Study on Isolation and Raman Spectroscopy of Glycinin in Soybean Protein

YIN Haicheng1,2*, HUANG Jin1, ZHANG Huiru1

1 College of Biological Engineering, Henan University of Technology, Zhengzhou 450001, China;
2 Grain & Corn Engineering Technology Research Center, State Administration of Grain, Zhengzhou 450001, China

Abstract: The secondary structures of soybean glycinin were investigated by Raman spectroscopy and its acidic and basic polypeptides were isolated. The results showed that the secondary structures of glycinin were mainly composed of 21.51% α-helix, 41.62% β-sheet, 24.70% β-turn, and 12.18% random coil. For the disulfide bridge (—S—S—), the ratios were 34.8% gauche—gauche—gauche (g—g—g), 32.1% gauche—gauche—trans (g—g—t), and 33.1% trans-gauche-trans (t—g—t). The typical acidic subunit A and basic subunit B were clearly separated by heat denaturation and reduction with β-mercaptoethanol, and their corresponding molecular masses were 42 and 38 ku, respectively. Raman spectroscopic analysis can be used to determine the secondary structural properties of glycinin. Further studies of the glycinin structures will be helpful for the utilization of soybean protein resources.

Keywords: Soybean glycinin; Secondary structures; Raman spectroscopy; Acidic and basic polypeptides

1 Introduction

The diversity and complexity of protein conformations are closely related to specific molecular structures and unique spatial conformations[1]; therefore, studies of protein structures are very important for understanding function and behavior mechanisms. Glycinin, with a relative molecular weight of 340–375 ku and comprising approximately 40% of the total soybean protein, is a common storage protein in soybean, has high allergenicity and immunogenicity, and is a health threat to humans and animals[2]. Up until now, glycinin is discovered of five subunits, A1aB2, A1bB1b, A2B1a, A3B4, A5B5, known as hexamer (6AnBn); each subunit consists of an acidic subunit A and basic subunit B linked by a disulfide bond, with A containing A1a, A1b, A2, A3, A4, and B containing B1a, B1b, B2, B3, B4, and B5[3]. Biochemical and molecular biological research has suggested that subunit types between the different soybean varieties have some differences, and the same variety in different regions has different characteristics such as amino acid sequences, spatial conformation, and physiological functions[4]. Additionally, various spectral characteristics between different protein molecules consisting of their functional groups and chemical bonds have been determined by Fourier transform infrared spectroscopy and Raman spectroscopy. Quantitative information of protein secondary structures using spectral analysis can explain the composition, characteristics, and altered patterns of protein molecules[5].

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*Corresponding author. E-mail: yhcht007@126.com
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Raman spectroscopy produces an inelastic scattering spectrum and is a powerful method for studying the structures and transformations of proteins; these transformations are caused by the vibration modes and changes in lattice vibration polarizabilities of protein molecules. The fingerprint spectra characterizing the vibrational levels in protein molecules can be used to determine the main chain conformation and secondary structures of a specific protein[6]. Long et al.[5] used Raman characteristic bands to determine quantitative information of the glycinin secondary structures. Li et al.[7] detected the spatial conformation of soybean 11S by Raman spectroscopy, which revealed that surface hydrophobicity of soybean 11S globulin was negatively correlated with the content of α-helix and β-fold and positively correlated with the content of β-rotation and irregular curls. In addition, Raman spectra can be used to detect non-polar functional groups, such as —C═C—, —C—C—, and —S—S—, as well as the vibrational dipole moment of functional groups or chemical bonds of soybean protein molecules[6]. Thus, Raman spectroscopy has been used to evaluate protein structures, conformational changes, and functional properties. In this study, the Yu-29 soybean variety was selected to isolate glycinin, and Raman spectroscopy was conducted to analyze the structures of this protein. This study provides a basis for improves the quality of soybean protein.

2 Materials and Methods

2.1 Materials

Defatted soybean powder was prepared from Yu-29 soybean by grinding, defatting with n-hexane, and removing the solvent at ambient temperature. Chemicals for SDS-PAGE including bisacrylamide, acrylamide, SDS, ammonium persulfate, dithiothreitol, and IPG strips were purchased from Beijing DingGuo Biotechnology Co. Ltd. (Beijing, China). Tris-HCl, glycerol, and 2-mercaptoethanol (2-ME) were purchased from Sigma (St. Louis, MO, USA). All reagents were of analytical grade.
2.2 Preparation of Glycinin

Glycinin was prepared as described by Wolf. In brief, the powder was stirred with distilled water in a 1:10 W:V ratio and the pH value of the suspension was adjusted to 8.0 with 1 mol/L NaOH solution. After 2 h, the suspension was centrifuged at 9 000 r/min for 30 min at 4 °C. The pH of the supernatant was further adjusted to 6.4 with 2.0 mol/L HCl and incubated in an ice bath for 20 h without stirring, and was then centrifuged at 10 000 r/min for 10 min at 4 °C. The following preparation and centrifugation were performed at 4 °C. The pellet (11S fraction) was suspended in PBS, pH 7.6, and the suspension was incubated in an ice bath overnight, and then adjusted to a saturated state with (NH4)2SO4 (51% saturation). The saturated solution was centrifuged at 6 500 r/min for 30 min; the supernatant solution was also adjusted to the 66% mass fraction with (NH4)2SO4. The insoluble protein fraction was separated by centrifugation at 6 500 r/min for 20 min; the precipitate was collected and suspended in PBS, pH 7.6, dialyzed, concentrated, and purified by column chromatography with Sepharose CL-6B (4×142 cm).

The purity of glycinin was determined by polyacrylamide gel electrophoresis (SDS-PAGE) and Quantity One software. The protein was dialyzed and adjusted to pH 7.0 with 1 mol/L NaOH, and then lyophilized and stored at −20 °C.

2.3 Separation of Acidic and Alkaline Subunits

The concentration of purified glycinin was adjusted to 5 mg/mL in PBS, pH 8.0, followed by addition of 20 mmol/L β-mercaptoethanol, heating to 90 °C for 30 min and then cooled down to room temperature. The suspension was centrifuged at 9 000 r/min for 30 min at 4 °C, and the clear supernatant solution and precipitate were used for the next extraction step. The precipitate was washed 3 times with PBS (pH 8.0), dissolved in PBS (pH 7.0) containing 0.1% SDS, and dialyzed against PBS (pH 7.0) for 24 h. The alkaline protein chain of glycinin was subsequently obtained. The pH of the clear supernatant solution was adjusted to 5.0 and centrifuged (at 9 000 r/min for 30 min at 4 °C) to remove the supernatant and then the acid peptide in the precipitate under the same conditions as in the alkaline peptide experiment was prepared. The concentration of PBS buffer used to separate the acid and alkali peptides was 0.025 mol/L.

2.4 SDS-PAGE

SDS-PAGE was used to evaluate purified glycinin according to the method of Laemmli[10] with a 12.5% separating gel and 5.0% stacking gel. Purified glycinin samples (10 mg/mL) were treated with buffer containing 10 mmol/L Tris-HCl, 2.5% SDS, 1.0 mmol/L EDTA, and 0.02% bromophenol blue. The samples were heated in a 100 °C water bath for 5 min and then cooled. A sample (15 μL) was added to each lane. Electrophoresis was performed at 100 V, and then at 200 V when samples entered the separating gel for 1.5–2.0 h. The gel was stained with 0.25% Coomassie Brilliant Blue (G-250) in 50% methanol and destained with 7.0% acetic acid with water–methanol–acetic acid (V:V:V) = 8:1:1.

2.5 Analysis of Raman Spectra

The crystal glycinin samples were prepared and plated on a concavity slide. The Raman spectrometer used was a JY-HR800 laser confocal microscope Raman spectrometer (Jobin Yvon, France) with an argon ion laser operated at 40 mW and at an excitation wavelength of 514.5 nm. Spectra were recorded with a spectral slit width of 50 μm, resolution of 1 cm−1, groove density of 1 600, 2 cm−1 interval from the 4 000 to 400 cm−1 scanning region, exposure time of 30 s, and number of repetitions of 10.

The average was automatically determined using a computer. Baseline calibration was performed by considering the phenylalanine peak (1 004 cm−1) as the normalization factor. The spectrum was mapped with LabSpec 5.0 and processed with OMNIC 8.0. Amide I was determined for volumetric analysis. Data regarding secondary proteins structures and amino acid side chains were obtained using Origin 8.5 software.

2.6 Statistical Analysis

All data are presented as the mean ± standard deviation (SD, n = 3). The relative amounts of secondary structures of glycinin were determined from the second derivative amide I spectra by manually computing the areas under the bands assigned to a particular substructures.

3 Results and Discussion

3.1 Glycinin Purity and Separation of Its Acidic and Basic Subunits

Glycinin obtained from the isoelectric point was filtered by gel filtration chromatography, the elution curve is shown in Fig. 1. The elution curve of glycinin showed two peaks: peaks I and II. The eluate was collected according to peaks I and II and analyzed by SDS-PAGE. Consistent with the findings of Yuan et al.[11], the results revealed that a typical acidic subunit A and basic subunit B were clearly separated (Fig. 2), and their corresponding molecular masses were 35–42 and 20 kDa, respectively. Previous studies showed that proteins, according to the typical structure, molecular weight, water solubility, ionization characteristics, and proteins specificity, could be highly purified by exchange chromatography, density gradient ultracentrifugation, and gel chromatography; purity could be evaluated by SDS-PAGE. These methods were used to obtain proteins crystals[12]. In this study, highly pure glycinin was produced by combining saturated (NH4)2SO4 and Sepharose CL-6B, and analyzed by gray scale scanning and Quantity One software, showing a purity of 94.9% (Fig. 3). The results were consistent with the investigation of Tsutomu et al.[13].

The acid and alkaline peptide chains of glycinin can be separated using an isoelectric point precipitation method[14]. Fig. 2 shows two bands with molecular weights of approximately 42 and 38 kU, which correspond to the acidic peptide chain of glycinin. Particularly, the two bands were respectively assigned to the A1 acidic peptide chain and A1, A2, and A4 acidic peptide chains. A single band with a molecular weight of 20 kU was found to be the base peptide chain.
3.2 Raman Spectrum Analysis of Glycinin

Protein structures can be predicted according to characteristic absorption peak intensity and peak position in the middle of Raman absorption peak\(^{[15]}\). The amide I band is observed due to the C=O and C—N stretching vibrations and amide III band is observed due to the C—N stretching vibrations and N—H bending vibrations on the plane and is often incidental to other vibrational bands in the Raman spectrum of proteins\(^{[16]}\). Thus, it is important to study amide I to evaluate the main skeleton and secondary structures of proteins\(^{[17]}\). The glycinin Raman peak at 400–2 000 cm\(^{-1}\) is shown in Fig. 4 and corresponding peak values are listed in Table 1. Major peaks obtained from curve fitting were observed at 1 667 and 1 242 cm\(^{-1}\) and attributed to the amide I and III regions, respectively. The peaks observed in the amide I region at 1 647, 1 650, 1 654, 1 657, and 1 659 cm\(^{-1}\) were assigned to \(\alpha\)-helix. The peaks at 1 666, 1 669, 1 671, 1 674, 1 677, and 1 679 cm\(^{-1}\) were attributed to \(\beta\)-sheet, but \(\beta\)-turn at 1 640, 1 644, 1 682, 1 684, 1 687 and 1 689 cm\(^{-1}\) and random coil at 1 661 and 1 664 cm\(^{-1}\) (Table 1 and Fig. 4). As a result, 19 single peaks were detected (Fig. 5a). Raman Spectral Analysis Package Version 2.1 was used for curve fitting of the amide I band (Fig. 5b), which indicated that the \(\beta\)-sheet, \(\alpha\)-helix, \(\beta\)-turn, and random approximately were glycinin secondary structures accounting for 41.62\%, 21.51\%, 24.70\%, and 12.18\%, respectively (Table 2). Similar results were previously reported for glycinin\(^{[18]}\) and other proteins containing \(\beta\)-sheets, \(\alpha\)-helices, and other structural elements\(^{[19]}\).

3.3 Side Chain Conformation of Glycinin

The doublet at 850 and 830 cm\(^{-1}\) in the Raman spectra of proteins containing tyrosyl residues was examined to determine its origin and detect Fermi resonance between the symmetric ring-breathing vibration and overtone of the out-of-plane vibration of the \(para\)-substituted benzene ring. This information could also reflect whether the tyrosine residues of proteins were exposed or buried\(^{[7]}\). When \(I_{850}/I_{830}\) was 1.25–1.40, 0.7, and 0.3–0.5, the residues representing tyrosine were completely exposed, ionized, and embedded in the surface of proteins molecules, respectively\(^{[20]}\). In this study, the characteristic band of tyrosine side chains in glycinin was \(I_{850}/I_{830} = 1.145\) at 642, 834, 855, and 1 204 cm\(^{-1}\). Furthermore, in accordance with the N (buried or exposed) calculation method described by Kalapathy\(^{[20]}\), the N-buried and N-exposed tyrosine residues account for 14.1\% and 85.9\% of the total amount of residues in glycinin, respectively (Table 3). Thus, the tyrosine residues evaluated in this study tended to be exposed.
Table 1 Tentative assignment of glycinin in Raman spectra

<table>
<thead>
<tr>
<th>Band position (cm(^{-1}))</th>
<th>Assignments</th>
<th>Structural information</th>
</tr>
</thead>
<tbody>
<tr>
<td>510</td>
<td>c–c–s–s–c–c</td>
<td>Gauche–gauche–gauche</td>
</tr>
<tr>
<td>528</td>
<td>c–c–s–s–c–c</td>
<td>Gauche–gauche–trans</td>
</tr>
<tr>
<td>622, 1 004, 1 032</td>
<td>Tyrosine</td>
<td>Aromatic amino acid residues</td>
</tr>
<tr>
<td>642, 834, 855</td>
<td>Tryptophan</td>
<td>Indole ring</td>
</tr>
<tr>
<td>756, 1 337, 1 554</td>
<td>C–C stretching vibration</td>
<td>Microenvironment polarity</td>
</tr>
<tr>
<td>935, 1 122, 1 448</td>
<td>Phenylalanine</td>
<td>Normalization index</td>
</tr>
<tr>
<td>830, 850</td>
<td>Fermi resonance</td>
<td>—</td>
</tr>
<tr>
<td>1 204</td>
<td>Tyrosine and phenylalanine</td>
<td>Aromatic amino acid residues</td>
</tr>
<tr>
<td>1 242</td>
<td>Amide III bands</td>
<td>—</td>
</tr>
<tr>
<td>1 618</td>
<td>Tyrosine and tryptophan</td>
<td>Aromatic amino acid residues</td>
</tr>
<tr>
<td>1 642, 834, 855</td>
<td>—</td>
<td>C=O and C—N stretching, and N—H rocking</td>
</tr>
<tr>
<td>1 622, 1 004, 1 032</td>
<td>Phenylalanine</td>
<td>Indole ring</td>
</tr>
<tr>
<td>756, 1 337, 1 554</td>
<td>Tryptophan</td>
<td>Aromatic amino acid residues</td>
</tr>
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</table>

Table 2 Raman bands and quantification estimation of secondary structures of glycinin in amide I band

<table>
<thead>
<tr>
<th>Raman spectra</th>
<th>β-sheet</th>
<th>α-helix</th>
<th>β-turn</th>
<th>Random coil</th>
</tr>
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<tbody>
<tr>
<td>Band position (cm(^{-1}))</td>
<td>1 666, 1 669, 1 671, 1 674, 1 677, 1 679</td>
<td>1 647, 1 650, 1 654, 1 657, 1 659</td>
<td>1 640, 1 644, 1 682, 1 684, 1 687, 1 689</td>
<td>1 661, 1 664</td>
</tr>
<tr>
<td>Secondary structures (%)</td>
<td>41.62 ± 1.02</td>
<td>21.51 ± 0.64</td>
<td>24.70 ± 0.37</td>
<td>12.18 ± 0.25</td>
</tr>
</tbody>
</table>

Table 3 850/830 and tyrosine residues analysis results of glycinin

<table>
<thead>
<tr>
<th>Glycinin</th>
<th>(I_{850}/I_{830})</th>
<th>(N_{\text{buried}})</th>
<th>(N_{\text{exposed}})</th>
<th>(N_{\text{exposed}}/N_{\text{buried}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Values</td>
<td>1.145</td>
<td>0.141</td>
<td>0.859</td>
<td>6.091</td>
</tr>
</tbody>
</table>

Fig. 5 Deconvoluted spectrum (1 640–1 690 cm\(^{-1}\)) region (a) and curve-fitted individual component bands (b) of amide I in glycinin Raman spectrum

Amino acids containing disulfide bridges (—S—S—) can provide information that aids in the reliable interpretation of the registered Raman spectra. Proteins containing —S—S— are typically identified by Raman spectroscopy, as the —S—S— stretching vibration is polarized and highlighted in the Raman spectra. Based on the regions between 500 and 550 cm\(^{-1}\) of the Raman spectra, the —S—S— of glycinin appeared in three different conformations. The primary conformation and position were gauche—gauche—gauche (g—g—g) indicated by the Raman spectra band at 512 cm\(^{-1}\), which was the dominant conformation in many proteins containing —S—S— bonds. Other secondary bands observed at 525 and 540 cm\(^{-1}\) were attributed to gauche—gauche—trans (g—g—t) and trans—gauche—trans (t—g—t) conformations, respectively\([21]\). In the present study, the multiple fitting results of the glycinin disulfide stretching vibration indicated that the g—g—g—g—g—g—t and t—g—t conformations are 34.8%, 32.1%, and 33.1%, respectively. Similarly, phenylalanine, tyrosine, tryptophan, and other aromatic amino acids containing —S—S— could provide relevant information regarding proteins side chains. Hartwig et al.\([22]\) demonstrated that Raman spectroscopy was a very sensitive method for differentiating the aromatic amino acids of phenylalanine, tyrosine, and tryptophan, in spite of the fact that their structures differed between the phenyl rings and indole ring, as well as the side-chain planarity in the nature. Characteristic Raman bands due to phenylalanine residues of glycinin were observed at 622, 1 004, 1 032, and 1 204 cm\(^{-1}\), with the sharp peak at 1 004 cm\(^{-1}\) corresponding to vibration of the phenyl ring, which is typically not chemically modified and affected by proteins conformational changes. Therefore, this characteristic peak is often used as a normalized structure factor. Another band observed at 1 032 cm\(^{-1}\) is assigned to the phenylalanine CH-in-plane bending of the stretching vibration. The intensities of Raman bands near 756 and 1 340 cm\(^{-1}\) is related to the hydration environment and lipophilicity of the tryptophan indole ring, which is present in various proteins.\([23]\).
The glycinin Raman spectra at 756, 1,337, and 1,554 cm\(^{-1}\) very clearly presented most of the characteristic bands (e.g., the tyrosine residue of glycinin, the buried and exposed tyrosine gauche–gauche–trans, and 33.1% trans–gauche–trans. In total, the main chain of glycinin was composed of 21.51% \(\alpha\)-helix, 41.62% \(\beta\)-sheet, 24.70 \(\beta\)-turn, and 12.18% random coil. And the C–C–S–S–C–C side chain contained 34.8% gauche–gauche–gauche, 32.1% gauche–gauche–trans, and 33.1% trans–gauche–trans. In total, the buried and exposed tyrosine residues accounted for 14.1% and 85.9%, respectively.

4 Conclusions

A Raman spectrum method was used to characterize the secondary structures of glycinin and each characteristic peak was identified. The experimental results showed that the main chain of glycinin was composed of 21.51% \(\alpha\)-helix, 41.62% \(\beta\)-sheet, 24.70 \(\beta\)-turn, and 12.18% random coil. And the C–C–S–S–C–C side chain contained 34.8% gauche–gauche–gauche, 32.1% gauche–gauche–trans, and 33.1% trans–gauche–trans. In total, the buried and exposed tyrosine residues accounted for 14.1% and 85.9%, respectively.

Conflict of Interest

The authors declare that there is no conflict of interest.

References