Article

Study on Antibacterial Activity and Structure of Chemically Modified Lysozyme

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Abstract: Egg white lysozyme was modified by chemical methods using organic acids. Caffeic acid and p-coumaric acid in organic acids were used as modifiers, and 1-Ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride and N-hydroxy succinimide were used as dehydration condensation agents during modification. A certain degree of modified lysozyme was obtained through appropriate modification conditions. The antibacterial properties and structure of the obtained two organic acid modified lysozymes were compared with natural enzymes. The results showed that compared with the natural enzyme, the activity of modified lysozyme decreased, but the inhibitory effect on Gram-negative bacteria was enhanced. The minimum inhibitory concentrations of caffeic acid modified enzyme and p-coumaric acid modified enzyme on *Escherichia coli* and *Pseudomonas aeruginosa* were 0.5 mg/mL and 0.75 mg/mL, respectively. However, the antibacterial ability of modified lysozyme to Gram-positive bacteria was lower than that of natural enzyme. The minimum inhibitory concentration of caffeic acid modified enzyme and p-coumaric acid modified enzyme to *Staphylococcus aureus* and *Bacillus subtilis* was 1.25 mg/mL. The peak fitting results of the amide-I band (1600 cm^{-1}-1700 cm^{-1}) absorption peak in the infrared spectroscopy showed that the content of the secondary structure of the two modified enzymes obtained after modification was different from that of natural enzymes.

Keywords: hen egg white lysozyme; chemical modification; antibacterial activity; secondary structure

1. Introduction

Lysozyme is a kind of naturally monomeric protein, which has good antibacterial effect and widely exists in nature. In nature, there are type C, type I, type G and other types of lysozyme [1]. Hen egg white lysozyme is C-type lysozyme which consisting of 129 amino acids. It is a glycoside hydrolase with a molecular weight of about 14400 and an isoelectric point of about 11 [2]. The chemical property of hen egg white lysozyme is very stable. When the pH value is in the range of 1.2 - 11.3, the structure is almost unchanged, and the stability to heat is also very strong in acid environment [3]. Lysozyme has the functions of antibacterial, antiviral, scavenging necrotic tissue, accelerating wound repair and regeneration, and is widely used in the food and pharmaceutical industries and it has been officially approved by many countries and organizations as a food preservative or preservative. But natural lysozyme is a non-broad-spectrum antibacterial agent that mainly acts on Gram-positive bacteria. Lysozyme achieves antibacterial activity by destroying the β-1,4 glycosidic bond between N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) in the bacterial peptidoglycan layer [4]. Due to the high content of peptidoglycan in the cell wall of Gram-positive bacteria, lysozyme has a good antibacterial effect on Gram-positive bacteria. However, gram-negative bacteria in vitro lipid outer membrane package and cell wall contains only a small amount of peptidoglycan, which to some extent affected the lysozyme on gram-negative bacteria antibacterial effect, limiting its development potential as a natural preservative [5].

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To change this limitation, Visalsok Touch et al. [6] used 2 mmol/L dithiothreitol to treat lysozyme for 0.5 - 4 h at pH 8.0 and 30°C, which enhanced the bactericidal effect of lysozyme on Gram-negative bacteria. Ibrahim et al. [7, 8] thought that structural modification of lysozyme with a membrane-fusing hydrophobic domain, such as saturated hydrophobic peptides or fatty acids, may facilitate the ability of lysozyme to access and move through the outer cell membrane and thus destroy Gram-negative bacteria, so Ibrahim added a hydrophobic peptide to the C-terminus of the lysozyme molecule, which increased the inhibitory effect of lysozyme on *Escherichia coli*, and Ibrahim et al. [9] also used long-chain fatty acids to modify natural lysozyme, so that the antibacterial ability of modified lysozyme to Gram-negative bacteria can be enhanced. Besides, Ibrahim et al. [10] also modified lysozyme with perillaldehyde, and synthesized Periliaedhyed-lysozyme1 polymer with inhibitory ability to *Staphylococcus aureus* and *Escherichia coli* k12, Andreas and Berkop-Schnurch et al. [11] used cinnamic acid and caffeic acid to modify lysozyme, and found that lysozyme can bind to these aromatic organic acids through covalent bonds, so as to achieve the purpose of expanding the antibacterial spectrum of lysozyme. However, the modified lysozyme obtained by this modification method will reduce the inhibitory effect on Gram-positive bacteria while inhibiting.

In order to further explore the effect and change of lysozyme on antibacterial ability after chemical modification by organic aromatic acids, this study focused on the inhibitory effect of caffeic acid and p-coumaric acid modified lysozyme on Gram-negative bacteria and the structural changes of the enzyme before and after modification.

2. Materials and Methods

2.1. Materials

Strains and reagents: *Staphylococcus aureus* ATCC43300, *Micrococcus lysodeikticus* ATCC4698, *Escherichia coli* ATCC25922, *Bacillus subtilis* ATCC6051, *Pseudomonas aeruginosa* ATCC27853, were provided by Biological Public Laboratory of Harbin Institute of Technology, Weihai. Hen egg white lysozyme, 1-Ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), caffeic acid, p-coumaric acid and N-hydroxy succinimide (NHS) were purchased from Shanghai Aladdin Biochemical Technology Company, and peptone, beef extract, agar powder, purchased from Sinopharm Chemical Reagent Company.

Main experimental equipment: ultra-clean worktable, high pressure steam sterilization pot, constant temperature incubator, constant temperature water bath, freeze dryer, high speed centrifuge, UV-Visible spectrophotometer (UV-1800, Shimazu, Japan), Fourier Transform Infrared Spectrometer with Attenuated Total Reflectance cell (TENSOR II, Bruker, Germany).

2.2. Preparation of Modified Lysozyme

30 mg caffeic acid and p-coumaric acid were dissolved in 5 mL 3 mol/L NaOH solution, adjusted to pH 7.5 with 5 mol/L HCl, and then 120 mg EDC and 80 mg NHS were added to each organic acid solution, respectively. After complete dissolution, the solution was placed at room temperature 25 °C for 40 min. Then 60 mg lysozyme was added to the solution. In this reaction system, the mass ratio of organic acid to lysozyme was 1:2, and the mixture was stirred in a water bath at 35 °C for 24 h. After the reaction was completed, the insoluble part of the system was removed by centrifugation (4000g, 15 min), and the supernatant was retained for subsequent experiments.

2.3. Sephadex G-25 Column Chromatography

Purification of modified lysozyme, 5 mL of the reaction solution sample was injected into Sephadex G-25 column and eluted with 0.1 mol/L, pH 6.2 phosphate buffer (PBS). The flow rate was 0.8 mL/min and the detection wavelength was 281 nm. Collection of peaks with lysozyme activity, freeze drying, removal of unreacted free caffeic acid, p-coumaric acid and other impurity molecules.

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2.4. Determination of Lysozyme Activity

2.4.1. Preparation of Micrococcus lysodeikticus suspension

Micrococcus lysodeikticus was inoculated in liquid medium and cultured at 28 °C and 200 rpm for 24 h. Under the condition of 5000g, 15 min, the bacterial solution obtained by expanding culture was centrifuged, and the final bacterial precipitation was washed three times with sterile saline, and the final bacteria were frozen with sterile glycerol as a protective agent. When the lysozyme activity was measured, the 0.1 mol/L, pH 6.2 PBS was used to dilute it into a certain concentration of bacterial suspension, so that the OD value at 450 nm was about 1.0.

2.4.2. Determination of lysozyme activity

5mg lysozyme was dissolved in 0.1 mol/L pH 6.2 PBS, so that the concentration of the enzyme solution to be tested was 1 mg/mL. After the substrate suspension and the enzyme solution to be tested were placed in a water bath at 25 °C for 30 min, the substrate suspension was taken out to determine its OD value under 450 nm, and then the enzyme solution to be tested was added and quickly shaken up. The OD_{450} value was measured once every 30 s for three consecutive measurements. At room temperature, the decrease of OD_{450} value per 30 s was 0.001 as an enzyme activity unit (25 °C, pH 6.2). The unit of enzyme activity per milligram (U/mg) can be expressed by Formula 1. The enzyme activity of natural enzyme and modified lysozyme was determined by this method. The highest enzyme activity was 100%, and the relative enzyme activity was the ratio of the measured enzyme activity to the highest enzyme activity.

$$\mathrm{U/mg} = \frac{(\Delta \mathrm{OD}_{450}/\ \mathrm{min}\ )}{mass\ of\ enzyme\ sample\ (mg)} \times 10^3 \qquad (1)$$

2.5. Determination of Antibacterial Ability

2.5.1. Preparation of Bacterial Suspension

On the super clean bench, a small amount of *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa* were picked with inoculation ring and inoculated on sterilized culture media respectively, and cultured at 28 °C for 24 h. Then the single colonies of various bacteria were picked with the inoculation ring and inoculated into the sterilized liquid medium. The liquid medium was placed in a constant temperature shaker at 28 °C for 24 h. The culture of each strain was diluted to 0.5 McFarland 's turbidity by McFarland 's turbidimetry, i.e. [12], the culture concentration was diluted to 1.5×10^8 CFU/mL.

2.5.2. Determination of Inhibition Zone

The 20 mg of natural hen egg white lysozyme, caffeic acid modified lysozyme and p-coumaric acid modified lysozyme were weighed respectively. In the preparation process of the sample solution, 1 mL 0.1 mol/L pH 6.2 phosphate buffer was used as the solvent, so that the final concentration of the sample solution was 20 mg/mL. Using the filter paper method, each test bacterial solution that had been purified and cultured and diluted to 1.5×10^8 CFU/mL was coated in a solid medium. After the bacterial solution on the surface of the medium was fully dried, the sterilized filter paper was placed on the surface of the medium, and 20 μL of the prepared enzyme sample solution was respectively added dropwise on the filter paper, and cultured at a constant temperature of 28 °C for 24 h. The diameter of the inhibition zone of each enzyme sample solution in the petri dish against different strains was measured.

2.6. Determination of Minimum Inhibitory Concentration (MIC)

A certain amount of natural enzyme and modified enzyme were dissolved in 0.05 mol/L pH 6.2 phosphate buffer solution, and the sample solutions with mass concentrations of 1.50 mg/mL, 1.25 mg/mL, 1.00 mg/mL, 0.75 mg/mL, 0.50 mg/mL and 0.25 mg/mL

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were prepared [13]. 200 μL, 1.5 × 10^8 CFU/mL *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* were inoculated in each series respectively, and each concentration was repeated three times. Incubation at 28 °C constant temperature incubator for 24 h, count the number of colonies. The sample solution was replaced by 0.05 mol/L pH 6.2 phosphate buffer as blank control. The colony growth on the plate was observed, and the concentration of the sample in the sterile growth plate was MIC.

2.7. Fourier-transform Infrared (FTIR) Spectroscopy and Protein Secondary Structure

Infrared spectra of enzymes were recorded using FTIR spectrometer which equipped with Attenuated Total Reflectance (ATR) accessory. D_2O has no interference absorption in the amide I band (1600 cm^{-1}-1700 cm^{-1}), and the amide I band (1600 cm^{-1}-1700 cm^{-1}) is an important wavenumber segment for the secondary structure information of the protein, so an appropriate amount of natural hen egg white lysozyme, caffeic acid modified lysozyme and p-coumaric acid modified lysozyme were dissolved in D_2O to prepare a D_2O solution and sampled for ATR-FTIR scanning [14-16]. ATR-FTIR Set parameters: Spectral resolution 4 cm^{-1}, Wavenumber scanning range 4000 cm^{-1}-400 cm^{-1}, Scan times 32. After obtaining the infrared spectrum, Peak Fit v4.12 software was used to analyze the l600 cm^{-1}-1700 cm^{-1} band belonging to the characteristic peak of amide I band in the infrared spectrum. Firstly, the baseline was corrected, and then the Gaussian method was used to deconvolution, and then the second derivative method was used to fit, and the multiple fitting was performed until it coincided with the original spectrum [17, 18]. After the fitting was completed, the characteristic peak position of each conformation and the ratio of the area of each characteristic peak to the total area were obtained, and the proportion of each conformation in the protein secondary structure was obtained.

3. Results and Discussion

3.1. Analysis of Column Chromatography Results

Separation and purification by Sephadex G-25 column, the elution curves are shown in Figure 1 and Figure 2. Elution peaks 1, 1', first outflow, the corresponding component is a macromolecular substance, elution peaks 2, 2', after the outflow, the corresponding is a small molecule substance, were collected related components, measuring enzyme activity, can be obtained elution peaks 1, 1', with enzyme activity, is a mixture containing lysozyme, elution peaks 2, 2', does not have enzyme activity, is not involved in the reaction of free small molecule substances, mainly not involved in the reaction of free caffeic acid, p-coumaric acid and other impurity molecules, so the collection of peaks 1, 1', with enzyme activity components freeze-dried.

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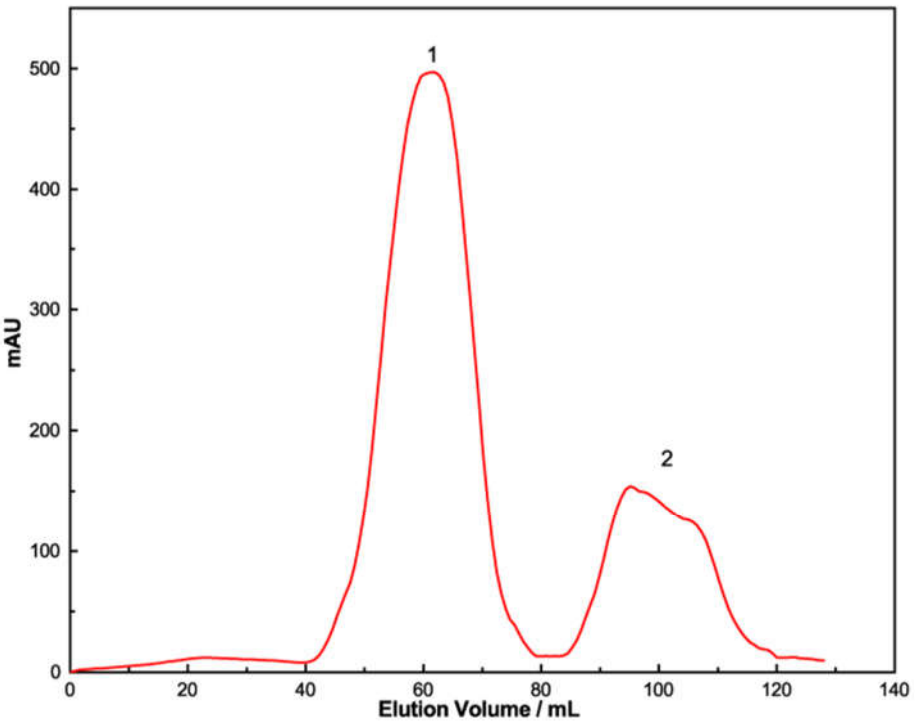

Figure 1. The gel chromatography spectra of caffeic acid modified enzyme.

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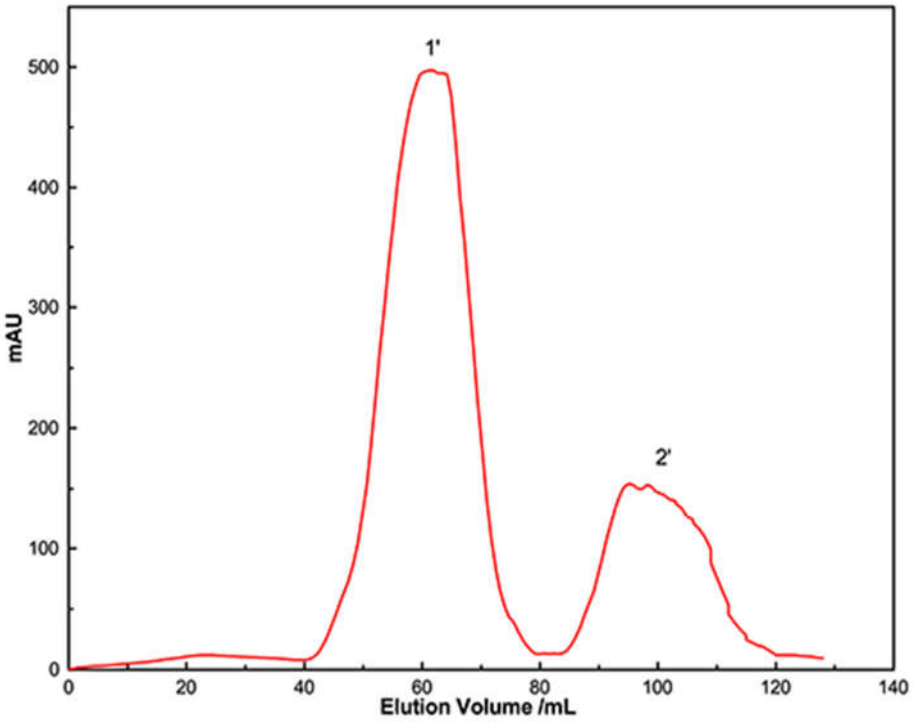

Figure 2. The gel chromatography spectra of p-coumaric acid modified enzyme.

3.2. Lytic Activity

Lysozyme activity measured with *Micrococcus lysodeikticus* as substrate is shown in Figure 3. In Figure 3, obviously, compared with the natural enzyme, the activity of the modified enzyme decreased. It was calculated that the activities of caffeic acid modified enzyme and p-coumaric acid modified enzyme were 69.2 % and 64.7 % of the natural enzyme, respectively. The secondary structure test showed that the content of each secondary structure of the enzyme changed before and after modification, and the change of enzyme structure may be the main reason for the decrease of its activity.

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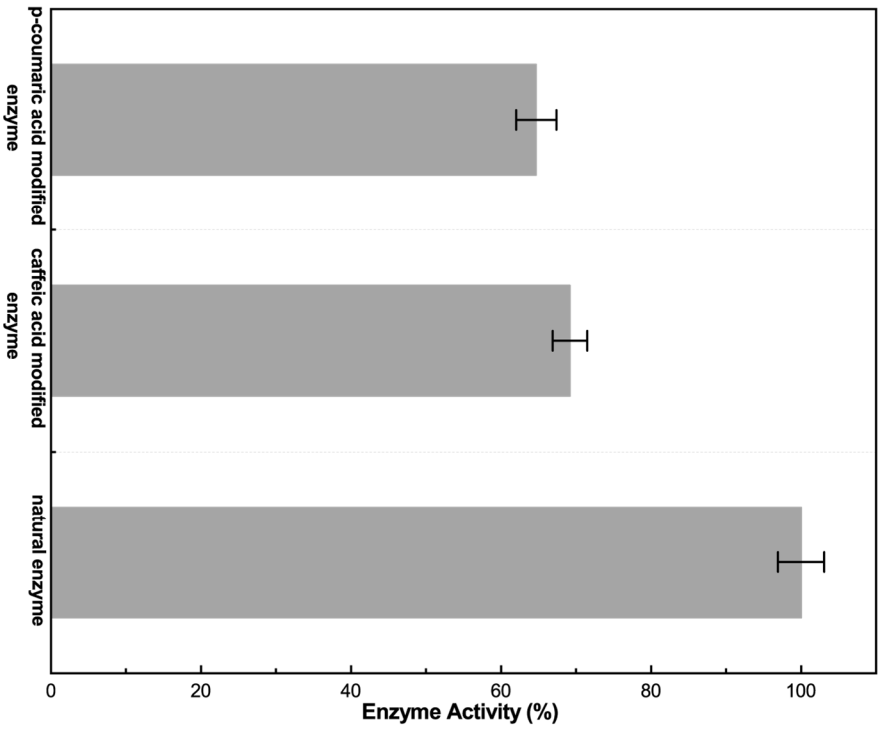

Figure 3. The relative enzyme activity of natural and modified enzyme.

3.3. Antibacterial Effect

The results of the inhibition zone diameter of natural lysozyme and modified lysozyme on different test strains are shown in Figure 4. It can be seen from Figure 4 that the inhibitory effect of caffeic acid modified enzyme and p-coumaric acid modified enzyme on Gram-negative bacteria is stronger than that of Gram-positive bacteria. The inhibition zone diameters of the two modified enzymes against Gram-negative bacteria were greater than 13 mm, which was significantly larger than that of the natural enzyme. Therefore, the antibacterial effect of the modified enzyme on Gram-negative bacteria was significantly enhanced compared with the natural enzyme, and the antibacterial ability of the caffeic acid modified enzyme was slightly stronger than that of the p-coumaric acid modified enzyme. The results showed that the antibacterial effect of modified lysozyme on Gram-negative bacteria was stronger than that of natural lysozyme, but the inhibition ability of modified lysozyme on Gram-positive bacteria was weaker than that of natural lysozyme to some extent. The inhibitory effect of the modified enzyme on Gram-negative bacteria was enhanced while the inhibitory effect on Gram-positive bacteria was weakened. The inhibition zone diameters of the two modified enzymes on Gram-positive bacteria were smaller than that of the natural enzyme.

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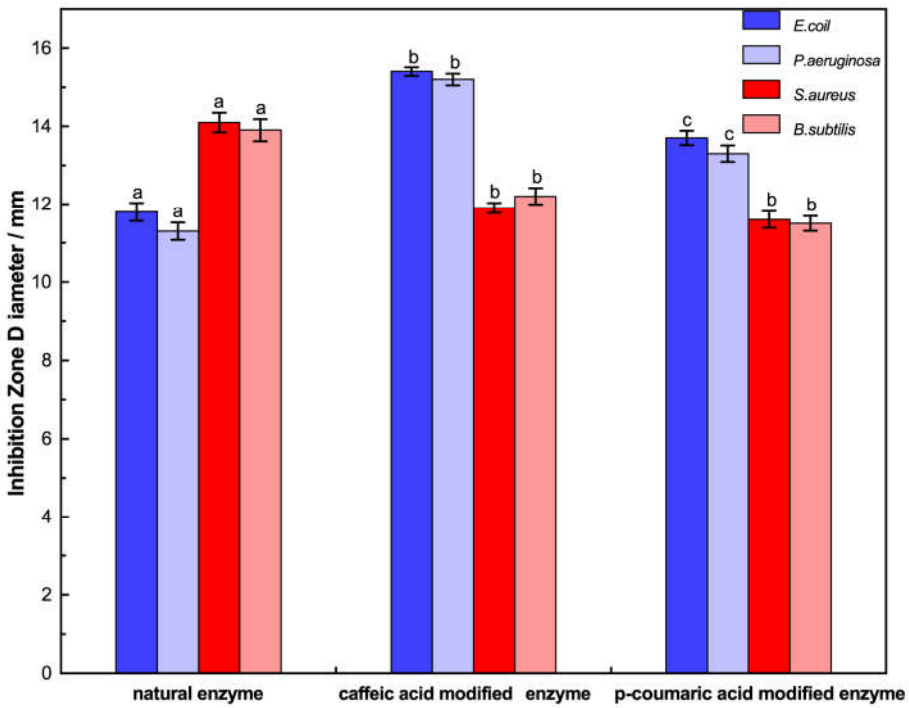

Figure 4. The bacteriostatic circle diameter of natural and modified enzyme samples to different bacteria (the values of different letters marked with the same strain were significantly different, $P < 0.05$).

3.4. Minimum Inhibitory Concentration (MIC)

The MIC results of natural lysozyme and modified lysozyme to different test strains are shown in Table 1. By analyzing Table 1, it can be seen from Table 1 that the two modified lysozymes can effectively inhibit the growth and metabolism of Gram-negative bacteria to a certain extent. The MIC results of natural enzyme and modified enzyme against Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) were as follows : natural lysozyme 1.50 mg/mL, caffeic acid modified lysozyme 0.50 mg/mL, coumaric acid modified lysozyme 0.75 mg/mL, while the MIC of natural lysozyme against Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) was 0.75 mg/mL, and the MIC of modified lysozyme against two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) was greater than 1.00 mg/mL. Through the determination of MIC, it was finally determined that the antibacterial spectrum of modified lysozyme was expanded to a certain extent.

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Table 1. Determination results of MIC of natural lysozyme and modified lysozyme on different test strains.

Bacteria	Sample	Blank	1.50 mg/mL	1.25 mg/mL	1.00 mg/mL	0.75 mg/mL	0.50 mg/mL	0.25 mg/mL
Escherichia coli	1	++	--	++	++	++	++	++
	2	++	--	--	--	--	--	++
	3	++	--	--	--	--	++	++
Pseudomonas aeruginosa	1	++	--	++	++	++	++	++
	2	++	--	--	--	--	--	++
	3	++	--	--	--	--	++	++
Staphylococcus aureus	1	++	--	--	--	--	++	++
	2	++	--	--	++	++	++	++
	3	++	--	--	++	++	++	++
Bacillus subtilis	1	++	--	--	--	--	++	++
	2	++	--	--	++	++	++	++
	3	++	--	--	++	++	++	++

1: natural enzyme, 2: caffeic acid modified enzyme, 3: p-coumaric acid modified enzyme.

++: bacterium growth, --: no bacterial growth.

3.5. Secondary Structure

Amide-I band (1600cm^{-1}-1700cm^{-1}) in the IR spectra of proteins is most sensitive to their secondary structure (α-helix, β-sheet, random coil, β-turn, etc.) [18, 19]. Therefore, the amide-I region was evaluated to estimate the proportion of different secondary structures in natural lysozyme and modified lysozyme. In D_2O solution, the amide I band profile can be decomposed into several components. The wavenumber distributions of the four secondary structures (α-helix, β-sheet, random coil, β-turn) of proteins in the amide I band in the D_2O solution are shown in the Table 2. By peak fitting, the peak separation results are shown in Figure 5. According to the distribution range of the secondary structure in Table 2, the relative proportion of the four secondary structures in Table 3 is obtained by calculating the area ratio. The relative proportions of secondary structure content of natural and modified enzymes are visually displayed in the Figure 6.

From Table 3 and Figure 6, it can be seen that the content of each secondary structure of the modified enzyme has changed compared with the natural enzyme. Compared with the natural enzyme, the content of β-sheet increased after different acid modification, from 28.10 % to 38.87 % and 32.62 %, respectively. The content of caffeic acid modified enzyme and p-coumaric acid modified enzyme random coil decreased compared with natural enzyme, and the decrease of p-coumaric acid modified enzyme was less than that of caffeic acid modified enzyme. The content of α-helix of caffeic acid modified enzyme increased significantly compared with native enzyme, but the content of α-helix of coumaric acid modified enzyme decreased compared with native enzyme. Compared with the content of natural enzyme β-turn, the content of caffeic acid modified enzyme β-turn decreased significantly from 23.27 % to 12.78 %, while the content of p-coumaric acid modified enzyme β-turn increased to 35.33 %. By analyzing the relative content of secondary structure of natural enzyme and modified enzyme, it was deduced that the change of modified enzyme structure may be the reason for the change of enzyme activity and antibacterial activity.

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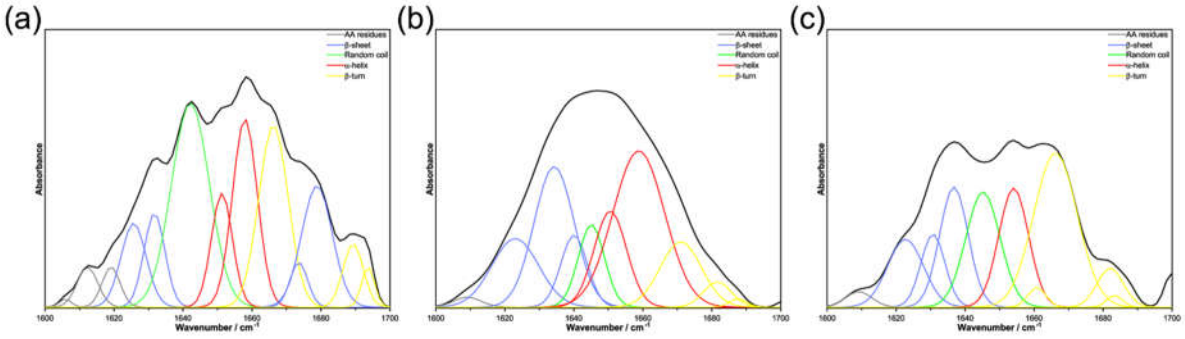

Figure 5. The amide I band fitting diagram of natural enzyme and modified enzyme: (a) natural enzyme (b) caffeic acid modified enzyme (c) p-coumaric acid modified enzyme.

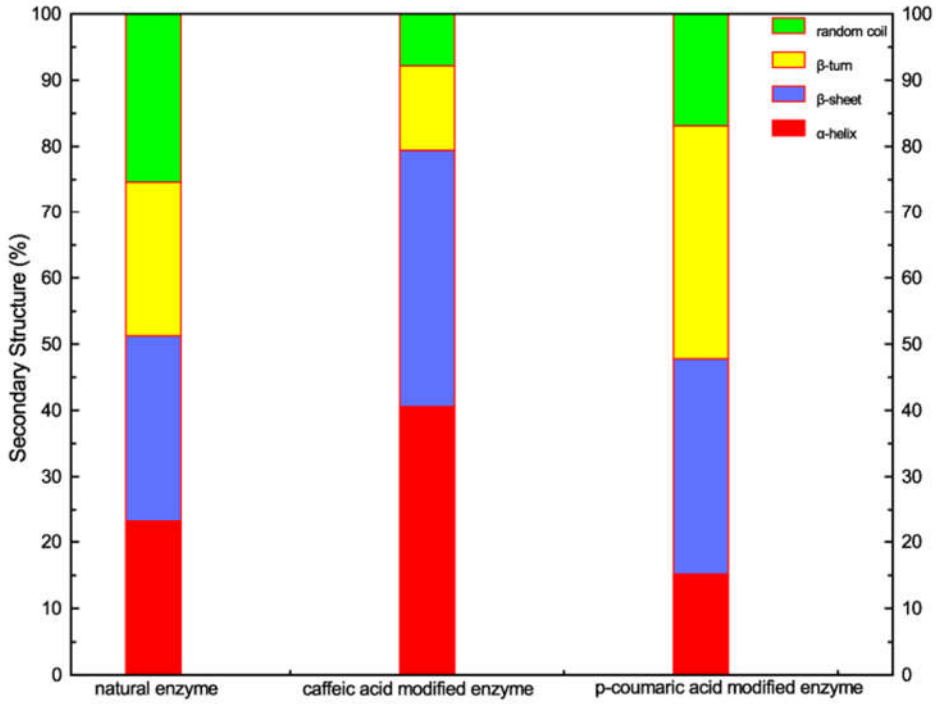

Figure 6. Visual secondary structure relative contents of natural enzyme and modified enzyme.

Table 2. The distribution range of the secondary structure in D_2O solution.

Secondary Structure	α-helix	β-sheet	β-turn	random coil
Wavenumber* (cm^{-1})	1649-1658	1620-1640&1675-1680	1659-1674&1681-1696	1640-1648

*Data are from Susi and colleagues [14, 15, 20, 21].

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Table 3. The relative proportion of the four secondary structures.

		Secondary Structure			
		α-helix	β-sheet	β-turn	random coil
Sample	natural enzyme	23.13%	28.10%	23.37%	25.41%
	caffeic acid modified enzyme	40.53%	38.87%	12.78%	7.82%
	p-coumaric acid modified enzyme	15.17%	32.62%	35.33%	16.88%

4. Conclusion

Compared with natural enzymes, although the enzyme activity of hen egg white lysozyme modified by caffeic acid and p-coumaric acid decreased, the antibacterial effect on Gram-negative bacteria was enhanced. The minimum inhibitory concentrations of caffeic acid and p-coumaric acid modified enzyme against Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) were 0.5 mg/mL and 0.75 mg/mL, respectively, while the inhibitory effect on Gram-positive bacteria was weakened. The minimum inhibitory concentrations of the two modified enzymes against *Staphylococcus aureus* and *Bacillus subtilis* were greater than those of natural enzymes. Compared with the natural enzyme, the content of each secondary structure of the modified enzyme changed to some extent. The change of the secondary structure of this enzyme is the reason for the change of enzyme activity and antibacterial activity. This study provided guidance and expanded ideas for the application of natural hen egg white lysozyme modification to expand its antibacterial spectrum and enhance the antibacterial ability of lysozyme against gram-negative bacteria in the pharmaceutical and food industry.

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