogeniser (FA25; Fluko Equipment Shanghai Co., Ltd., Shanghai, China). The pH of solution was adjusted to 8.0 with a few drops of 1.0 mol/l NaOH. The phosphorylation process was started by the addition of STP (8%, w/w on protein basis) and thorough mixing. The reaction mixture was kept at 40°C for 2.5 h and then dialysed for 12 hours. Phosphorylated SPI was obtained by freeze drying (Zhang *et al.* 2007).

For microwave-assisted phosphorylation (MAP), SPI solution (6%, w/v) was prepared and homogenised at 10 000 rpm for 2 minutes. The pH of the solution was adjusted to 8.0 with a few drops of 1.0 mol/l NaOH. STP was added and the solution was placed in the microwave oven, after which the modification was conducted at varying microwave power (300–750 W) and processing time (1–4 min). The sample was dialysed overnight, and the pH was adjusted to 7.0 when the reaction was terminated. Phosphorylated SPI was obtained via freeze drying (Hai & Chi 2009).

Determination of phosphorylation degree. The determination of the degree of phosphorylation was carried out in aliquots (5 ml) of the reaction mixture which were taken at the end of phosphorylation. After the removal of protein by precipitating with drops of 10% trichloroacetic acid (TCA), pyrophosphate in the supernatant was precipitated as zinc pyrophosphate by adding 2 ml of 1 mol/l zinc acetate at pH 3.8–3.9. Zinc pyrophosphate was then dissolved in ammonium buffer prior to the titration of zinc ions with EDTA disodium salt, using Solochrome Black T as indicator. This method is especially suitable for the determination of pyrophosphate in the presence of *ortho-* and *trimeta-*phosphates (Sung *et al.* 1983).

Determination of emulsibility. Phosphate buffer solution (0.2 mol/l pH7.0) was used to prepare SPI solution (1 mg/ml). 10 ml corn oil was added into 30 ml of SPI sample solution, the mixture was then homogenised at 25°C for 1 min at 10 000 rpm to form a homogeneous emulsified liquid. The average grain diameter of the emulsified liquid droplets was measured using Zeta Potential particle size analyser. Optic preferences were as follows: specific refraction of corn oil and water were 1.4673 and 1.33, respectively, the absorption parameter was 0.001. The average droplet diameter d_{32} was measured to represent the emulsifying activity as soon as the solution was prepared, then it was measured 1 h later and marked as d_{43}. The D-value Δd_{43} was used to represent the emulsifying stability d_{32} and d_{43} were calculated by the instrument according to the following formula where n_i means the amount of emulsified liquid droplets, d_i means droplets diameter

$$d_{32} = \sum_i n_i d_i^3 / \sum_i n_i d_i^2$$

$$d_{43} = \sum_i n_i d_i^4 / \sum_i n_i d_i^3$$

Determination of solubility. SPI sample was weighed to prepare 1% (w/v) protein solution. The solution was centrifuged for 10 min at 4000 rpm at pH 7.0. The supernate was collected and its protein content was determined by Coomassie Brilliant Blue method. Total protein content was determined by micro-Kjeldahl method. Dissolubility was indicated by the percentage of the supernatant protein from total protein.

Determination of apparent viscosity. 0.02 mol/l, pH 7.4 phosphate buffer was used to prepare 2% (w/v) SPI solution. A digital viscometer was used to determine the apparent viscosity at 25°C.

Determination of Zeta potential. The defined amount of NaCl (0.01 mol/l) was added into 0.02 mol/l, pH 7.4, Tris-HCl buffer. SPI full solution was added after solution degassing. 0.45 μm micro porous membrane was used for filtration before using Zeta Potential particle size analyser for determining zeta potential at 25°C.

Determination of surface sulfhydryl groups. 4 ml of 0.1 mol/l Tris-glycine buffer (pH 8.5, containing 0.01 mol/l EDTA) was added to 1 ml of 4% protein solution, 125 μl DTNB reagent (20 mg DTNB dissolved in 5 ml, 0.1 mol/l, pH 8 Tris-glysine buffer) was then added into the mixture after keeping it warm (at 40°C) for 30 minutes. The colour development proceeded at 25°C for 10 min before determining the OD value at 412 nm using the extinction coefficient of 13 600 l/(mol·cm) to calculate the content of sulfhydryl groups.

Determination of total sulfhydryl groups. Tris-glysine used contained 0.25% SDS, protein concentration was changed to 0.4%, the other factors were just the same as in the determination of surface sulfhydryl content.

Determination of surface hydrophobicity. 0.02 mol/l, pH 7.4, phosphate buffer was used to dilute the protein solution to 0.1–2 mg/ml. 20 μl ANS solution (using 0.02 mol/l, pH 7.4, phosphate buffer) was added into 4 ml of diluted samples of different concentrations as fluorescent probe and kept at room temperature for 1 hour. The fluorescence intensity of the samples was determined by fluorescence spectrophotometry at the excitation wavelength of 338 nm and emission wavelength of 496 nm. The slope of fluorescence intensity vs. protein concentration represented the surface hydrophobicity of the protein molecules.

Analysis of FTLR. Protein samples were taken and mixed with KBr, triturated and preformed, the whole band of (500–4 000 cm^{-1}) was scanned at 20°C in FTIR spectrometer. Infrared spectrogram of each sample was a superposition of 32 times scan. The spectrum from 1600 cm^{-1} to 1700 cm^{-1} was chosen and analysed with Peak Fit v 4.12 software. It was deconvoluted by Gaussian after correcting the baseline, then fitted by the second derivative. The proportion of the secondary structure in each section was calculated on the basis of the area of each subpeak.

Analysis of fluorescence spectra. The sample solutions were adjusted to 1% protein concentration (0.1 mol/l phosphate buffer, pH 7.4). The measurements were carried out in 1 cm quartz cuvette. The slit of excitation and emission was 5 nm, the excitation wavelength was 280 nm, the scanning wavelength range was 300–420 nm.

Data analysis. All of the experiments were performed in triplicate, one-way analysis of variance was analysed by SPSS v 17.0 (SPSS Inc., Chicago, USA), pair wise comparison of the means was analysed by Tukey procedure (P = 0.05).

RESULTS AND DISCUSSION

Microwave-assisted phosphorylation conditions

Different MAP conditions affect the reaction efficiency of SPI phosphorylation, while the phosphorylation degree is closely related to protein emulsibility (Hai & Chi 2009), so this article analysed the phosphorylation degree under the conditions of different microwave power and processing time. Figure 1 shows that at microwave power 300 W, the extension of the processing time resulted was escalating in the degree of SPI phosphorylation. However, it firstly increased and then decreased while the microwave power ranged from 450 W to 700 W. This indicated that the processing time should not be overlong with microwave power being relatively high. The microwave heating at 600 W for 3 min resulted in the phosphorylation degree increasing to 35.72 mg/g which was a little higher than with SPI made by common phosphorylation methods (35.58 mg/g),

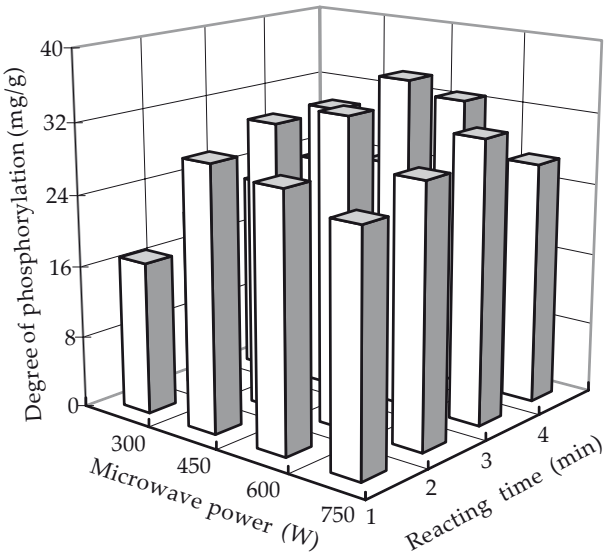

Figure 1. The effect of microwave treatment on degree of phosphorylation of SPI

but the reaction efficiency increased significantly. The sample of the highest degree of phosphorylation was chosen as the research object, and that was MAP-SPI (power 600 W, processing time 3 min). SPI, SPI heated by microwave at 600 W for 3 min, and common phosphorylation SPI were used as blank control group, microwave control group, and phosphorylation control group, respectively. The change of physicochemical characteristics of MAP-SPI would be deeply researched.

Analysis of emulsifying properties

Emulsifying properties of SPI are a very important feature in nature. During the process of emulsion formation, proteins are quickly adsorbed to the surface of oil droplets, thus reducing the surface tension, viscoelastic adsorbed membrane is formed around the oil droplets via intermolecular covalent interaction at the same time, which prevents the oil droplets from gathering and stabilises the emulsion (Dalgleish 1997). The droplets size directly reflects the emulsifying properties of the protein, which was evaluated by determining the average diameter of emulsion droplets d_{32} and d_{43} in this experiment. The smaller was the average particle size d_{32} of emulsion droplets, the better was the emulsifying activity of SPI. The smaller was the change of the average particle diameter d_{43} in a certain period of time, the more stable was the emulsion, that is, the higher emulsifying stability the protein possessed (Jafari *et al.* 2007).

Figure 2 shows that the average particle diameter of SPI emulsion droplets was 3.95 μm and it decreased to 3.63 μm after microwave processing. However, the average particle diameter of SPI modified by common phosphorylation and MAP decreased significantly ($P < 0.05$) to 2.12 μm and 1.91 μm, respectively. When the degrees of phosphorylation were almost the same, emulsifying activity of MAP-SPI was higher than that of common phosphorylation SPI, thus suggesting that the increase of the emulsifying activity of the product modified by MAP resulted from the combined reaction of phosphorylation and microwave treatments. The emulsifying stability was of importance in representing the emulsifying properties, this experiment analysed how the average particle diameter of the emulsion droplets d_{43} changed. The results in Figure 2 show that Δd_{43} of SPI emulsion heated by microwave treatment was maximum, which meant its stability being relatively poor. It might be due to the enhancing interaction between protein molecules after the treatment with microwaves, which made the protein molecules gather easily. However, Δd_{43} of SPI emulsion modified by common phosphorylation and MAP reduced significantly ($P < 0.05$), the latter being the minimum. This means the emulsion droplets changed relatively little, while the emulsifying stability was improved obviously. The MAP method was more efficient in improving the emulsifying activity of SPI compared with common phosphorylation.

Analysis of dissolubility and viscosity

The emulsifying property of SPI does not exist separately, it is closely related to other functional properties, especially dissolubility and viscosity. Preferable dissolubility and appropriate apparent viscosity are the prerequisite for a good emulsifying property. Therefore, these two important properties were analysed in this experiment. The results are shown in Figure 3.

It could be seen that the solubility of SPI treated by microwave obviously did not change ($P > 0.05$).

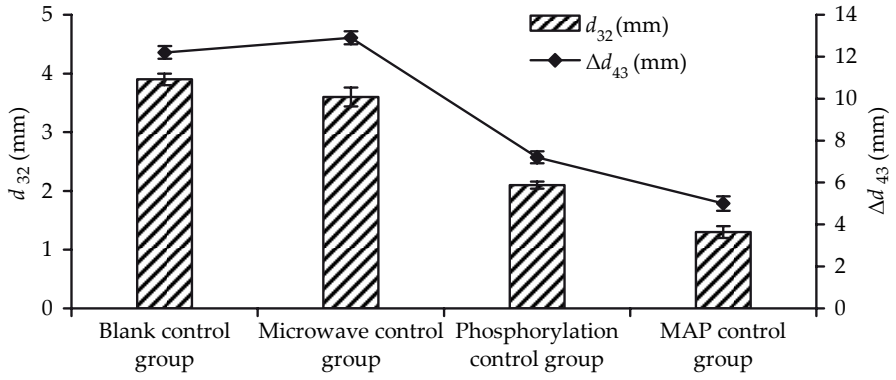

Figure 2. Effect of phosphorylation methods on emulsifying properties of SPI

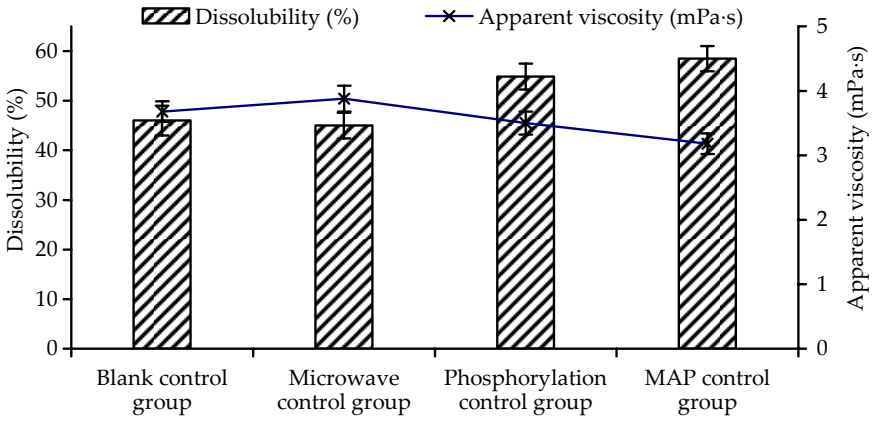

Figure 3. Effect of phosphorylation methods on solubility and apparent viscosity of SPI

However, the solubility of SPI modified by both phosphorylation and MAP changed significantly ($P < 0.05$). This may have been caused by a certain amount of phosphate groups attaching to the phosphorylated protein, and these phosphate groups could form a large number of hydrogen bonds with water molecules; the phosphate groups could not only increase the electronegativity of the protein system but also enhance the protein molecular electrostatic repulsion, which made it easier for the protein molecules to disperse in solution. As a result, the solubility of the phosphorylated protein improved.

The change of apparent viscosity reflected the change of protein intermolecular forces. The more powerful the molecular attractive force, the stronger the molecular friction and, consequently, the higher the apparent viscosity of the protein (Schreiber 2002). Figure 3 reflects the improved viscosity of SPI treated with microwave, which may have been caused by the stretch of the protein molecular structure resulting from the heating effect of the microwave treatment, which enhanced the intermolecular and frictional forces of the protein, and thus the apparent viscosity improved. However, the apparent viscosity of SPI modified by phosphorylation and MAP decreased to different degrees. The attaching of PO_4^{3-} groups may change the protein molecular structure and surface charge, thereby changing the hydration and interaction of the protein.

Analysis of molecular characteristics

The emulsifying properties of SPI are influenced by multiple internal factors, including mainly several molecular characteristics such as structure, hydrophobicity, flexibility, charge density, and so on. We analysed the Zeta potential, content of sulfhydryl groups, and surface hydrophobicity, the results are shown in Table 1.

Zeta potential can indicate the change of the surface charge density of the protein. Since isoelectric point of SPI (pH 4.5) was lower than that of the buffer solution (pH 7.4), SPI was electronegative (Malhotra & Coupland 2004). As shown in Table 1, there was no significant difference in Zeta potential between blank control group and microwave control group ($P > 0.05$), which means that the surface charge density of SPI did not change during the microwave treatment. Compared with the blank control group, Zeta potential of SPI modified by phosphorylation and MAP obviously decreased ($P < 0.05$), but the difference was not

Table 1. Effect of phosphorylation methods on molecular characteristics of SPI

	Zeta potential (mV)	Surface sulfhydryl groups (μmol/g)	Total sulfhydryl groups (μmol/g)	Surface hydrophobicity
Blank control group	−42.25 ± 0.49^a	6.22 ± 0.08^a	47.99 ± 0.23^a	38.78 ± 0.65^a
Microwave control group	−43.00 ± 0.99^a	7.41 ± 0.08^b	53.92 ± 1.06^b	45.72 ± 0.07^b
Phosphorylation control group	−63.10 ± 0.42^b	8.45 ± 0.16^c	57.01 ± 0.66^c	54.33 ± 0.55^c
MAP control group	−60.85 ± 0.92^b	8.68 ± 0.30^c	58.02 ± 0.58^c	60.85 ± 0.90^d

$^{a–d}$means with different letters within the same column are significantly different ($P < 0.05$)

obvious between themselves. During the process of phosphorylation, STP reacted with the ε-amino of lysine, which reduced the surface positive charge of the protein, on the other hand the negative charge increased owing to the attaching of PO_4^{3-} groups. As a result, the surface charge density of the protein changed, electronegativity, dissolubility, and dispersibility increased, all being the basis for the improvement of the emulsifying properties.

Non-protein sulfhydryl (-SH) and disulfide bonds (-S-S) are important functional groups of SPI. Some processing methods such as heating, high pressure, oxidation and reduction, etc., may lead to the change of sulfhydryl groups and disruption of disulfide bonds. This greatly affects the functional properties of proteins (Damodaran & Anand 1997). The changes in the content of sulfhydryl groups of SPI modified by microwave, phosphorylation, and MAP, respectively, are shown in Table 1. It appears that contents of surface sulfhydryl groups and total sulfhydryl groups of SPI modified by the three methods increased to different degrees, which indicates that a certain amount of disulfide bond were broken and exposed during the protein processing, which led to an increase of molecular extension, the expansion of molecular contact area in oil/water interface, and improvement of emulsifying properties.

Hydrophobicity is one of the important properties that influences physicochemical and functional characteristics of proteins (Chuan & Yan 2007). ANS was adopted as an fluorescent probe to determine the hydrophobicity of SPI in this experiment. The results are shown in Table 1. Compared with the blank control group, hydrophobicity of the microwave control group, phosphorylation control group, and MAP control group improved 1.17, 1.40, and 1.57 times, respectively, meaning that the internal hydrophobic groups of the protein were exposed, thus contributing to the molecular orientation arrangement in oil/water interface and improving the emulsifying properties.

The analysis of molecular characteristics of SPI processed by different methods is presented in this article, these important molecular characteristics were all based on the characterisation of some specific groups, to some extent explaining the molecular mechanism by which MAP could improve the emulsifying properties of SPI. The following experiment would study the changes in modified protein molecular space structure via spectral analysis.

Infrared spectroscopic analysis

Infrared spectroscopic analysis is a very useful method to observe the changes in protein molecular space structure after phosphorylation. Four samples of infrared spectrum are shown in Figure 4.

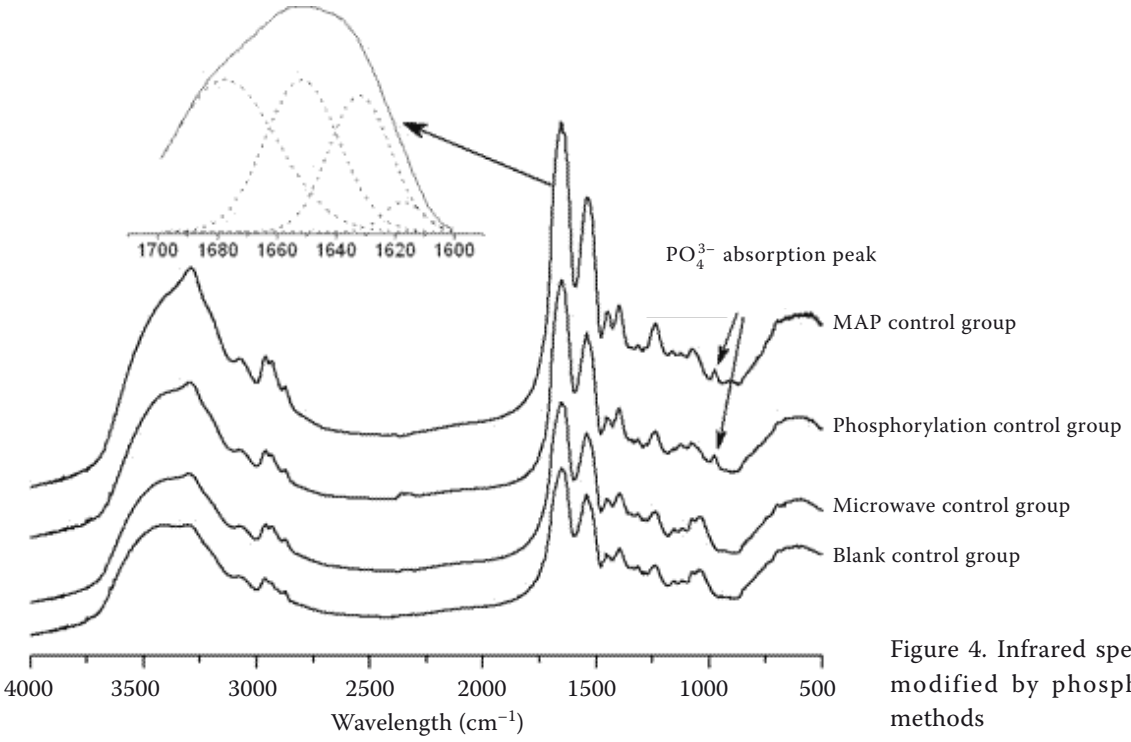

Figure 4. Infrared spectra of SPI modified by phosphorylation methods

Compared with the blank control group, the peak shape and peak position of infrared spectrum of SPI treated by microwave changed slightly. However, only some specific absorption peaks of the samples modified by phosphorylation and MAP changed which made it clear that phosphorylation was only effective with some particular amino acid residues and ineffective with the most. The sample modified by phosphorylation had a distinctive peak at the wavelength of 984 cm^{-1} while that modified by MAP at the wavelength of 978 cm^{-1}. However, the absorption peak of PO_4^{3-} was situated between 920 cm^{-1} and 1100 $cm^{-1.}$ Therefore, it can be inferred that the peaks at 984 cm^{-1} and 978 cm^{-1} were generated by PO_4^{3-} (Matheis & Whitaker 2007). The peaks of the other two samples were sharp between 3300 cm^{-1} and 3400 cm^{-1}, the reason may be that the stretching vibration of N-H was at the wavelength of 3300 cm^{-1} to 3500 cm^{-1}, the primary amine (-NH_2), which had two absorption peaks, formed the secondary amine (-NH-) after reacting with STP; moreover, the secondary amine had only one absorption peak at the wavelength ranging from 3310 cm^{-1} to 3350 cm^{-1} (Meng *et al.* 2003), thus forming the peak. As confirmed by infrared spectra, a large number of PO_4^{3-} groups were attached during the process of SPI modification by STP, thus free amino groups decreased while protein molecular net negative charge increased. The reaction mechanism was the esterification of amidophosphoric acid of lysine residues. This corresponds with the research of Hirotsuka *et al.* (1984).

Infrared spectrum is the most frequently-used method of analysis which determines the secondary structure of proteins. It is sensitive to changes of the peptide chain structure (Zhao *et al.* 2008). During the research of protein secondary structure, spectral peaks in amide band I (1600–1700cm^{-1}) were in common used for identification, which, from recently, is a relatively well-developed method. 1610–1640 cm^{-1} is beta sheet, 1640–1650 cm^{-1} is random coil, 1650–1658 cm^{-1} is alpha helix, and 1660–1700 cm^{-1} is beta turn (Zhang *et al.* 2003). Amide bands I of SPI modified by different methods were fitted in this article (Figure 4), and the unit ratio of protein secondary structure is shown in the following Table 2.

It could be seen in Table 2 that the main molecular conformation of SPI are beta sheet and random coil, and almost no alpha helix or beta turn. After the treatment with microwave, the amount of alpha helix showed no change while that of beta sheet decreased. However, both the beta turn and random coil increased to different degrees. This indicated that non-polar groups of SPI treated by microwave were exposed, the structure becoming loose; this may have resulted from the heating effect of the mikrowave treatment. Compared to SPI, SPI modified by phosphorylation and MAP revealed a significant increase both in beta sheet and in beta turn ($P < 0.05$), while a significant decrease was observed in alpha helix and in random coil ($P > 0.05$). This may have been caused by the increase of electronegativity of the phosphorylated protein system, which enhanced the electrostatic repulsion between the protein molecules leading to hydrogen bonds between non-polar groups of the protein formed afresh and, as a result, the content of beta sheet increased while that of random coil decreased. This proved that the protein structure became compact and the ordering enhanced after phosphorylation. This was closely related to the increase of dissolubility and decrease of viscosity of the modified proteins. Besides, secondary structures of SPI modified by phosphorylation and MAP diverse significantly ($P > 0.05$) except beta turn which may have been caused by the combined action of the microwave treatment and phosphorylation that led to the content of the secondary structure of SPI modified by MAP being intermediate between the microwave control group and phosphorylation control group.

Table 2. The contents of the secondary structure of SPI modified by phosphorylation methods

	α-helix	β-sheet	β-turn	Random coil
Blank control group	15.34 ± 0.39[a]	43.61 ± 0.65[a]	8.42 ± 0.45[a]	32.63 ± 0.53[a]
Microwave control group	15.16 ± 0.25[a]	37.41 ± 0.51[b]	11.18 ± 0.18[b]	36.25 ± 0.66[b]
Phosphorylation control group	9.37 ± 0.18[b]	55.33 ± 0.52[c]	15.32 ± 0.25[c]	19.98 ± 0.49[c]
MAP control group	11.57 ± 0.31[c]	50.96 ± 0.54[d]	14.45 ± 0.42[c]	23.02 ± 0.50[d]

[a-d]means with different letters within the same column are significantly different ($P < 0.05$)

Analysis of fluorescence spectra

Fluorescence analysis has advantages such as high sensitivity, strong selectivity, simple operation etc., that can be used to study the conformation of proteins in solution. The fluorescence-emission spectra excited at 280 nm are mainly emitted by the residues of tryptophan and tyrosine (Tao *et al.* 1995), alternate name of which is endogenous fluorescence spectra, and these can reflect the changes of protein space structure directly. Fluorescence intensity and λ_{max} of the samples can reflect the degree of oxidation of tryptophan residues and the change of the microenvironment (Hidalgo & Kinsella 1989).

Figure 5 clearly shows that the microwave treatment had a certain effect on the fluorescence emission spectrum. The fluorescence intensity decreased a little while the maximum emission wavelength (λ_{max}) revealed no obvious change. This means that the microwave treatment made the structure of SPI stretch, which is in accordance with the above mentioned research. Phosphorylation and MAP affected the fluorescence emission spectrum of SPI significantly, a certain degree of blue shift occurred in λ_{max} that moved from 342 nm (blank control group) to 336 nm; moreover, the fluorescence absorption intensity enhanced obviously, which accounted for the enhancement of the hydrophobic properties of Trp residues. Trp residues are always embedded in the interior of the protein molecules (Royer 2006), which may be caused by the shielding effect of PO_4^{3-} that makes the tertiary structure of protein become tight. Compared to the phosphorylation control group, MAP treatment reduced the fluorescence intensity of SPI, which probably resulted from the fact that the microwave treatment can promote the stretch of the protein structure.

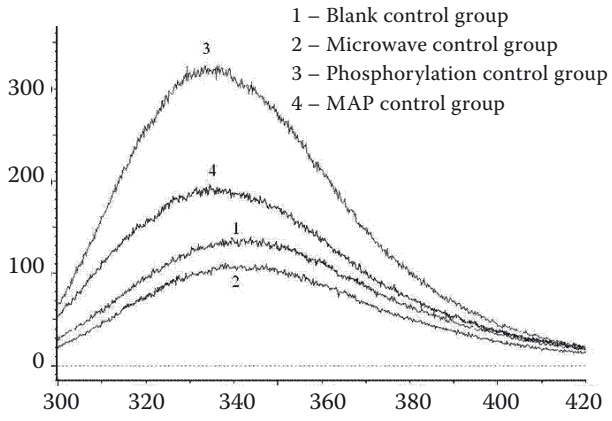

Figure 5. Fluorescence emission spectra of the four samples with excitation at 280 nm

CONCLUSIONS

The preparation time applied in the microwave-assisted phosphorylation of SPI was much shorter than in the common phosphorylation of SPI. The functional properties, molecular characteristics, and the structure of SPI modified by the two phosphorylation methods are similar. Microwave radiation can speed up the reaction rate of SPI phosphorylation, while the microwave-assisted phosphorylation is a new, efficient, and better way to prepare modified SPI with good functional properties.

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