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Improvement of Water Resistance of Zein Film Modified by Autoclave Treatment

Xiaoyu Li[1], Ruihong Li[1], Chengtong Jing[1], An Tu[1], Weicai Sang[1], Shuang Dong[1]*
[1](*School of Agricultural Engineering and Food Science, Shandong University of Technology, China*)

Abstract:
Background*: Zein film has good film forming properties, but raw zein film tends to absorb moisture under high humidity condition, resulting in decreased transparency, which limits its application.*
Materials and Methods*: In order to improve the water resistance, zein film was modified by autoclave treatment. The water absorption, transparency, mechanical properties and enzymatic degradation resistance, as well as the structural properties using Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), X-ray diffraction spectroscopy (XRD) were investigated.*
Results*: The physicochemical properties of zein film showed that the water resistance and transparency of zein film were significantly improved after autoclave treatment, while the mechanical properties were not significantly improved ($p > 0.05$). Enzymatic degradation experiments showed that the degradation rate of zein film after autoclave treatment was decreased from 17.46±0.61% to 14.03±1.05%. ATR-FTIR analysis showed that the ratio of β-turn in the secondary structure of modified zein film decreased, and the ratio of α-helix, β-sheet and random coil structure increased. XRD pattern showed that the autoclave treatment could induce a more closely packed crystallinity.*
Conclusion: *Results from this work indicated that autoclave treatment of zein film was an effective modification method to improve the application of zein film.*
Key Word*: zein film; autoclave treatment; water resistance; structural properties.*

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I. Introduction

Zein is the main storage protein in corn seed and the main by-product during industrial corn starch production, accounting for about 50~60% of the total protein content [1]. Zein can be divided into four groups according to different solubility and sequence homology, among which α-zein accounts for 70~85% of the total amount of zein content, and γ-zein is the second most abundant derivative (10~20%) [2]. Zein is constituted of mainly non-polar amino acids (e.g., leucine, proline, alanine and phenylalanine), while lacks in lysine and tryptophan. Such amino acid composition pattern of zein favors the solubilization in hydroalcoholic solutions, that is insoluble in water and anhydrous ethanol, but soluble in 60~95% ethanol solution and organic solvents such as acetone and acetic acid [3]. In addition, zein contains more sulfur-containing amino acids and the molecules are connected by strong disulfide hydrophobic bonds, which form a favorable structure and molecular basis for the formation of zein film. The amphiphilic properties of zein allow the aggregation of the protein into colloidal particles which are able to form gliadin film without additional plasticizers [4]. Zein film are safe, non-toxic, oxygen-isolating, oil-isolating and degradable [5], and have great development prospects in food packaging, tissue engineering, cell culture, material grafting [6] and microfluidic devices [7-8]. However, raw zein film tends to absorb moisture under high humidity condition, further leading to the poor transparency and mechanical properties. To broaden the application field of zein film, it is urgent to find an effective modification approach and improve the water resistance of zein film.

The modification methods to improve the water resistance of zein films mainly include physical modification, chemical modification and biological modification. Chemical modification is generally carried out by adding some exogenous substances to modify the protein. This method can effectively improve the properties of zein film and improve its physical properties and stability [9-10]. Studies have shown that the addition of polysaccharides, polyphenols, proteins, plasticizers and surfactants can effectively improve the mechanical properties of zein films and reduce water vapor permeability and water solubility of the films [11-12]. But chemical modification process is not easy to control, by-products that are not easy to remove. Biological modification mainly uses protease to degrade macromolecular proteins and improve their functional properties [13]. However, this method largely depends on the activity of the enzyme, which is greatly affected by the external

environment, and it is not easy to operate and control in the actual production [14]. Physical modification refers to heating, freezing, ultrasonic, ultraviolet, radio frequency microwave, ultra-high pressure and so on [15-16]. Physical modification generally does not change the primary structure of the protein and the modification process is simple, less time-consuming, safe and non-toxic. Chen et al. [17] and Sun et al. [18] prepared zein films by hot extrusion method, which showed that the modified zein films had better thermal stability, and the performance of blocking oxygen and water vapor was relatively improved. Moreover, the study of Cheng et al. [19] showed that the heat treatment could affect the distribution of protein molecules in the film. Higher temperature could effectively improve the distribution of molecules in the film, making the structure more compact, which might lay foundation for the improved water resistance of zein film.

The autoclave treatment method used in this study is one of the physical modification approaches. Steams is produced in the hot and humid environment, with its great latent heat and strong penetration capacity, making it easy to denature protein [20]. From the study of whey protein edible film, with the extension of heating time and the increase of heating temperature, the covalent cross-linking degree of the film was increased, which improved the water resistance of the film and tensile properties [21]. In addition, the study of Masamba et al. [22] also confirmed that a higher preparation temperature was a good processing condition for improving the mechanical and water barrier properties of zein composite films.

In this study, zein film was modified by autoclave treatment and the influence of temperature and treatment time of autoclave treatment on water resistance properties of zein film was investigated via water absorption and transparency as indicators. In addition, functional properties (mechanical property and enzymatic degradation resistance) of zein film after treatment were characterized, as well as the structural properties using Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), X-ray diffraction spectroscopy (XRD), to further elaborate the modification mechanism from multiple perspectives. This study could provide a theoretical basis for improving the water resistance of zein films and promote the development and utilization of high-value corn starch by-products.

II. Material And Methods

Materials

Zein (purity of 92.8%) was purchased from Gaoyou Rixing Pharmaceutical Co., Ltd. Trypsin was purchased from Hangzhou Lianke Biotechnology Co., Ltd. Other chemical reagents, e.g., absolute ethanol, glycerol and so on, were of analytical grade.

Preparation of zein film

In this experiment, a casting method was used to prepare zein film according to the method of Qu, et al. [23]. Certain amounts of zein powder were weighed and dissolved in 80% ethanol solution to prepare a film-forming solution with a concentration of 15% (w/v). Glycerin, 20% of protein mass (w/w), was added as plasticizer to improve the flexibility of zein film . The film-forming solution was incubated in a water bath at 60℃ for 10 min, followed by constant stirring by magnetic stirrer at a speed of 500 rpm for 30 min, to ensure complete dissolution of zein and glycerin.

Certain volumes of the film-forming solution were immediately poured into the Petri dishes, and placed horizontally at room temperature (25℃) for 48 h. After ethanol volatilization, zein films were peeled off from the molds and equilibrated in a chamber with relative humidity of 50±2% and temperature of 25 °C for one week.

Autoclave treatment

The autoclave treatment of zein films were carried out via an autoclave sterilizer (LDZX-50FBS, China) at different conditions, termed as $T_{126\text{-}40}$ (126°C, 40 min), $T_{126\text{-}30}$ (126°C, 30 min), $T_{121\text{-}30}$ (121°C, 30 min) and $T_{115\text{-}20}$ (115°C, 20 min), respectively. After treatment, the treated films were cooled at room temperature (25℃) and then equilibrated in a chamber relative humidity of 50±2% and temperature of 25 °C for a week for further analysis.

Thickness

The electronic digital caliper (500-196-30, China) was used to measure the thickness of zein film with an accuracy of 0.01 mm. During measurement, six positions on film samples were selected and the average value was used to calculate the film transparency.

Water absorption

According to the method reported by Ahammed, et al. [24], the treated films were cut into rectangular strips of 1.0 cm×2.5 cm and dried to a constant weight (W_1). The films were immersed in distilled water (40 mL) for 24 h at room temperature (25.0±0.1℃). After that, the excess water were wiped up from surface by filter paper, weighed again and recorded as W_2. The immersed films were dried to a constant weight (W_3). The water absorption was calculated by the following formula:

$$\text{Water absorption} = (W_2 - W_3)/W_1 \times 100\% \quad (1)$$

Where W_1 is initial weight of the film sample (g); W_2 is wet weight after immersion in distilled water (g); W_3 is dry weight after immersion (g).

Transparency

The films were cut into rectangular strips of 1.0 cm×2.5 cm and vertically placed in the ultraviolet spectrophotometer (756PC, China) to measure the absorbance of zein film at the wavelength of 600 nm. Each sample was measured three times and the transparency of film sample was calculated according to the following formula:

$$\text{Film transparency} = T/A_{600} \quad (2)$$

Where T is the thickness of the film (mm); A_{600} is absorbance value at 600 nm.

Mechanical properties

Using a texture analyzer (TA.XT-plus, China) to measure its mechanical properties. The film samples were cut into rectangular strips of 1.0 cm×6.0 cm, and clamped between grips. The initial height was set at 30 mm and the tests were run with the initial cross-head clearance and probe test speed of 50 mm and 50 mm/min, respectively.

Enzymatic degradation resistance

The film samples (2.0 cm×4.0 cm) were dried (50℃) to constant weight and accurately weighed as W_1. PBS buffer solution of 10 mL (containing 0.25% trypsin, pH 7.4) was added to completely immerse the films and the film samples were incubated at 37℃ for 4 h. After that, the surface excess water was wiped out with filter paper and dried (50℃) to the constant weight (W_2). The enzymatic degradation rate of zein film was calculated according to the formula [25]:

$$\text{Enzymatic degradation rate} = (w_1 - w_2)/w_1 \times 100\% \quad (3)$$

Where W_1 is the initial weight of the film (g); W_2 is the weight of the enzymatic degradation film (g).

Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

The molecular structures of zein films were characterized by an Attenuated total reflectance-Fourier transform infrared spectrometer (Nicolet5700, USA). Spectra of raw and treated film samples were collected in the range of 4000-675 cm^{-1}. Each spectrum collected at a resolution of 4 cm^{-1} with an accumulation of 32 scans, and the air was used as the blank background.

X-ray diffraction (XRD)

The X-ray diffractometer (D8, Bruker, Germany) was operated at 40 kV and 40 mA with Cu Kα radiation (λ= 0.154 nm). The raw and treated film samples were leveled placed and scanned from 3° to 45° (2θ) at a speed of 2°/min. The diffractograms were analyzed using JADE 6.5 software.

Statistical analysis

All performances and analyses were conducted in triplicate. Results were reported as mean ± standard deviation (SD) and statistically analyzed using SPSS (version 19.0) software.

III. Result

Water absorption

Fig.1 showed the water absorption rate of zein film under different treatments. As could be seen from Fig.1, the water absorption of untreated zein film was 148.23±6.08%, significant higher than all treated samples ($p<0.05$). In contrast, the water absorption rate was greatly reduced (18.95~28.38%) after autoclave treatment. The lowest water absorption rate was observed for $T_{126\text{-}30}$, though there were no significant difference among the treated samples statistically ($p>0.05$). The results indicated that autoclave treatment could markedly reduce the water absorption of zein film, which further improved the film's water resistance. This was consistent with the research results of Li, et al. [26].

The main reason of the decrease of water absorption might be attributed to the protein denaturation after treatment. During this autoclave treatment, peptide chains of zein were partially folded and the internal sulfur-containing amino acids would be exposed, resulting to form new disulfide bonds [27]. At the same time, high temperature promoted the crosslinking reaction of disulfide bonds and reduced the binding ability of zein to water molecules. These new formation of disulfide bonds made the structure more compact [28]. Therefore, the hydrophobicity of zein film was significantly increased after autoclave treatment.

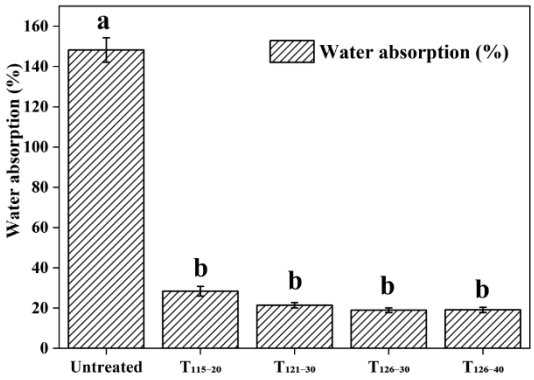

Fig.1 Water absorption of zein films

Film transparency

Good transparency of zein film is favorable in terms of food packaging, microfluidic devices and culture medium. Fig.2 showed the results of film transparency of raw and treated zein films after soaking in water. From the results, the untreated film showed the lowest transparency of 67.51±1.75. After autoclave treatment, the transparency after soaking in water was significantly increased ($p < 0.05$), which demonstrated the autoclave treatment was an effective method in improving the transparency of zein film. Furthermore, combining with the results in section 3.1, there was negative correlation between the transparency and water absorption rate. For instance, the highest transparency of zein films was observed at $T_{126\text{-}30}$, while it was lowest in water absorption (Fig.1). During the autoclave treatment, with the increase of treatment temperature, protein molecules were suggested to pack more closely. The poor fluidity of the polymers was suggested to be the main reason making more difficult for the water molecules to enter [29].

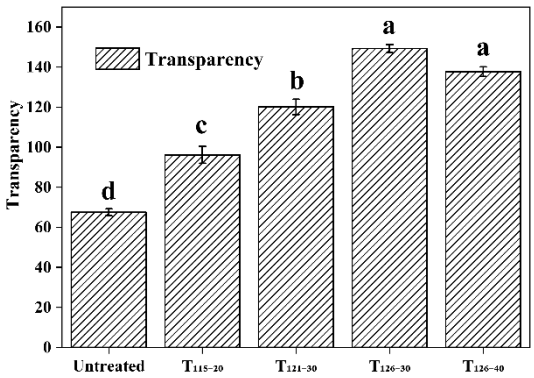

Fig.2 Transparency of zein films after soaking in water

In order to visually examine the transparency, Fig.3 showed the detailed visual changes of zein film before and after soaking in deionized water for 24 h. It could be seen from the photographs that the untreated zein film had poor transparency after soaking, that the background image was hard to identify. Soaking in water caused the raw zein film to absorb water and swell, resulting in a decrease in transparency. However, the clarity of background images was greatly improved after autoclave treatment, indicating the transparency of treated zein films was significantly improved. Zein was insoluble in water, and the interaction between zein and its molecules would be increased when the zein film was immersed in water, leading to the aggregation of zein molecules. Therefore, it would lead to the reduction of scattering degree, and therefore the transparency of zein film [30]. After autoclave treatment, zein molecular structures became more compact, which could effectively reduce the aggregation of zein molecules.

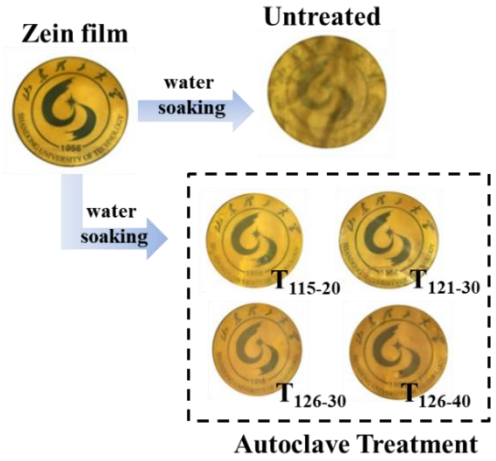

Fig.3 Photographic observation of transparency changes of zein films after water soaking

Mechanical properties

Fig.4 showed the tensile strength (TS) and elongation at break (EB) of zein film under different treatment conditions. It could be seen from Fig.4 that the highest tensile strength of zein film was observed at $T_{126\text{-}30}$ (0.065±0.013 MPa). Compared with the untreated zein film, there was no significant change among the other three samples ($p > 0.05$). As for the elongation at break, the highest value was obtained at $T_{121\text{-}30}$ (3.80±1.25%), though there was no significant difference compared with the untreated zein films ($p > 0.05$). But when the treatment temperature was higher than 121°C, the elongation at break tended to be weaken with the increase of treatment intensity. In general, the mechanical properties of the modified zein films were not significantly improved. The main reason might be that the mositure contained in zein film was volatilized due to the effect of high temperature during autoclave treatment [30], and the glycerol in zein film partially was precipitated due to the reduction of solvent. Glycerol is a kind of high hygroabsorbency molecule with a certain plasticizing effect. The addition of glycerol led to the reduction of intermolecular force along the polymer chain, which effectively improved the flexibility and fluidity of protein chain, accordingly reducing the brittleness and avoiding the shrinkage during processing and storage [31]. Therefore, glycerol precipitation in the treatment process would lead to the increase of brittleness and decrease of tensile strength of the film.

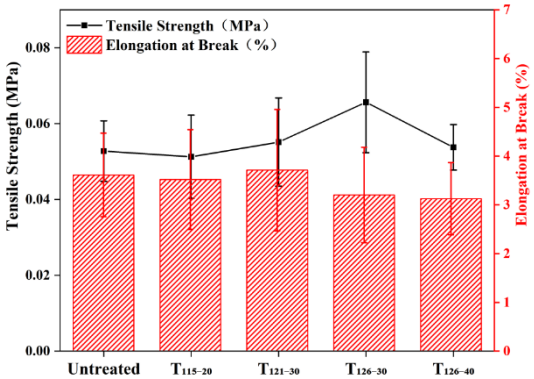

Fig. 4 Mechanical properties of zein films

Enzymatic degradation resistance

As shown in Fig.5, the untreated zein film showed a higher rate of enzymatic degradation rate (17.46±0.61%). After treatment, the enzymatic degradation rate showed a slight downward trend and the lowest value was observed at $T_{126\text{-}40}$ (14.03±1.05%), significantly lower than that of untreated zein film ($p < 0.05$). It could also be seen from the results of $T_{126\text{-}30}$ and $T_{126\text{-}40}$ (Fig.5), the enzymatic degradation decreased gradually with the treatment intensity. This might be due to the structural change of zein film after autoclave treatment.

The arrangement of protein molecules became more compact and the active sites of enzyme might be folded up, thus reducing the enzymatic degradation rate [32].

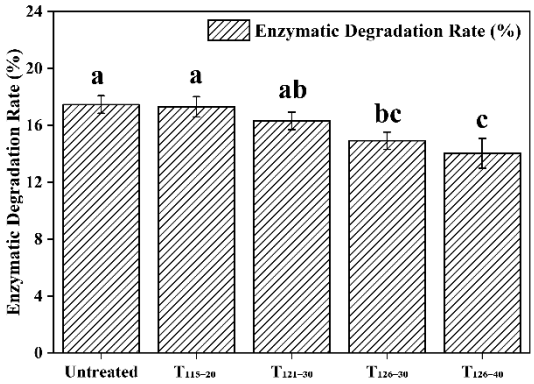

Fig. 5 Enzymatic degradation rate of zein films

ATR-FTIR

Infrared spectroscopy were mainly used to analyze the conformation changes of proteins via some characteristic absorption bands, including amide I (1700~1660 cm^{-1}), amide II (1540~1520 cm^{-1}) and amide III (1300~1220 cm^{-1}). The amide I region is mainly generated by C=O stretching vibration, the amide II region is due to N-H bending and C-H stretching vibration, and the amide III region is attributed to the combination of C-N stretching and C=O bending vibration. The amide III region generally represents the change of side chain and hydrogen bond [33]. Fig.6 showed the infrared spectrum of zein films. As shown in Fig.6a, in general, the spectrums of treated zein films were similar to that of untreated. Moreover, no new peak was observed, indicating that no new chemical groups were generated in zein films. As shown in the partial enlarged detail (Fig.6b), the absorption intensity from 3600 cm^{-1} to 3100 cm^{-1} of treated zein films were all higher than the untreated and the highest peak intensity was observed at T_{126-30}, corresponding to the vibration stretch of O-H [34]. The possible reason for the significant enhancement of absorption of zein film after treatment could be that the content of O-H group in zein film increased after autoclave treatment.

In addition, Fig. 6c & d the region ranging from 1480 to 1200 cm^{-1}represented the fingerprint region of protein, mainly due to the vibration and tautomerism of C-H and N-H single bonds. In Fig.6d, the amide I region represented the change of protein peptide bonds, and the increase of peak intensity in this region indicated the increase of disorder in the protein conformation. The amide II zone was attributed to the forming environment of hydrogen bonds [35]. It could be seen from Fig.6d that the contents of N-H and C=O in the zein films after autoclave treatment are higher than those in the untreated zein films.

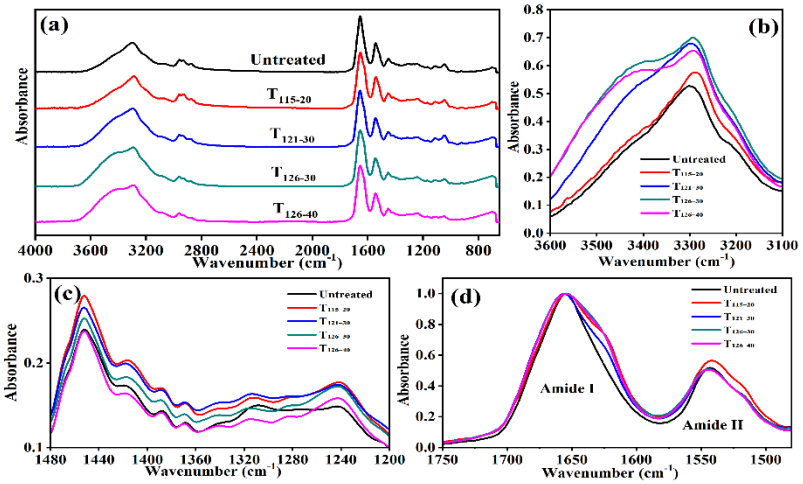

Fig.6 Infrared spectra of zein films

The amide I corresponding region was used to calculate the percentage of the secondary structure elements in zein film and the results were shown in Table 1. Compared with the untreated zein films, the content of random coil structure was increased from 17.07% to 18.33%, while the content of β-turn after 126℃-30min treatment was decreased greatly from 47.45% to 45.64%. Moreover, α-helix and β-sheet were also showed a slight increase after treatment. It was suggested that autoclave treatment would change the secondary structure contents of zein. Possible reasons could be that the processing of high pressure, temperature and humid environment would enhance protein intermolecular and intramolecular hydrogen bonding, thereby promoting the formation of α-helix, β-sheet and random coil secondary structure. The decrease of β-turn content would reduce the flexibility of zein film [36], which was corresponding to the mechanical properties results in section 3.3. The increased content of α-helix and β-sheet structure might result in the increasing hydrophobicity of zein films, which was in agreement with the results above.

Table 1 Relative percentage of secondary structural units of zein films

Secondary structure units	Untreated	$T_{126\text{-}30}$
β-sheet	26.12%	26.47%
random coil	17.07%	18.33%
α-helix	9.36%	9.55%
β-turn	47.45%	45.64%

3.6 XRD

XRD could provide the atomic structure information of crystalline proteins, and was the method most close to the atomic resolution of the three-dimensional structure analysis of protein which could be used to explain the interaction of components in the film [37]. As shown in Fig.7, all the spectra exhibited generally similar diffraction patterns. The untreated films showed a wide diffraction peak at about 19° (2θ) and a small peak at about 9° (2θ), which was consistent with the results obtained in other literatures [38]. The treated films showed two obvious diffraction peaks at around 9° and 19° (2θ), respectively.

According to Bragg's formula ($2d\sin\theta=n\lambda$), the interplanar spacing (d) could be calculated. The interplanar spacing of the treated zein film at around 9° (2θ) peak was at the range of 9.30~9.53 Å, longer than that of the untreated films (7.96 Å). With the increase of treatment temperature, the interplanar spacing of treated zein films increased to different degrees. Meanwhile, at the same temperature, the interplanar spacing decreased with the extension of time. According to previous studies, the interplanar spacing corresponding to the first peak is mainly related to the horizontal accumulation of α-helix structure in protein or the average distance between adjacent helices [39]. It indicated that the d-spacing between inter-helix was more sensitive to the environment of high pressure and humidity, which was conducive to the formation of a more uniform film.

A wide diffraction peak appeared at about 19° (2θ), the calculated crystal plane spacing of untreated zein films was 4.50 Å, and the interplanar spacing of all zein films at this position (4.46~4.58 Å) was not changed significantly [40]. It had been reported that the shorter spacing around 4.50 Å was associated with the mean skeleton distance within the α-helix structure of zein. The results above suggested that autoclave treatment could not affect the main chain of α-helix skeleton. In the process of treatment, the spiral main chain structure of the zein remained stable.

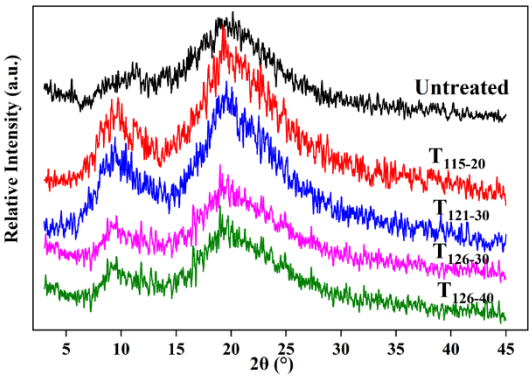

Fig.7 XRD spectra of zein films

IV. Conclusion

In this work, autoclave treatment under different temperature and time was applied to modify zein film to improve water resistance. The treated zein films had significant lower water absorption (18.95~28.38%) and higher transparency (96.23~149.29), indicating improved water resistance. The mechanical properties including TS and EB were not significantly changed ($p > 0.05$), while the enzymatic degradation resistance was enhanced with the treatment intensity. Moreover, ATR-FTIR analysis showed that α-helix, β-sheet and random coil ratio increased slightly and β-turn structure ratio was obviously decreased after modification. XRD analysis showed that the α-helix main chain structure of zein film was not destroyed, but the crystal plane spacing distance between adjacent spirals increased, autoclave treatment helped to form more uniform films. This study could provide a theoretical basis for improving the water resistance of zein films modified by autoclave treatment and improve the application value of zein films in food packaging.

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