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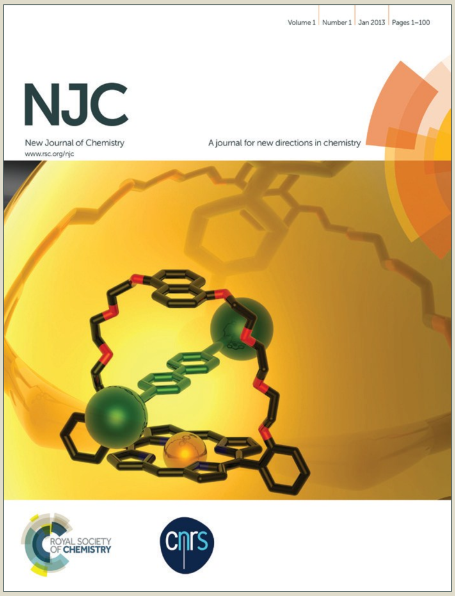

Effect of Pretreatment on the Enzymatic Synthesis of Rosin Acid Starch

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Abstract

A new esterified starch, rosin acid starch (RAS), had been synthesized under mild conditions, using rosin as a starting material directly with Novozym 435 as catalyst. In order to improve the efficiency of the enzymatic reaction, native cassava starch (NCS) was pretreated by heat-moisture, ultrasonic, gelatinized, microwave and alkali treatment before the enzymatic esterification. Scanning electron microscopy (SEM) and X-ray diffraction (XRD) analysis showed the morphology and crystallinity of the NCS were changed according to the pretreated methods. Compared with NCS, the surface of ultrasonic and heat-moisture pretreatd starch (PS) maintained smooth

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and circular morphology structure, and the PS kept high crystalline. The surface of alkali, microwave and gelatinized PS was changed from smooth to crude, with reducing crystalline, but they kept some kinds of granule shape. The activated effect of pretreatment on the degree of substitution (DS) of RAS was as follows: alkali RAS (0.0808) > microwave RAS (0.0174) > gelatinized RAS (0.0153) > ultrasonic RAS (0.0099) > heat-moisture RAS (0.0073) > Native RAS (0.0053). The esterified products from PS were confirmed by FT-IR spectroscopy and Nuclear magnetic resonance (NMR) analysis.

Keywords: Pretreatment; Enzyme Biocatalysis; Starch; Biosynthesis; Enzyme Technology

1. Introduction

As a ubiquitous and very abundant biopolymer, starch is considered as the most promising candidate to replace traditional petroleum-based products in many industrial applications [1,2]. Many efforts have been made to improve the performance of the starch [3]. In our group, rosin acid starch (RAS), a novel starch ester, was synthesized recently [4], using rosin as a starting material directely, the esterification reaction was catalyzed by lipase (Novozym 435) under mild conditions. Rosin acid constitutes the main component of rosin gum, a kind of natural product obtained from conifer exudates, which is a mixture consisting primarily of abietic acid, levopimaric acid and pimaric acid, with a typical molecular formula $C_{19}H_{29}COOH$ [5]. There are two chemically reactive centers in the rosin acid molecule, the double bond and the carboxyl group [6]. The characteristic bulky hydrophenanthrene ring structure in rosin acid molecule is similar to petroleum-based cycloaliphatic and aromatic compounds, which provides substantial hydrophobicity, rigidity and chemical stability [7]. The study about the physicochemical properties of the RAS shows that esterified starch with rosin acid significantly improves its performances in hydrophobicity, viscosity and emulsifying properties [8]. Starch ester materials can improve cereal products by increasing their production efficiency [9], and can be used as free-flowing powders in coffee whiteners, whipped topings or whipped creams, and ice cream, for their improving emulsifying properties. Moreover, the properties of RAS depended on the degree of substitution (DS) [10,11]. Our previous research showed that the swelling power and solubility in water of the esterified starch were decreased as the DS increased [10]. The RAS might be used as an ingredient where viscosity and hydrophobic interactions were desired, such as surface coating materials, flavoring agents in food industry and biomedical materials.

However, the reaction efficiency of the enzymatic synthesis of RAS was relatively lower. The DS value of the esterified starch was just from 0.031 to 0.102 for 4 h reaction in our previous work [10]. Horchani et al. [12] have achieved a DS of 2.86 by using microwave to heat in the solvent-free lipase-catalyzed synthesis of long-chain starch esters. Lu et al.[13] have report the DS of the palmitic acid starch reaches to 0.144 for 3 h reaction when the enzymatic esterification took place in ionic liquid mixtures consisting of 1-butyl-3-methyl-imidazolium acetic ([BMIm]Ac) and 1-butyl-3-methyl-imidazolium tetraflouroborate ([BMIm][BF4]). It is indicated that the [BMIm]Ac can destroy the semicrystalline structure of native starch granules by disrupting hydrogen bonding between hydroxyl groups in starch molecules, results in improving the reaction efficiency. Considering the fact that the starch granule is composed of noncrystalline region and alternating crystalline, chemical reagents are blocked from contacting with the molecules in the crystalline region, which leads to the low reactivity of native starch [14,15]. Therefore, pretreatment of starch should be a very important method to improve the chemical reactivity of native starch and the efficiency of the subsequent esterification reaction.

Starch pretreatment methods mainly include physical, chemical and biological degradation. Shariffa et al. [16] have studied enzymatic degradation of cassava starch and sweet potato starch for the sythesis of laurate starch. Compared with native starch, the dextrose equivalent value of heat-treated starch increased significantly, i.e., 36-50% and 27-34% for tapioca and sweet potato starch; Zhang et al. [17] have investigated the effects of pre-gelatinized pretreatment on the structure and characteristic of corn starch. Their results indicated that large quantities of microcrystals were formed with the reduction of moisture content, and the X-ray diffraction pattern of pregelatinized starch was a combination of an unsharp crystalline and noncrystalline diffraction peak. Lewandowicz et al. [18] found that the crystal structure of potato and tapioca starches was changed from type B to type A after the microwave radiation treatment. Huang et al. [19] have investigated ultrasound pretreatment on the corn starch. They found the amorphous area of ultrasound-treated corn starch is slightly destroyed, and the reaction efficiency increases. Ball-milling treatment can also affect the crystal structure of starch and improve their chemical activity in the grafting or esterification reaction [15,20]. In the study of enzymatic synthesis of corn starch palmitate, Geng et al. [21] find that the size of starch decreases and the crystalline pattern changes after treated by NaOH/Urea, which results in the improvement of the DS. Therefore, in order to improve the reaction efficiency of the enzymatic synthesis of rosin acid starch, starch pretreatment should be taken into account.

In this research, NCS was pretreated by heat-moisture, ultrasonic, gelatinized, microwave and alkali treatment before reacting with rosin acid. The efficiency of the esterification reaction was determined by analyzing the DS of RAS. The morphological and structural features of NCS, PS and RAS were investigated by SEM, FT-IR and ^{1}H NMR analysis. The effect of starch pretreatment on the synthesis of RAS was discussed.

2. Materials and methods

2.1 Materials

Gum rosin was supplied by Guangxi Wuming Chaoyan Rosin Plant, China, and was used directly for the esterification reaction. Cassava starch (approximately 17% amylose and 83% amylopectin) was purchased from Guangxi Cenxishi Sanjiao Food Scuffled. Novozym 435 with an activity of 10 unit/mg was purchased from Novo Industries, Denmark. DMSO, methanol and acetone were analytical grade purchased from Chengdu Kelong Chemical Reagent Co., China.

2.2 Pretreatment of cassava starch

2.2.1 Alkali pretreatment

According to the method of Geng et al. [21], cassava starch (4 g) was dissolved in NaOH/urea solution (6 g NaOH and 3 g urea in 100 ml deionized water) completely, and then neutralized with HCl. The starch was washed with 100 ml 95% of ethanol twice after being precipitated by the same solvent. Finally, the precipitate was dried at 70 ℃ for 24 h.

2.2.2 Gelatinized pretreatment

According to the literature [22], cassava starch suspension at the same concentration of 50% (m/v) was gelatinized in boiling water bath, and then cooled to room temperature. The starch was precipitated by adding 3 volumes of 95% ethanol to the gelatinized solution, then the precipitation was collected and dried at 70 ℃ for 24 h.

2.2.3 Ultrasonic pretreatment

According to the method described by Isona et al. [23], 30 ml cassava starch suspension at the same concentration of 30% (m/v) was sonicated on ice bath at 100 W for 30min using a JY92-Ⅱultrasonic disruptor (Xinzhi, Ningbo, China). The starch was washed with ethanol twice after precipitated by the same solvent. Finally, the precipitate was dried at 70 ℃ for 24 h.

2.2.4 Heat-moisture pretreatment

According to the method described by Kayode et al. [24], cassava starch (10 g) was put into a 100ml conical flask with cotton plug bottle, and then placed in an autoclave and treated at 120 ℃. Finally, the PS was dried at 70 ℃ for 24 h.

2.2.5 Microwave pretreatment

According to the literature [25], NCS suspension at the same concentration of 6% (m/v) was placed in the EM-2511EH microwave oven (SANYO, Hefei，China), and treated with high heating power for 10 min. The starch suspension was cooled to room temperature. The starch was washed with ethanol twice after precipitated by the same solvent. Finally, the precipitate was dried at 70 ℃ for 24 h.

2.2.6 General procedure for esterification reaction

Reference [26], 0.25g PS was dissolved in 50 ml DMSO in round flask, after adding 0.44 g rosin acid. To the mixtures, 15% immobilized lipase (Novozym 435) (m/m, relative to starch) was added to initiate the reaction. Reaction was carried out at 45 ℃ with a magnetic stirrer for 4 h and stopped by filtering the reaction mixture through 200 mesh filter to remove the immobilized lipase. The RAS was collected after the precipitation by adding 150 ml of methanol, then washed with methanol and dried at 70 ℃ for 24 h.

2.3 Analysis of reaction products

2.3.1 Determination of the DS

The DS, which was defined as the average molar ratio of attached rosin acyl groups per anhydrous glucose unit (AGU), was determined by a titration method. 1 g sample was dissolved in 50 ml DMSO, and then 20 ml 0.2 mol/L NaOH was added. Which made the mixture was stirred for 4 h at 50 ℃. Excess NaOH was back-titrated with 0.1 mol/L HCl solution by using phenolphthalein as indicator. The DS value of starch ester was calculated as follow:

$$DS = \frac{162 \times C(V_0 - V)}{m - 285 \times C(V_0 - V)}$$

Where 162 is the molecular weight of the AUG, 285 is the molecular weight of the rosin acid, V_0 and V is the titration volume of HCl consumed in NCS and RAS respectively, C is the molar concentration of HCl, and m is the sample quantity.

2.3.2 SEM Analysis

The morphologies of NCS, PS and RAS were observed by using a Supra 55 (Zeiss, Germany) scanning electron microscopy (SEM). Before the test, the samples were mounted into the specimen stubs with double-sided tape, and then coated with a thin layer of gold to make the sample conductive. SEM was performed under high vacuum at an accelerating voltage of 5 kV. The photographs were taken by using automatic image capture software.

2.3.3 X-Ray Diffractometry

The X-ray diffraction patterns of the NCS, PS and RAS samples were measured using a D/MAX 2500 V diffractometer (Rigaku, Tokyo, Japan) under the following conditions: Cu Kαradiation, Ni filter disk, 30 mA, 40 kV. The scattering angle (2θ) was varied from 4° to 60° with a step width of 0.02°.

2.3.4 FT-IR measurements

FT-IR spectra were recorded on a MAGNA-IR 550 spectrometer (Nicolet Instruments Corp., Maldison, WI). The samples were mixed with dry KBr at a ratio of 1: 300. The spectra were recorded in a transmittance mode to scan from 4000 to 500 cm^{-1} with a resolution of 4 cm^{-1}.

^{1}H NMR analysis

The ^{1}H NMR spectra were recorded on Bruker Avance 600 MHz spectrometer while the sample was dissolved in deuterated dimethyl sulfoxide (DMSO-d_6) at 30 ℃. The spectra were obtained at 30 ℃ with a pulse angle of 30°, a delay time of 10 s, and an acquisition time of 2 s. All chemical shifts were reported in parts per million (ppm).

3. Results and discussion

3.1 The effect of pretreatment on the morphology and crystallography of cassava starch

Appropriate pretreatment can disrupted the hydrogen bonds of starch and sequentially destroyed the crystalline structure of starch granules, which results in the change of starch granular structure [27-29]. Therefore, by investigating the difference in morphology and crystallography between the NCS and the PS, the information about structure changing by pretreatment can be obtained, and finally its effect on esterification can be deduced.

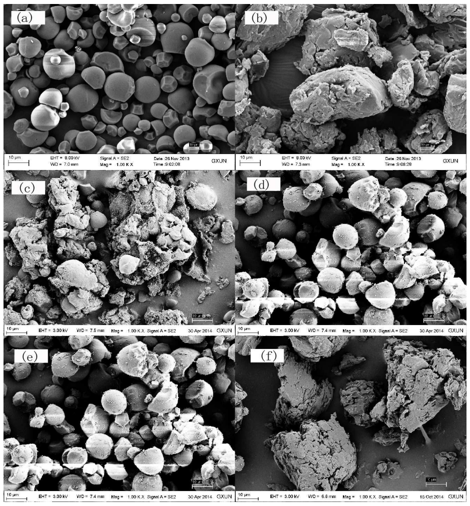

Fig. 1 SEM images of Figure (a) NCS, (b) alkali PS, (c) gelatinized PS, (d) Ultrasound PS, (e) heat-moisture PS, (f) microwave PS

NCS granules were polygonal or irregular in shape with the structurally circular and smooth surface (Fig.1a) [30]. The shape and surface of the pretreated starch had changed in varying degrees according to the pretreatment method. The morphology was changed more significantly as pretreated by alkali, gelatinized and microwave , and effect of ultrasonic and heat-moisture pretreatment on the morphology was slight (Fig.1b,c,f). Compared with NCS, the surface of alkali and microwave PS changed from slick to rough, however, and the ultrasonic and heat-moisture PS kept the similar granule shape with a little broken on the surface (Fig.1d,e).

Pretreatment changed crystallinity of the starch granule as well. The NCS exhibited an A-type crystalline pattern (Fig.2 a), which showed strong reflections at about 14.98, 16.96, 18.02, and 23.02° and weaker peaks at 11.66, 19.92, 26.72, and 30.46°, that is in agreement with the report of Kasemwong [31]. The XRD pattern of ultrasonic PS and heat-moisture PS was very similar to NCS (Fig.2 d, e), that indicated these pretreatments exerted little affect to the inner bond of starch, and made little change in the crystallinity of the starch granule. However, after being gelatinized or microwave pretreatment, the major peaks at 14.98, 16.96, 18.02, and 23.02° disappeared and two new reflections appeared at 13.14 and 19.72° (Fig.2 c, f). The change of the number and intensity of XRD peaks illustrated the crystals structure was changed and the degree of crystallization was reduced after gelatinized or microwave pretreatment. Alkali pretreatment completely changed the crystallinity of the starch granule, as the XRD pattern of alkali PS showed in Fig.2 b. There were only dispersive broad peaks indicating the starch granules were converted from semicrystalline structure into amorphous state. In summary, compared with the NSC, the crystallinity of PS was decreased, the degree of crystalline was as follows: alkali PS < microwave PS < gelatinized PS < ultrasonic PS < heat-moisture PS < NCS.

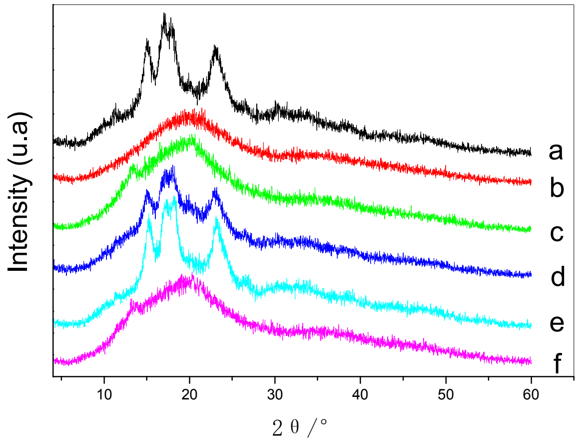

Fig. 2 X-ray diffraction of (a) NCS, (b) alkali PS, (c) gelatinized PS, (d) Ultrasound PS, (e) heat-moisture PS, (f) microwave PS

The interaction of hydrogen bond plays an important role in the stability of starch granules in the suspension [32]. The effect of gelatinized, heat-moisture and microwave pretreatment should be majorly due to the temperature effect, and ultrasonic pretreatment would include thermal, mechanical and cavitation effect. Take the gelatinized pretreatment as an example, when the temperature of starch suspension was higher than the gelatinisation temperature, hydrogen bond of starch were broken so that water molecule penetrated into starch granule easily, and the hydration of free hydroxyl groups made it swell [33,34]. These processes make the polysaccharide chain of starch granule more stretch in solution. When re-precipitated by esthanol, the original hydrogen bonds can't recover, and the morphology and crystallinity of starch has changed. After being gelatinized pretreatment, the high crystallinity of starch chain becomes stretched partly, leads to freer hydroxyl being released. Therefore, the purpose of activating starch and making the follow-up esterification reaction easily could be achieved [14,35]. However, the temperature effect of the five pretreatment methods was different, so that their influence on the hydrogen bonding of starch was discrepant, and and resulted in the difference of the morphology and crystallinity. The results (Fig. 1 and Fig. 2) was indicated alkali pretreatment was more significant than others, and it more conducive to subsequent esterification reaction.

3.2 The effect of starch pretreatment on the esterification reaction

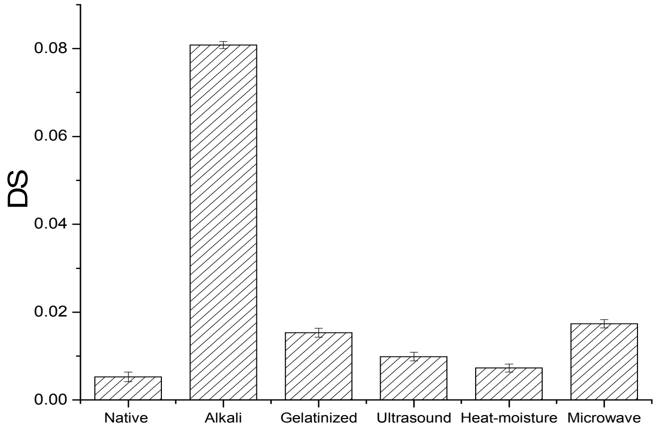

Fig. 3 The effect of pretreated starch on the DS of RAS

Alkali PS, microwave PS, gelatinized PS, ultrasonic PS, heat-moisture PS and NCS were used as substrate, together with rosin acid, for the enzymatic esterification reaction. The DS of esterified products were determined by titration assay, and the results were shown in Fig. 3. The DS of rosin acid starch from NCS was the lowest (DS=0.0053), which indicated the reactivity of NCS was very low. All pretreatments had increased the DS of esterified products, which means pretreatments in the experiment had really improved reactivity of starch. Among them, the alkali pretreatment was the best, which increased the DS to 0.808, about 152 folds higher than non-pretreated substrate. The DS of esterified products achieved 0.0174 and 0.0153 separately after pretreated by microwave and gelatinized, about 3.28 and 2.89 folds higher than NCS. However, ultrasonic and heat-moisture pretreatments produced smaller activative effect on starch, and the DS of esterified products from the ultrasonic and heat-moisture pretreated starch was just 0.0099 and 0.0073. Comparing the result of esterification and the morphology and crystallography analysis, it can be deduced that the value of DS was relative to the morphology and crystalline of the starch granules. The more uncoiling or dissociation of double-helical regions in the starch granule, and more breakdown of crystalline structure produced by pretreatment, the more reaction activity the starch would be, and therefore the higher DS of esterified product [13].

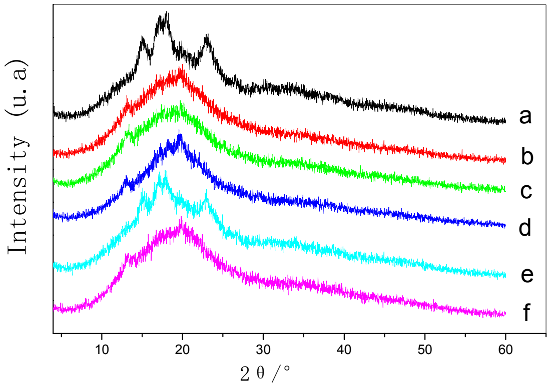

Fig. 4 X-ray diffraction of (a) Native RAS, (b) alkali pretreated RAS, (c) gelatinized pretreated RAS, (d) Ultrasound pretreated RAS, (e) heat-moisture pretreated RAS, (f) microwave pretreated RAS

The morphology and crystallography of the esterified products were also investigated, the results were shown in Fig.5 and Fig.4. The XRD pattern of RAS produced from NCS, ultrasonic and heat-moisture pretreated starch was similar to their original substrates, which indicated the enzymatic esterification did not change the inner crystalline structure of the starches but just took place on the surface of the granule, so that the surface of these RAS changed from slick to rough but kept some kinds of granule shape (Fig. 5 a, d, e).

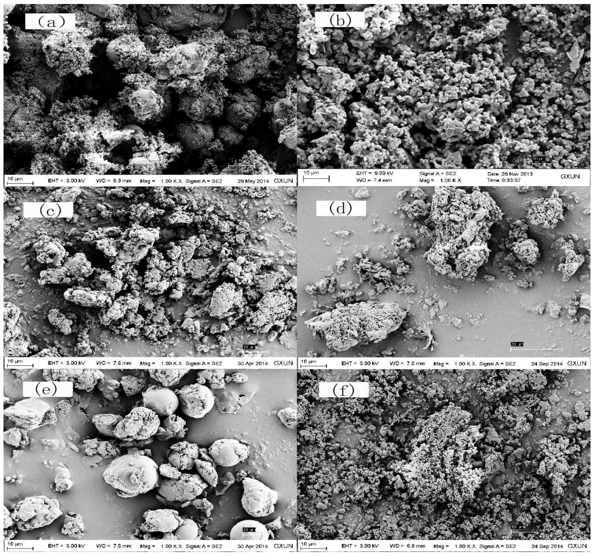

Fig. 5 SEM images of Figure (a) Native RAS, (b) alkali pretreated RAS, (c) gelatinized pretreated RAS, (d) Ultrasound pretreated RAS, (e) heat-moisture pretreated RAS, (f) microwave pretreated RAS

The morphology of RSA synthesized from alkali, microwave and gelatinized pretreated starches exhibited a significant difference with those of NCS and PS (Fig.5 b, c, f). The starch granules were completely destroyed, losing their individuality and smoothness, exposing the internal laminated structure. Therefore, the esterification reaction occurred not only in the non-crystalline regions of starch, but also inside the original crystalline regions. The XRD analyses showed that the crystallography of the esterified products from alkali and microwave and the gelatinized pretreated starches were similar to their original substrates. The results indicated that the esterification process did not further damage the crystalline of the pretreated starches although they were firstly dissoluted in polar DMSO. Furthermore, the integration with hydrophobic rosin acid onto the polysaccharide backbone would disrupt the regular hydrogen bonds in starch but did not lead to newly crystalline pattern. There is an interesting phenomenon about ultrasound pretreated starch and the subsequent esterified product, as showed in Fig. 1 and Fig. 2, ultrasound treatment showed only slight effect on shape and crystallinity, however, ultrasound pretreated RAS showed very similar morphology to alkali, gelatinized or microwave pretreated RAS (figure. 4 and figure. 5), The shape and crystallinity of pretreated starch was affected by the pretreatment mothods, however, the shape and crystallinity of esterified starch was affected by the pretreatment process and the esterification reaction. the effect of ultrasound treatment on shape and crystallinity changed not significant, but the crystallography of ultrasound treatment was lower than NCS; While the esterification was occured in DMSO, the esterification reaction efficiency was improved by pretreated starch, and the RAS was obtained by alcohol sedimentation method, further the crystallinityof ultrasound RAS was decreased by this process. Therefore it showed very similar morphology to alkali, gelatinized or microwave pretreated RAS. In fact，as can be shown in figure 5, there were similar to their original substrates, but the shape and crystallinity was different, and it associated with the damage of hydrogen bond.

3.3 FT-IR analysis RAS with different DS

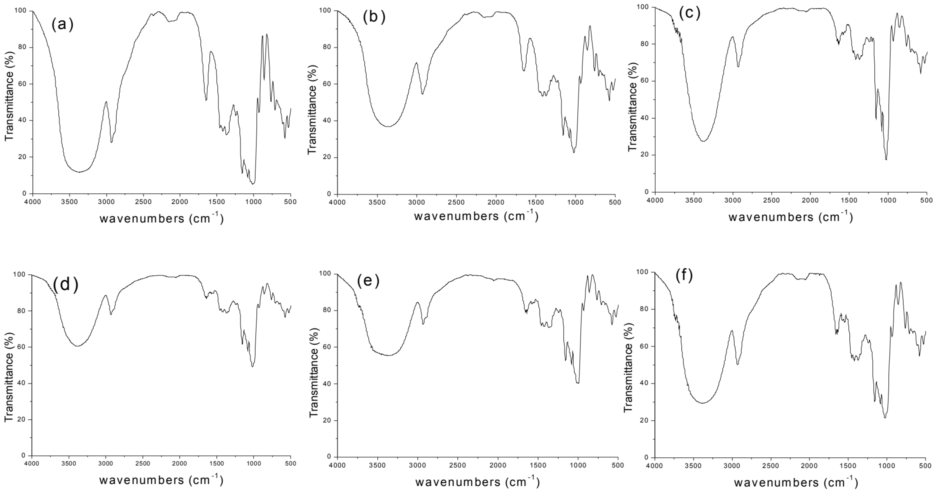

Figure.6 FT-IR spectra of (a) NCS, (b) alkali PS, (c) gelatinized PS, (d) Ultrasound PS, (e) heat-moisture PS, (f) microwave PS

Fig. 6 illustrated the FT-IR spectra of NCS and PS. In the spectrum of NCS (Fig.6a), the C-O stretching vibration of AGU showed several discernible absorbencies at 1157 cm^{-1}, 1081 cm^{-1}, and 1016cm^{-1}. Meanwhile, the O-H and the C-H stretching vibrations give strong signals at 3332 cm^{-1}and 2932 cm^{-1} respectively. The spectrum was similar to the result of Shariffa [36]. Compared with NCS, there were neither band generate nor disappear in the spectrum of PS (Fig. 6b-f)., which means the covalent bonds in NCS were not changed during pretreatment process.

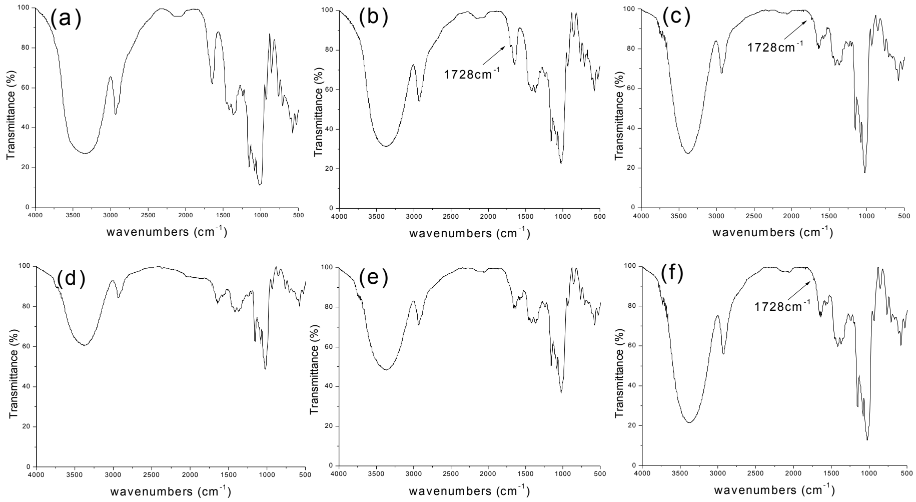

Fig.7 FT-IR spectra of (a) Native RAS, (b) alkali pretreated RAS, (c) gelatinized pretreated RAS, (d) Ultrasound pretreated RAS, (e) heat-moisture pretreated RAS, (f) microwave pretreated RAS

In FT-IR spectrum of RAS synthesized from alkali, gelatinized and microwave (Fig. 7b, c, f), there was a new band at 1728cm^{-1}, which was attributed to the symmetric deformation vibration of carbonyl C=O [37]. This new absorption indicated esterified starch products were formed during the lipase-catalyzed esterification process. However, the bands of symmetric carbonyl vibration were hardly seen in the curves of the RAS synthesized from ultrasonic and heat-moisture pretreated starches (Fig. 7d, e), although the titration analysis showed rosin acid existed in hydrolysate of esterification product. A reason for this phenomenon would be the ester content in the esterified products was too low to be detected by FT-IR, for the DS of RAS synthesized from microwave and heat-moisture pretreated starches were just 0.099 and 0.0073 in the experiment.

Moreover，the results of FT-IR analysis were in agreement with those of SEM and XRD analysis. Compared with NCS, gelatinized, ultrasound, heat-moisture and microwave pretreatment, alkali pretreatment made obvious change in the morphology and structure of starch (Figure.1 and Figure.5), meaning much more uncoiling or dissociation of double-helical regions in the pretreated starches that led to higher reaction activity of the pretreated starched, and resulted in higher ester content in the esterified products[38].

3.4 ^{1}H NMR analysis

NMR reflects a sample of the chemical structure, chemical and other properties of important means, has become a research structure and property of the starch and modified starch important tool. Figure.8 showed that the typical ^{1}H NMR spectrum of (a) NCS, (b) RAS (DS=0.0153) and (c) RAS (DS=0.0808). Chemical shifts of the protons at 3.644 ppm were assigned to H-3, 3.575 ppm to H-5, 3.302 ppm to H-2, and 3.13 ppm to H-4. The chemical shifts of H-1 and OH-2, 3, 6 were assigned to peaks at 4.616 and 5.526 ppm [39]. Compared with the spectrum of NCS, there are three new proton signals at 1.127, 1.225 and 2.081 in ^{1}H NMR spectrum of RAS, which are assigned to protons of CH_3, CH_2 and CH in rosin acid [40]. At the same time, it can be found that the intensity of those three peaks increased with the increasing of the RAS DS. According to the results of FT-IR and ^{1}H NMR spectrum analysis, it can be confirmed that RAS was successfully synthesized.

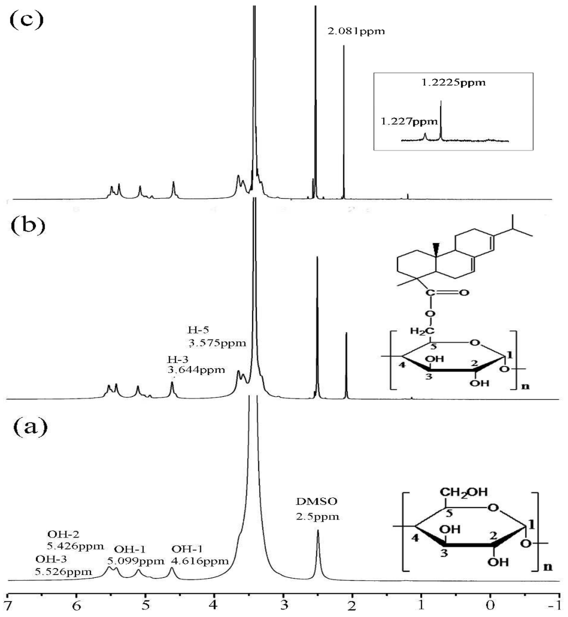

Figure.8 ^{1}H NMR spectra of NCS (a), RAS DS=0.0154(b) and RAS DS=0.0808 (c)

4. Conclusion

The reaction efficiency of rosin acid starch prepared by enzymatic esterification was related to the reactivity of starch, which was affected by the crystal structure of starch granules. Starch pretreatment affected the hydrogen bond group and the crystalline state in varying degrees according to the pretreated methods, that lead to different DS of the rosin acid starch. Among the pretreatment methods in this research, ultrasonic and heat-moisture pretreatment produced slight change to the morphology and crystalline structure of native cassava starch. However, pretreatment of alkali, microwave and gelatinized changed the native cassava starch surface from smooth to crude, and reduced the crystalline obviously. Due to the activation effect of pretreatment, the DS of rosin acid starch was improved as follows: alkali rosin acid starch (0.0808) > microwave rosin acid starch (0.0174) > gelatinized rosin acid starch (0.0153) > ultrasonic rosin acid starch (0.0099) > heat-moisture rosin acid starch

(0.0073) > native rosin acid starch (0.0053). Considering the degree of esterification reaction was related to the morphology and crystalline structure of the pretreated starch, much more pretreated methods such as enzyme degradation and gas explosion should be carried out for rosin acid starch synthesis in the next step.

Acknowledgments

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Graphical Abstract

Esterification reaction efficiency was improved by pretreatment of starch, and the high DS of RAS was achieved.