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Selective Chemical Modification of Soy Protein for a Tough and Applicable Plant Protein-Based Material

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Soy protein, one kind of most abundant plant protein has gained great attention and popularity due to its various merits, such as biocompatibility, biodegradability, and renewability in recent years. However, the poor mechanical properties and high moisture sensitivity of soy protein-based materials in practical use limit their further application in lot of fields. In this paper, we tried to overcome these shortcomings through an aqueous reagent, tetrakis(hydroxymethyl) phosphonium chloride (THPC), to modify the amino groups of Lys and Arg residues in soy protein. The solid state 13C CP/MAS NMR and 31P NMR spectroscopy confirm the successful chemical reaction though the hydroxymethyl arm replacement of THPC both qualitatively and quantitatively. The FTIR spectroscopy, rheological test, TEM and AFM observation indicate the change of the coherent tertiary structure, and thus increase the interconnections among the molecular chains of soy protein after the modification. Finally, we obtained a modified soy protein film with the good combination of tensile strength and extensibility in both dry (10±2 MPa and 25±3%) and wet (5±1 MPa and 200±20%) state. In addition, the modified soy protein film shows an unexpected antimicrobial property that adds the merit of the final product. We believe that the method we reported in this article provides another practical approach to improve the properties and broaden the application areas of natural soy protein-based materials, for example, as an antimicrobial package film.

Introduction

Petroleum-based plastic films and bags are one of the most important daily necessities that can be found everywhere in our daily life. However, environmental pollution from these materials have become a serious issue, particularly when they are used as one-time packaging material, because most of them are considered to be difficult for recycling and degrading in nature environment.1,2 In recent years, with the long term and increasing trend to reduce environmental impact caused by human activities, special attention is being paid to explore renewable biomass-based materials to replace conventional petroleum-based materials.3-5 The development of biomass-based films from proteins for the use of packaging materials as well as other biomaterials has attracted more and more attention by researchers both in academia and industry because of the availability, renewability, biocompatibility, and biodegradability of various proteins.6 In addition, as most of proteins have the excellent film-forming ability, considerable work can be found to develop protein films as biodegradable packaging materials in past few years.3-7 Furthermore, the application of plant proteins is considered having another advantage because it could reduce the risk of spreading diseases from animal proteins, such as bovine spongiform encephalitis (mad cow disease).8

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 (β-conglycinin, approximately 35%).9 Owing to its sustainability, abundance, low cost, and superior film-forming ability, SPI has attracted great interest for the development of environmentally friendly materials.10 There are different kind of SPI-based materials, such as plastics,11,12 gels,13-15 films,16-18 additives or coatings,19,20 and biomedical materials21,22 reported in the literature, but most of them cannot be used widely and commercially because of their weak mechanical properties, poor processability and relatively high moisture sensitivity. In order to improve those properties, a variety of modification methods have been developed to overcome the drawbacks of the SPI-based material, such as bulk, physical, enzymatic, and chemical modification, but most of them were simply crosslinking with aldehydes or blending with other synthetic/natural polymers.23-29

In our previous work,20 we found a new modification method by performing a controllable Atherton-Todd reaction on the amino groups of Lys and Arg residues in soy protein backbone. Such a method did not introduce any crosslinking agent and plasticizer that are almost unavoidable in the previous work reported in the literature, but destroyed the original globular nature of SPI in order to increase the molecular interaction among protein molecules. Only by modifying 0.5% of total amino acid residues in SPI (which almost does not change the protein nature of SPI at all), we can increase the tensile strength of dry SPI film to ~35 MPa, which meets the requirement in real application. However, such a SPI film does not
show a satisfactory tenacity, as its elongation at break is only about 2.5% in dry state, which still has some limitations for the further use.

In this article, we aim to keep the previous idea of chemical modification on few amino acid residues, but select another appropriate reactant to balance the tensile strength, tenacity, and flexibility of the resulting SPI films in dry state. As we think such a reactant is better not only has the same effect as the diethyl chlorophosphite (DECP) we used before, but also can act as a crosslinking agent. Therefore, tetrakis(hydroxymethyl) phosphonium chloride (THPC, see Scheme S1), an inexpensive, amine-reactive, aqueous tetra-functional crosslinking agent, which has been used as a crosslinking agent for polypeptide-based biomaterials by Chung et al. \textsuperscript{31,32} becomes our candidate. Through a Mannich type condensation, THPC can react with primary and secondary amines, and if uses more than one hydroxymethyl arm to react, it can form covalent crosslinks and three-dimensional structure in aqueous solution.

\section*{Experimental Section}

\subsection*{Materials}

SPI powder (protein content > 90\%) was obtained from Shenyuan Food Co. Ltd., China. Tetrakis(hydroxymethyl) phosphonium chloride (THPC, 80\%) and dithiohretiol (99\%) was purchased from Sigma, USA. Triethylamine (TEA, 99\%), anhydrous ethanol (99.7\%), carbon tetrachloride (99.9\%), and guanidine hydrochloride (99\%) were obtained from Sinopharm Co. Ltd., China. β-Glycerol phosphate disodium salt pentahydrate (β-GP) was purchased from Aladdin, China. All chemicals were analytical grade and used without further purification.

\subsection*{Preparation of SPI solution}

The preparation of SPI solution follows the procedure as described in our previous work. \textsuperscript{33} 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 guanidine hydrochloride aqueous solution and then stirred at room temperature for 3 h, while adding 25 mmol/L dithiohretiol to break the disulfide bonds. After dialysis against NaOH aqueous solution (pH=10, diluted from 2 mol/L 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 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.

\subsection*{Chemical modification of SPI}

The modification method was derived from our previous work on the modification of another natural polymer chitosan. \textsuperscript{34,35} In brief, 32.6 g 6.0\% (w/w) SPI solution (equivalent to 1.57 mmol primary amino groups in 2 g SPI) as the mole percentage of Lys and Arg residue was 6.3\% and 6.5\% from the amino acid analysis result of our SPI sample) was put into a four-neck flask under the nitrogen atmosphere. Then, 15 mL anhydrous ethanol and 10 mL TEA was added into SPI solution under stirring in circulated ethanol bath at −5°C. Afterward, different amount (0.33, 1.55 and 3.1 mL) THPC solutions were slowly dropped into the solution within 30 min, and the reaction was allowed to continuously carry out for another 3 h at the same temperature. Finally, the reaction system was slowly heated to the room temperature and stirred for 24 h to yield a clear yellow solution. These clear yellow solutions were dialyzed against deionized water in a dialysis tube with the cut-off value of 14 kDa for 48 h till the pH of the solution was approximately 7.4, and then were freeze-dried to obtain the final products. The mole ratio of added THPC to –NH\textsubscript{2} in Lys and Arg was chosen to be 1, 5, and 10, so we labeled the corresponding products as SPTC1, SPTC5, and SPTC10, respectively. In general, we used SPTCn to represent the modified SPI samples throughout the text.

\subsection*{Preparation of modified SPI (SPTCn) films}

SPTCn film was prepared by casting 3 mL of 4\% (w/w) SPTCn solution into a 4 × 4 cm polystyrene plate and dried at about 25°C and 50\% relative humidity. The thickness of SPTCn film was approximately 260 \textmu m.

\subsection*{Characterizations}

Quantitative \textsuperscript{31}P NMR spectra were acquired with a DMX 500 spectrometer (Bruker, Switzerland) at a phosphorous frequency of 200 MHz using the inverse gated 1H decoupling technique. The one pulse experiments were performed with the 90° pulse length of 13.6 \textmu s. The delay before the application of 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 \textsuperscript{31}P NMR spectra were calibrated against 90\% phosphoric acid. Solid state \textsuperscript{31}C CP/MAS NMR spectra were recorded on an AVANCE III 400WB spectrometer (Bruker, Switzerland) operating at carbon frequency of 100 MHz. The rotors which contain the samples were spun at about 10 kHz, and the contact time, acquired 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 \textsuperscript{13}C NMR chemical shifts were calibrated using the carboxyl peak of glycine (176.03 ppm) and converted to the value from tetramethylsilane. The Fourier transform infrared (FTIR) spectra were collected by a Nicolet 6700 FTIR spectrometer in transmission mode. For the measurement of protein solutions, samples were dissolved in D\textsubscript{2}O to make a 1\% (w/w) solution and placed between a pair BaF\textsubscript{2} windows using a 50\% thick Teflon spacer in a liquid cell. To eliminate spectral contribution due to atmospheric water vapor, the instrument was continuously purged with dry air. For each measurement, 64 interferograms were co-added and Fourier-transformed employing a Genzel-Happ apodization function to yield spectra with a nominal resolution of 4 cm\textsuperscript{-1}. All rheological experiments were performed in strain controlled mode with a Physica MCR 301 rheometer (Anton Paar GmbH, Austria) using a cone-and-plate geometry of 1° incline, 60 mm diameter (CP 60/1). To minimize evaporation, a solvent trap was employed and a low viscosity mineral oil was applied around the sample. Dynamic frequency sweep experiments were performed in the range of 0.1−100 rad/s at 1% strain. TEM images were observed with a FEI-600 transmission electron microscope (FEI, USA) operated at 120 kV. Samples were dissolved in deionized water and then sonicated for 15 min to make a 0.5\% (w/w) solution. Before observation, the solutions were absorbed onto 200 mesh formvar/carbon-coated copper grids and stained with 2\% (w/w) uranyl acetate for 60 s. AFM images were recorded on a Multimode 8 Digital Instruments atomic force microscope (Bruker, USA) in tapping mode, using a Si\textsubscript{3}N\textsubscript{4} cantilever with a spring constant of 50 N/m and a resonance frequency of 340 kHz. 4\% (w/w) sample solutions were diluted 1000 times with deionized water, and then 20 \mu L of those diluted solutions were dipped onto freshly cleaved mica (-1 cm\textsuperscript{2}) attached to a magnetic steel disc (serving as sample holder). The samples were subsequently air-dried at room temperature overnight. The mechanical properties of films were measured with an Instron 5565 universal testing machine. The sample size was 0.5
× 3 cm, the gauge length was set at 1 cm, and the cross-head speed was 6 mm/min. All the tests were carried out at room temperature if it is not specified.

**Results and Discussion**

**Chemical modification of SPI**

According to our previous research on the chemical modification of SPI,\(^3\) we got two enlightenment. One is that the modification reaction should be taken place in a homogenous environment during the whole process, as it would be beneficial to improve reaction efficiency. Thus, we need to keep the pH of reaction system away from the isoelectric point of SPI. Another is that the amino acid residues with primary amino groups (–NH\(_2\)) are readily phosphorylated compared to those with other functional groups in SPI because the phosphoryl rate for homologous nucleophiles are inversely related to their pK\(_a\) values, i.e., the amino acid residues with low pK\(_a\) have the high reactivity.\(^6\) Therefore, to use another phosphorylated reagent THPC to react with the amino group on Lys and Arg in SPI is still a practical and efficient way to do the modification. In the meantime, we use TEA to provide a relatively stable weak alkalic environment in the reaction system, ensuring SPI can form a stable solution in such an environment.

First of all, we use NMR spectroscopy to prove the successful modification of SPI. Fig. 1 shows the solid-state \(^{13}\)C CP/MAS NMR spectra of pristine SPI and modified SPTCn samples. The spectral features of those samples are similar to SPI reported by Mizuno et al.\(^3\) and Kealley et al.\(^9\) with major signals corresponding to carbonyl (165–180 ppm), aromatic (115–130 ppm), α carbons (45–65 ppm), β carbons (25–45 ppm), and methylene and methyl groups remote from the backbone (15–25 ppm). This means the molecular structure of SPI did not have an obvious change after chemical modification. However, in addition to these non-site specific peaks conserved for SPI, there was one new peak appeared at 48 ppm in the spectra of SPTCn samples (curve b–d), which is attributed to –CH\(_2\)– of hydroxymethyl arms in THPC reacted on SPI. Furthermore, the intensity of this peak area increases with increase in the amount of THPC added in the reaction system, indicating that the amount of THPC modified on the SPI macromolecular chains is also increased.

![Fig. 1 Solid state \(^{13}\)C CP/MAS NMR spectra of SPI and SPTCn samples. (a) pristine SPI, (b) SPTC1, (c) SPTC5, (d) SPTC10.](image)

However, \(^{13}\)C CP/MAS NMR spectra only show that THPC was successfully reacted on the polypeptide chains through a hydroxymethyl arm replacement, but cannot provide detailed information about the replacement, for instance, the reacted hydroxymethyl arm numbers. Fortunately, we can use quantitative \(^{31}\)P NMR spectroscopy get those information because the reagent THPC we selected has the phosphorus element. The \(^{31}\)P NMR spectra (Fig. 2) shows several new peaks in the range from +25 ppm to –40 ppm in SPTCn samples (curve b–d) compared to pristine SPI sample (curve a), which is directly related to the situation of hydroxymethyl arm replacement of THPC. However, the peaks located in the range from +2.5 ppm to –2.5 ppm overlap seriously, which should come from the phospholipid in SPI raw material itself\(^2\) and the phosphorus signal of reacted THPC on SPI. According to Chung's work,\(^2\) we pretty sure the peaks located at –23.7, –29.9, and –36.8 ppm are the phosphorus signals of those reacted THPC removed two hydroxymethyl arms, three hydroxymethyl arms, and four hydroxymethyl arms, respectively. Meanwhile, we have the reason to assume the newly appeared peak at 1.5 ppm to the phosphorus signal of reacted THPC removed one hydroxymethyl arm. In addition, no signal of THPC (50.1 ppm) is found in the \(^{31}\)P NMR spectra, suggesting the unreacted THPC was completely removed after dialysis process.

![Fig. 2 Quantitative \(^{31}\)P NMR spectra of SPI and SPTCn samples. (a) pristine SPI, (b) SPTC1, (c) SPTC5, (d) SPTC10.](image)

Although we confirm the chemical modification of THPC on the SPI polypeptide chains, we still cannot directly calculate peak area to obtain total phosphorus content as well as the percentage for different count of unreacted hydroxymethyl arms in every sample because of the seriously overlap peaks in the range from +2.5 ppm to –2.5 ppm. We cannot find a good way to solve this problem until we think of using β-GP as an external added “internal standard” in SPTCn D\(_2\)O solution. From Fig. 2, we can see β-GP only has one peak at about 4.6 ppm,\(^9\) which does not overlap with any other peaks of SPI or SPTCn samples in the \(^{31}\)P NMR spectra. As β-GP has no reactivity with SPI, so we have the possibility to calculate the corresponding phosphorous content in SPI or SPTCn samples from quantitative \(^{31}\)P NMR spectra according to the known amount of added β-GP. Table 1 is the total phosphorous content in samples and the corresponding added phosphorous content come from chemical modification. It is expected that more THPC reacted on SPI when larger amount of THPC was added in the reaction system. In addition, we can estimate the percentage of different count of unreacted hydroxymethyl arms in SPTCn samples, as shown in Table S1. In each SPTCn
sample, almost more than 85% of reacted THPC has two or three unreacted hydroxymethyl arms. From the phosphorous content and the percentage of different count of unreacted hydroxymethyl arms in SPTCn samples, we can get the number of amino acid residues reacted with THPC is from 0.86% to 1.05%. This value meets our requirement of experimental design, i.e., only a few amino acid residues to be modified in order to maintain the protein nature of SPI.

### Table 1 Phosphorous mass content in SPI and SPTCn samples (n=3)

<table>
<thead>
<tr>
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<th>SPI</th>
<th>SPTC1</th>
<th>SPTC5</th>
<th>SPTC10</th>
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<tbody>
<tr>
<td>Total P (%)</td>
<td>0.07±0.01</td>
<td>0.18±0.02</td>
<td>0.20±0.03</td>
<td>0.22±0.03</td>
</tr>
<tr>
<td>Added P (%)</td>
<td>0</td>
<td>0.11±0.01</td>
<td>0.13±0.02</td>
<td>0.15±0.02</td>
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### Characterizations of SPTCn samples

From the results of NMR spectroscopy, we confirmed that THPC was readily reacted on Lys and Arg residues on SPI chains, and the reaction was quite complicated. After reaction, the molecular interactions among protein should be changed because some basic amino acid residue were consumed (varying the hydrogen bond and electrostatic interactions within SPI molecules). In addition, according to the reaction mechanism between THPC and primary amine, and the fact that a certain percentage (>20%) of THPC reacted with SPI by using more than one hydroxymethyl arm (Table S1), we assume THPC may serve as a crosslinking agent to interconnect SPI molecular chains.

First, we use FTIR spectroscopy to monitor the secondary structure of SPTCn samples to see if it changes after chemical modification. Fig. S1a is the FTIR spectra of SPI and SPTCn D_2O solutions, in which all of them show a broad peak centered at 1640 cm\(^{-1}\) in amide I region. Specifically, there were no obvious difference between SPI and SPTC1, but for SPTC5 and SPTC10, there appears a shoulder peak at about 1620 cm\(^{-1}\). Such a difference can be seen clearer in their second derivative spectra (Fig. S1b). Both SPI and SPTCn samples show a main peak at 1640 cm\(^{-1}\) (random coil) and a small peak at 1645 cm\(^{-1}\) (α-helix).\(^{40, 41}\) After chemical modification, SPTCn samples show a new peak at 1621 cm\(^{-1}\) attributed to β-sheet conformation.\(^{40, 41}\) and such a peak is really sharp in SPTC5 and SPTC10 samples. This clearly demonstrates that the secondary structure in SPI changes in a certain extent, i.e., the molecular chains become more extended, after THPC modification.

Afterward, we tested the rheological behavior of SPI and SPTCn solutions to further reveal the molecular interactions in samples. Fig. 3 shows a shear-thinning behavior for all samples, and the viscosity of SPI and SPTC1 is almost no difference, which is very low. However, the viscosity increases significantly for SPTC5 and SPTC10 samples. The one for SPTC5 is about two order of magnitude higher than that of SPI/SPTC1, and SPTC10 one shows additional one order of magnitude larger. Similar behavior can be seen in the storage modulus G’ of the samples (Fig. 4). G’ is very low and almost the same for SPI/SPTC1, and increase for about two order of magnitude for SPTC5, and then increase for another two order of magnitude for SPTC10. Moreover, the G” value for SPI is higher than G’ in all angular frequency range, showing a liquid-like viscoelastic behavior. For SPTC1, at very beginning G’ is higher than G”, but it soon shows a crossover at about 2 rad/s. With regard to SPTC5 and SPTC10, G’ is higher than G” at almost entire angular frequency we tested (SPTC5 appears a crossover at about 100 rad/s). This suggests there are networks existed in SPTC5 and SPTC10 solutions,\(^{42}\) and it can explain why SPTC5 and SPTC10 show higher viscosity than SPI and SPTC1 samples. In a word, after modification, the interactions among the polypeptide chains of soy protein become stronger than before.

![Fig. 3](image_url) Apparent viscosity versus shear rate of SPI and SPTCn solutions at 25 °C. (a) pristine SPI, (b) SPTC1, (c) SPTC5, (d) SPTC10. Sample concentration: 40 mg/mL.

![Fig. 4](image_url) Storage modulus G’ and loss modulus G” as a function of frequency for SPI and SPTCn solutions at 1% strain and 25°C. Sample concentration: 40 mg/mL.

According to the reaction mechanism of THPC and rheological behavior of SPTCn solutions, we think there would exist network structures in SPTCn samples via the intermolecular interaction and/or crosslinking effect from THPC reaction. In order to have a more direct evidence and impression, we took TEM and AFM images of SPI and SPTCn samples. Fig. 5 is the TEM images by dropping dilute sample solutions on the formvar/carbon-coated copper grids. We can see SPI forms individual small particles with few aggregates, but with increase in THPC modification extent, the individual small particles gradually connect with each other and tend to form a network. For SPTC5 and SPTC10 samples, the formation of networks is very clear (Fig. 5c, d). In the meantime, AFM images show the similar morphology (Fig. 6), confirming the protein chains have the tendency to form network after chemical modification. In addition, we do believe the formation of network for SPTCn samples is due to the change of molecular structure after chemical modification, not the concentration effect, because the concentration for AFM
observation (0.004%) is more than 100 times dilute than that for SEM observation (0.5%).

Fig. 5 TEM images of SPI and SPTCn samples. (a) SPI, (b) SPTC1, (c) SPTC5, (d) SPTC10. Sample concentration: 0.5%.

Fig. 6 AFM images of SPI and SPTCn samples. (a) and (e) SPI, (b) and (f) SPTC1, (c) and (g) SPTC5, (d) and (h) SPTC10. (a)–(d) are height images, while (e)–(h) are phase images. Sample concentration: 0.004%.

SPTC5 film with remarkable mechanical properties

As described previously, the purpose of this modification is to destroy the original globular nature of SPI, and hence increase the molecular interaction among protein molecules. The characterizations shown above indicate the resulting SPTCn samples meet our requirement. We may prepare a strong and ductile SPI material through the enhanced molecular interactions among the polypeptide chains and the possible crosslinking by THPC in SPTCn samples. After screening, we found SPTC5 was the best candidate to prepare an applicable material (for instance, film). For SPTC1, the structural change after modification seems not enough to improve its mechanical properties. For SPTC10, we find the film is easy to shrink and corrugate during drying process (Fig. S2), which may result from the relatively high crosslinking degree. In contrast, SPTC5 film has a smooth surface and is transparent, which has the potential to be an excellent film material.

Fig. 7 shows the mechanical properties of SPTC5 film, and the inset picture gives the direct impression for its excellent tensile strength and flexibility. In our previous work, we have mentioned that the pristine dry SPI film is very brittle and very easy to dissolve in water. Here we can see our SPTC5 film shows both good performance in dry and wet state. The dry SPTC5 film has a breaking strength of 10±2 MPa with an elongation at break of 25±3% (n=6). Although the breaking strength of current film is not as good as the modified SPI film by other chemical modification method we reported previously (35±5 MPa), its elongation at break is far more large than that (2.5±0.5%). Obviously, this SPTC5 film has a better balance between strength and extensibility, thus holds much higher breaking energy (1.27 MJ/m²) compared with the previous one (0.42 MJ/m²). The difference of the mechanical properties between the current and previous modified SPI film may due to the different chemical modification on the protein structure. As described in our previous work, we point out that the phosphoryl modification consumes some Arg and Lys residues, decreasing the electrostatic interactions between the basic and acidic amino acid residues, and increasing the steric hindrance by the big diethoxy phosphoryl groups during the protein folding, therefore, the polypeptide chains are hard to form a tight globular structure as they do in the pure soy protein, and thus create some entanglement between the individual protein molecules. These molecular chain entanglement between the modified SPI molecules are surely stronger than the friction force between the unmodified ones, thus increase the breaking strength and elongation at break of the modified SPI film. In the current work, crosslinking between the protein molecules is added besides the effects mentioned above. It can be imagined that the covalent bond is much stronger than the main entanglement, so SPTC5 film can endure more strain, showing the large extensibility than the modified SPI films obtained in our previous work. On the other side, the crosslinking between the protein molecules also makes the former tight globular structure loser, resulting a little decrease of the breaking strength than before. In the meantime, SPTC5 film also exhibits a good mechanical properties in wet state, in which the breaking strength is 5±1 MPa and the elongation at break is 200±20% (n=6). These value are also superior to the former modified SPI film (3.8±1.5 MPa and 125±5%, respectively). Therefore, we are confident to believe that SPTC5 film meet the requirement as a practical material.

Fig. 7 Mechanical properties of SPTC5 film at wet and dry state (50% relative humidity) at 25°C (inset: bending film at dry state).
As we design for the modification, we use THPC to react with the Lys and Arg residues in SPI in order to destroy the inherent globular tertiary structure of SPI, and then build up new interactions among those “destroyed” protein molecules. Meanwhile, as THPC can react with more than one \(\sim \text{NH}_2\) groups on Lys/Arg residues, it also has the possibility to as a crosslinking agent to interconnect the protein chains. In order to prove there is really some crosslinking in the SPTC5 film, we put it into a mixture solution of guanidine hydrochloride and dithiothreitol, which can efficiently break the hydrogen bond and disulfide bond. It is shown that the SPTC5 film can be stably maintained in such a solution (Fig. S3a) as in deionized water (Fig. S3b) for at least five days, and is strong enough to be lifted by forceps. This phenomenon could be an evidence to the existence of covalent bond among the protein chains in SPTC5 film.

Apart from the excellent mechanical properties of SPTC5 film, it also shows the antimicrobial property, which adds its merit for a practical material. We know that SPI-based material is very easy to breed bacteria or fungi because protein itself is the nutrient for these bacteria/fungi, therefore its practical application is seriously weakened. However, our SPTC5 film can prevent the bacteria/fungi grow on its surface. Fig. 8 shows the comparison of an ordinary SPI film and SPTC5 film in an environment with constant temperature (25 °C) and humidity (≥95 %) for 10 days. Obviously, the SPI film grows many bacteria/fungi but SPTC5 film has almost no change. Although the exact reason for the antimicrobial property of our modified SPI film still needs further investigation, we do not think the modified SPI can directly kill the bacteria or fungi. We assume it is because the structural change of soy protein with the linkage of THPC, which makes the bacteria or fungi not like to close to the surface of the film as some other polymer material surfaces reported in the literatures,34,45 but it surely needs further evidence to confirm in the future. Considering the remarkable mechanical and antimicrobial properties of SPTC5 film, we believe it has a great potential to be used as a natural polymer-based package material.

![Fig. 8](image)

**Fig. 8** Photographs of pristine SPI film (a) and SPTC5 film (b) in an environment with constant temperature (25°C) and humidity (≥95%) for 10 days to see the bacteria/fungi growth.

**Conclusions**

In this article, we used phosphorous contained reagent THPC to react with the amino groups in Lys and Arg residues on SPI polypeptide chains. The chemical modification is successful, and the final phosphorous amount added on SPI is 0.11%–0.15% (w/w), which equivalent to approximately 0.86% to 1.05% number of amino acid residues in SPI has been modified. The modified SPTCn samples were fully characterized with FTIR spectroscopy, rheology, TEM, and AFM. The results indicate that the secondary structure, the apparent viscosity, the storage and loss modulus (\(G'\) and \(G''\)), as well as the morphology of them are changed correspondingly compared to those of pure SPI. We suggest such a modification alters the inherent interactions between the amino acid residues, increases the steric hindrance within the polypeptide chains, and introduces slight crosslinking among molecular chains of soy protein. Therefore, the globular structure of soy protein is destroyed and the polypeptide chains can be interconnect with each other. The increase of apparent viscosity and the modulus by rheological test and the \(\beta\)-sheet conformation by FTIR spectroscopy in the SPTCn solutions supported such an assumption. In addition, the fact that the SPTCn films can be stably existed in the mixture solution of guanidine hydrochloride and dithiothreitol is an additional evidence for the new covalent band formation (crosslinking). As a noticeable application, a tough and antimicrobial soy protein film without any additive crosslinking agent and plasticizer can be obtained through such a modification. Although we are not aiming to develop a specific biocompatible material, the mechanical properties of the soy protein film in both dry and wet state were good enough for the practical applications, such as a natural polymer-based packing material. In a word, the chemical modification method of soy protein reported here 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**