Robust soy protein films obtained by slight chemical modification of polypeptide chains†

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Soy protein based materials are of great interest because of the merits of biocompatibility, biodegradability, renewability, etc. However, the poor mechanical properties and high water sensitivity limit their further application in many fields. In this paper, we tried to overcome these shortcomings through a slight chemical modification of the polypeptide chains of soy protein. $^{31}$P NMR and solid state $^{13}$C CP/MAS NMR spectroscopy confirmed that the diethoxy phosphoryl groups were successfully grafted onto soy protein chains with a molar grafting ratio of 0.15–1.18%, which almost did not change the nature of soy protein. The isoelectric point and rheological behavior of the modified soy protein sample varied with the grafting ratio, indicating that the tertiary structure of the protein was changed after phosphoryl modification. The FTIR spectra of the modified soy protein suggested that the increase of $\beta$-sheet conformation from the slight chemical modification could be the reason for the change of the globular structure of soy protein. Finally, we obtained a robust soy protein film as expected, and we did not use any crosslinking agent and plasticizer that were almost unavoidable in the previous studies reported in the literature. The tensile strength and the elongation at break of our soy protein films were 35 ± 5 MPa, 2.5 ± 0.5% in the dry state, and 3.8 ± 1.5 MPa, 125 ± 5% in the wet state, respectively. We believe that the method we developed in this communication provides a practical approach to improve the mechanical properties and broaden the applications of natural soy protein based materials.

Introduction

In recent years, scientists have paid much attention to the use of biodegradable materials for coatings, packaging, tissue regeneration, drug delivery, etc. The development and the increasing usage of natural polymers reduce the need of petroleum-based synthetic polymers that are difficult to degrade and recycle, thus minimizing the negative effects on the environment and ecology.

Materials based on protein hold many merits, for instance, excellent functional properties and/or high nutritional value. Over the past few decades, several proteins such as silk fibroin, gelation, casein, albumin, and whey protein have been utilized as films, hydrogels, microparticles, and nanoparticles. However, most studies were based on animal proteins. In recent years, people started to think of plant proteins, such as soy protein, gliadin, and zein, because the application of plant proteins could reduce the risk of spreading diseases such as bovine spongiform encephalitis (mad cow disease). Soy protein isolate (SPI), the most important component of soybean, contains two major components differentiated by sedimentation coefficient, 11S (glycinin, approximately 52% of the total protein content) and 7S ($\beta$-conglycinin, approximately 35%). Owing to its sustainability, abundance, low cost and functionality, SPI has attracted great interest for the development of environmentally friendly materials. There have been several soy protein-based materials, such as plastics, gels, films, additives or coatings, and biomedical materials, reported in the literature. However, most of these were made by simply crosslinking with aldehydes or blending with other synthetic/natural polymers to overcome their fundamental limitations, such as water sensitivity, poor processability, and in particular, low mechanical strength.

Chemical modification of proteins is very important in the preparation of related materials. One of the most useful modification methods for proteins is phosphorylation. Phosphate groups can attach to the oxygen of Ser, Thr, Asp and Tyr residues, and via nitrogen to Lys, Arg and His residues. In SPI, plenty of those polar amino acid residues can be modified through phosphorylation. The phosphorylation modification increases the solubility and decreases the pI, leading to changes of the functional properties of the proteins, which contribute a lot to broaden the application of protein materials.
In this article, diethoxy phosphoryl groups were grafted onto amino groups of Lys and Arg residues in SPI by Atherton–Todd reaction. As far as we are aware, there is no report on such a method to modify the structure of SPI. We hope to reduce the electrostatic interaction between the residues, change the tertiary structure of soy protein as a globular protein, and thus improve the structures and mechanical properties of soy protein materials through this controllable phosphoryl modification.

Experimental

Materials

SPI powder (protein content >90%) was obtained from Shenyuan Food Co. Ltd., Shanghai, China. Diethyl chlorophosphate (DECP, 95%) was purchased from Sigma, USA. Dithiothreitol (99%) was purchased from Aladdin, Shanghai, China. Triethylamine (TEA, 99%), anhydrous ethanol (99.7%), carbon tetrachloride (99.5%) and guanidine hydrochloride (99%) were obtained from Sino-pharm, Shanghai, China. All chemicals used in this work were of analytical grade and were used without further purification.

Characterization

Quantitative $^{31}$P NMR spectra were acquired with a DMX 500 spectrometer (Bruker, Switzerland) at a phosphorus frequency of 200 MHz using the inverse gated $^1$H decoupling technique. The one pulse experiments were performed with a 90° pulse length of 13.6 $\mu s$. The delay before the application of the pulse was 6.15 s and the acquisition time was 0.13 s. The spectral width was 80 ppm with the number of data points 4k. The number of scans was 1k. The chemical shifts of $^{31}$P NMR spectra were calibrated against 9% phosphoric acid. Solid state $^{13}$C CP/MAS NMR spectra were recorded on an AVANCE III 400WB spectrometer (Bruker, Switzerland) operating at a carbon frequency of 100 MHz. The rotors that contain the samples were spun at about 10 kHz, and the contact time, acquisition time and repetition time were 2 ms, 0.03 s and 5 s, respectively. The spectral width was 300 ppm with the number of data points 2k. The number of scans was 1k. The $^{13}$C NMR chemical shifts were calibrated using the carboxyl peak of glycine (176.03 ppm) and threitol to break the disulfide bonds. A $^{31}$P NMR chemical shift of another natural polymer chitosan.

Preparation of SPI solution

The preparation of soy protein solution was reported in our previous paper. Briefly, raw SPI powder was extracted in a Soxhlet apparatus with anhydrous ethanol and acetone for 24 h to remove phospholipids. Then, it was dissolved in 6 mol L$^{-1}$ guanidine hydrochloride aqueous solution and then stirred at room temperature for 3 h, while adding 25 mmol L$^{-1}$ dithiothreitol to break the disulphide bonds. After dialysis against NaOH aqueous solution (pH = 10, diluted from 2 mol L$^{-1}$ NaOH aqueous solution) for two days and then deionized water for another day at room temperature, the solution was centrifuged at a speed of 9000 r min$^{-1}$ for 10 min to obtain a clear supernatant. The solution was then concentrated to 6.0% (w/w) by using reverse dialysis against 10% (w/w) polyethylene glycol solution.

Chemical modification of SPI by Atherton–Todd reaction

The modification method was derived from our previous work on the modification of another natural polymer chitosan. From the amino acid analysis result of our SPI sample, the mole percentage of Arg and Lys residue was 6.5% and 6.3% respectively. Therefore, 32.6 g 6.0% (w/w) SPI solution (equivalent to 1.57 mmol primary amino groups in 2 g SPI) was put into a fourneck flask under a nitrogen atmosphere. Then, 15 mL anhydrous ethanol and 10 mL TEA was added into the SPI solution under stirring at $-5^\circ$ C. Different amounts (22, 44, 88, and 132 $\mu$L) of DECP and 2 mL carbon tetrachloride mixtures were slowly dropped into the solution within 1 h. The reaction was continuously carried out for another 4 h at the same temperature. Afterwards, the reaction system was slowly heated to room temperature and stirred for 24 h to yield a clear yellow solution. The mole ratio of added DECP to $-\text{NH}_2$ was chosen to be 0.1, 0.2, 0.4, and 0.6, respectively. Thus the corresponding products (in general we used SP$n$ to represent the modified SPI samples throughout the text) were labeled as SP100, SP200, SP400, and SP600.
The clear yellow solution was dialyzed against deionized water in a dialysis tube with the cut-off value of 14 kDa for 48 h until the pH of the solution was approximately 7.4. Finally, the solution was freeze-dried to obtain the final product. The chemical modification route of SPI was shown in Scheme 1.

Preparation of the modified SPI films

Three milliliters of 8% (w/w) modified SPI (SPn) solution was cast into a 3 × 3 cm polystyrene plate and dried at about 25 °C and 50% relative humidity. The thickness of the SPn film was approximately 0.3 mm. Afterwards the film was immersed in anhydrous ethanol and water for 1 min in turn twice to make the film insoluble and soft.

Results and discussion

Phosphoryl modification of SPI

In general, the rate of phosphoryl reaction depends on the rate of nucleophilic attack. The phosphoryl rates for homologous nucleophiles are inversely related to their pK values, so normally an amino acid residue in protein with a low pK has a high reactivity. Therefore the amino acid residues with amino and hydroxyl groups are readily phosphorylated compared to those with other functional groups in SPI. In addition, we chose Atherton–Todd reaction that is specific to the primary amino group, so diethoxy phosphoryl groups should be only grafted onto Lys and Arg residues in SPI.

$^{31}$P NMR spectroscopy usually serves as a convenient and efficient tool to analyze the product of phosphoryl reaction. The $^{31}$P NMR spectra of our products showed new peaks at 12.2 ppm (Fig. 1, curves b–e) compared to SPI (Fig. 1, curve a), indicating that phosphoryl groups were successfully grafted onto SPI, as the peak of phosphorus in DECP was located at 6.5 ppm. However, there were still other peaks in $^{31}$P NMR spectra for both SPI and modified SPn samples, and we considered they probably came from phosphatidylcholine (−0.34 ppm), other phospholipids (0.53 ppm), and dimethyl phosphonic acid (20.2 ppm) in raw SPI, respectively. We have also confirmed that SPI chains have been chemically modified. As we knew that the phosphorus content in raw SPI was 0.087%, we can use it as an “internal standard” to calculate the corresponding phosphorus content in modified SPn samples from quantitative $^{31}$P NMR spectra (Fig. 1, curves b–e). The phosphorus content is shown in Table 1, and it increased with the increase in the DECP amount in the reaction system. In addition, we can further calculate the grafting ratio, and the results showed that the modified amino acid residues were very few. This actually was our strategy for the chemical modification, namely the modification should change the nature of soy protein as little as possible.
Characterization of the modified SPn

The chemical modification on SPI changed the number of polar amino acid residues, so the pI of the modified SPn should be different from the unmodified SPI sample. Table 2 shows that the pI of SPn decreases with the increase in the amount of diethoxy phosphoryl groups grafted onto the polypeptide chain. Such a result was similar to the studies on the succinylation and acetylation of soy protein by Franzen and Kinsella.38

The decrease of pI in the modified SPn samples indicated that the structure of soy protein was truly changed, so we used FTIR spectroscopy to further monitor their secondary structures. The FTIR spectrum of SPI D2O solution showed a broad peak centred at 1640 cm⁻¹, while the one from the modified SPn had a similar shape but a slight increase of absorption at about 1620 cm⁻¹ (Fig. S1a, ESI†). The difference of those two spectra can be seen clearer in their second derivative spectra (Fig. S1b, ESI†). Both SPI and the modified SPn showed a main peak at 1640 cm⁻¹ (random coil) and a small peak at 1645 cm⁻¹ (α-helix),42,43 but the modified SPn showed another sharper peak at 1621 cm⁻¹ attributed to β-sheet conformation.42,43 This clearly demonstrated that the secondary structure of SPI was somewhat changed after the phosphoryl modification.

The FTIR spectra revealed that the modified SPn had a more extended chain conformation (β-conformation) than the original SPI, and it also can be reflected through their rheological behavior. The shear viscosity of the modified SPn solutions increased with the increase in grafting ratio (Fig. S2, ESI†). The viscosity of the samples with a relatively low grafting ratio (SP100 and SP200) was about one order of magnitude higher, and that of those with a relatively high grafting ratio (SP400 and SP600) was more than two orders of magnitude higher than that of raw SPI. In the meantime, the storage modulus $G'$ and loss modulus $G''$ showed a similar regular pattern to that of the viscosity (Fig. 3). Moreover, for SPI, SP100, and SP200, the $G''$ values were higher than the $G'$ value in all the angular frequency range, showing a liquid-like viscoelastic behavior. However, with regard to SP400 and SP600, $G'$ was higher than $G''$ at low angular frequency, and then showed a crossover at high angular frequency (15–20 rad s⁻¹), suggesting that weak networks existed in their solutions.44 This also explained why SP400 and SP600 had the higher shear viscosity than other samples. In a word, the results obtained from the rheological tests suggested that with the increase in the degree of modification, the interactions among the soy protein molecular chains became more and more strong.

Fig. 4 shows the TEM images of SPI and modified SPn morphologies when their dilute solutions were dried on the formvar/carbon-coated copper grids. SPI formed individual small round particles with few aggregates (Fig. 4a), which accords with its globular protein nature. In contrast, the modified SPn samples seemed much easier to aggregate. SP100 has an increased number of circular aggregates with a size of about 30 nm, and some of these aggregates connected with each other (Fig. 4b). SP200 and SP400 displayed a further increase of circular aggregates and further interconnection of these aggregates (Fig. 4c and d). In these aggregates, we can find the round

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<th>Phosphorus content and molar grafting ratio of the modified SPn</th>
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<td>SPI</td>
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<td>P (%)</td>
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<td>Grafting ratio (%)</td>
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<th>Isoelectric point (pI) values of soy protein and SPn</th>
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Fig. 3 Storage modulus $G'$ and loss modulus $G''$ as a function of frequency for SPI and the modified SPn solutions at 1% strain and 25 °C. (a) SPI, (b) SP100, (c) SP200, (d) SP400, and (e) SP600. Sample concentration: 100 mg mL⁻¹.

Fig. 4 TEM images of SPI and modified SPn samples. (a) SPI, (b) SP100, (c) SP200, (d) SP400, and (e) SP600. The area located as 1 and 2 in (e) represents the SP600 sample and carbon coating, respectively.
particles appear to have been transformed to worm-like shapes. We assumed this was because our chemical modification changed the molecular interactions within the protein chains, preventing them from keeping the natural tightly round morphology. The increased freedom of protein chains made them come into contact and connect with each other, and subsequently formed the interconnected circular aggregates as shown in Fig. 4b–d. For SP600, there was even a transparent thin film formed on the carbon coating of the TEM sample holder (Fig. 4e), indicating the increased interconnection of the polypeptide chains.

From the evidence of FTIR spectra, rheological behavior, and TEM images shown above, it is strongly suggested that the globular tertiary structure of soy protein has been destroyed at a certain level after phosphoryl modification, and the molecular chain became more extended. This gives the basis for the preparation of a strong SPI material as the polypeptide chains can be entangled and/or interpenetrated with each other.

**Modified SPn films with remarkable mechanical properties**

There is little report on the pure SPI films in the literature because it is too brittle in the dry state and is water soluble. In order to make use of the SPI film, it has to be chemically crosslinked (for instance, with aldehydes and epichlorohydrin) and/or with a plasticizer (for instance, with glycerol). Despite the disadvantages of the crosslinking agent and plasticizer on the toxicity, poor biocompatibility, and the change of the nature of protein itself (if the degree of crosslinking and the content of plasticizer were high, which often can be seen in the literature), the mechanical properties and the water sensitivity of those SPI films were still not satisfied.

Here we used SP200 to prepare the film as we considered that 0.26% change of amino acid residues would have little effect on the whole nature of soy protein. Moreover, as the reactant DECP is water soluble, it can be completely removed through the dialysis process if there is any unreacted DECP remaining. \(^{31}\)P NMR spectra of SPn samples did not show any visible peak at 6.5 ppm (Fig. 2), proving no DECP remained in the products. Fig. 5 gives a direct impression of the difference between the pure SPI film and the modified SP200 film. The dry SPI film was very brittle, and cracked by itself even at 50% relative humidity (Fig. 5a). In the meantime, it dissolved in water readily and cannot maintain its shape (Fig. 5b). In contrast, the modified SP200 film showed good performance both in dry and wet states. The dry SP200 film can be lifted by forceps, and the wet one was rather stable and ductile. Fig. 6 shows their typical stress–strain curves. The tensile strength of the dry SP200 film was 35 ± 5 MPa with an elongation at break of 2.5 ± 0.5% \((n = 6)\), which can meet the requirement of real applications. For the wet film, it showed a rather large breaking strain of 125 ± 5% with an acceptable strength of 3.8 ± 1.5 MPa \((n = 6)\), also providing considerable application potentials.

The improvement of the mechanical properties of the modified SPn films was also thought to be due to the change of the secondary and tertiary structures of soy protein. We know soy protein is a globular protein, so the interaction between the molecules in the unmodified SPI film may be only the friction force between the individual globular proteins. There are large amounts of polar amino acid residues in SPI, and the results of the amino acid analysis of our SPI samples indicated that the percentage of five polar amino acid residues (Arg, Lys, His, Asp, and Glu) was 47.1%, almost half number of the total amino acid residues. The phosphoryl modification consumed some Arg and Lys residues, decreasing the electrostatic interactions between the basic and acidic amino acid residues. In addition, the big diethoxy phosphoryl groups increased the steric hindrance during the protein folding. Therefore, the polypeptide chains were hard to form a tight globular structure as they did in the pure soy protein. The FTIR spectra of the modified SPn films (figure not shown) demonstrated the increase of β-sheet conformation compared to a pure SPI film that was similar to...
the difference between the SPI and SPn solutions (Fig. S1, ESIt). This could be the evidence that the phosphoryl modification enhanced the intermolecular interaction among protein molecules during the film formation and thus improved the mechanical properties.

**Conclusions**

In this article, the diethoxy phosphoryl group was successfully grafted onto the soy protein chains via Atherton–Todd reaction with different grafting ratios (0.15–1.18%). After modification, the isoelectric point, the apparent viscosity, the storage and loss modulus ($G'$ and $G''$), and the conformation of protein were changed correspondingly compared to those of pure SPI. We suggested that such a modification reduced the electrostatic interactions between the acidic and basic amino acid residues, and increased the steric hindrance within the polypeptide chains of soy protein. Therefore, the globular structure of soy protein was destroyed and the polypeptide chains became more free to interconnect with each other. The increase of the apparent viscosity and the modulus of SPn solution observed by rheological testing and the β-sheet conformation in both SPn solution and film by FTIR spectroscopy supported such an assumption. As a noticeable application, a robust soy protein film without any crosslink agent and plasticizer can be obtained through such a modification. The mechanical properties of the soy protein film in both dry and wet states were good enough for the potential application as a biomaterial because only a few amino acid residues (for instance, less than 0.5%) have been modified. In conclusion, the phosphoryl modification of soy protein provides a practical route to improve the mechanical properties of soy protein materials and broaden the application area of such a cheap, abundant and sustainable natural material.

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**Notes and references**