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ScienceDirect
Carbohydrate Polymers 71 (2008) 441–447
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Synthesis and anti-hyperlipidemic activity of a novel starch piperinic ester

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Received 18 April 2007; received in revised form 8 June 2007; accepted 15 June 2007
Available online 26 June 2007

Abstract

A novel starch piperinic ester (SPE) with anti-hyperlipidemia activity was synthesized by coupling a carboxylic group of piperic acid and a hydroxyl group on the backbone of starch. The synthetic process includes three steps. Firstly, piperic acid was obtained by hydrolyzing Piperine that was extracted from seeds of *Piper longum* L. (traditional medicine of Mongolian). Then, piperic acid was reacted with Carbonyldiimidazole (CDI) in DMSO under N_2 at 70 °C for 4 h. Finally, starch piperinic ester was obtained by the reaction of activated piperic acid with water soluble starch at 80 °C under N_2 for 24 h. The structure of the novel copolymer was characterized by FTIR, ^{13}C NMR and ^{1}H NMR. Anti-hyperlipidemic activity of SPE was assayed by pharmacological testes and the results indicated that the SPE had high anti-hyperlipidemic activity and would be one kind of new potential candidate of anti-hyperlipidemia pro-drug.

Keywords: Polysaccharide; Starch; *Piper longum* L.; Anti-hyperlipidemia; Piperidine; Piperinic ester

1. Introduction

Arteriosclerosis of the coronary and peripheral vasculature remains one of the leading causes of death in the world. Many prospective studies have demonstrated that a univariate association exists between hyperlipidemia and the risk of arteriosclerosis (Assmann, Gullen, & Schulte, 1998). Epidemiological studies have demonstrated the strong causal relations between the levels of lipid parameters and hyperlipidemia. The increases of total serum cholesterol (TC), triglyceride (TG), low-density lipoproteins cholesterol (LDL-C), as well as decrease of serum high density lipoproteins cholesterol (HDL-C) have been considered to be significant risk factors for hyperlipidemia and arteriosclerosis (Castelli, 1996; Assmann et al., 1998; Asztalos & Schaefer, 2003). The LDL-C transfers cholesterol towards extrahepatic organs as major carrier and HDL-C transports cholesterol from the periphery tissues to the liver for catabolism. HDL-C plays a pivotal role in the reverse cholesterol transport (Asztalos & Schaefer, 2003). Thence, most of the studies in the area of precaution and treatment of arteriosclerosis pay more attention to the way of lowering plasma lipid. Simvastatin, derived from fungal fermentation, was effective in treating patient with hyperlipidemia. Simvastatin was chosen as a positive drug in this study.

Piperine, a major hydrophobic alkaloid of *Piper longum* L. and *Piper nigrum*, were reported to have pharmacological/toxicological effects (Bajad, Bedi, Singla, & Johri, 2001; Yang et al., 2002). For long time, much more attentions have been paid to studying natural piperidine and its

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doi:10.1016/j.carbpol.2007.06.014

derivatives extracted from Plants for chemistry and pharmacology (Jensen, Hansen, & Bool, 1993; Tabuneng, Bando, & Amiya, 1983; Wu, Sun, Pei, Lu, & Pan, 2004). Recently, the excellent anti-hyperlipidemia activity of piperine has been demonstrated in our Lab (Gereltu et al., 2004), but there were several side effects for oral administration to wistar rats. In our present research work, we studied the possibility of maintaining or improving anti-hyperlipidemic activities and reducing side effects of piperic acid with chemical modification.

Polysaccharides are prospective candidates of carriers or matrix for hydrophobic drug. Recently, considerable attention has been focused on the chemical modification of polysaccharides for medical application such as drug carriers for the controlled release (Aiping, Tian, Lanhua, Hao, & Ping, 2006; Li et al., 2005; Tim & Thomas, 2005). It have been demonstrated that chemical modified polysaccharide cannot only improve activities, but also lower toxicities, and decrease side effects of drug (Ying et al., 1999; Gereltu, Guiyun, Huricha, Hedeki, & Toshiyuki, 2003). Water soluble starch usually showed a good biodegradability and low toxicity, which was used as a hydrophilic carrier for delivery system of hydrophobic drug. In 1976, nicotine acidic ester bounding starch was synthesized and pharmacologically tested on rats for the anti-hyperlipidemia activity (Puglisi, Caruso, Paoletti, Ferruti, & Tanzi, 1976). The nicotine acidic ester bounding starch prolonged anti-lipolytic activity of nictonic acid and no rebounding of free fatty acids (FFA) in plasma. Later, Rusconi et al. prepared a series of nicotine acidic esters bounding starch with a different degree of esterification, and the physico-chemical characteristics were examined (Luisa, Andear, & Paolo, 1981).

For the purpose of decreasing side effects or toxicities of piperine by chemical modification, we tried to prepare polymer derivatives of piperine from starch and piperine. In this paper, we describe the synthesis of new copolymers composed of hydrophobic piperic acid, which was grafted by means of ester bridges, and polysaccharide (water soluble starch) backbone. These starch-piperic acid graft copolymers were prepared by coupling the carboxylic group of piperic acid and the hydroxyl group on the starch backbone. The resulting starch piperinic ester was characterized by FTIR and NMR, and anti-hyperlipidemia activity of starch piperinic ester was examined by pharmacological testes. The results indicated that starch piperinic ester had high anti-hyperlipidemic activity, and had no obvious toxicity for oral administration to wistar rats. Therefore starch piperinic ester would be one kind of new potential candidate of anti-hyperlipidemia prodrug.

2. Experimental

2.1. Materials and general methods

Soluble starch (α-Amylose, AR) was purchased from Tianjin Chemical Reagent Plant, China. Carbonyldiimidazole (CDI) was the commercial products of Aldrich Chemical Company. Dimethylsulfoxide (DMSO). DMSO was distilled after drying with calcium hydride for 24 h under a nitrogen atmosphere. Water soluble starch with a medium molecular weight (Mn = 12,000, Mw/Mn = 1.38) was prepared by hydrolysis of commercial starch in the presence of H_2SO_4 as catalyst, according to the method reported previously (Gao, Katsuraya, Mimura, Nakashima, & Uryu, 1998). Molecular weight of the water soluble starch was determined by GPC in pH 6.8 phosphate buffer solution at 25 °C. Other reagents were commercially available and used as received.

The Fourier transform infrared (FTIR) spectrum of starch piperinic ester was recorded using Bio-Rad FTS 6000 spectrometer at room temperature using KBr dick. ^{13}C NMR and ^{1}H NMR spectra were recorded on a Bruker AV 400 MHz in DMSO-d_6 at 25 °C, Chemical shifts were given in parts per million from tetramethylsilane. The percentage of piperic acid group was calculated from the peak intensities of proton signal ($\delta = 6.052$ ppm) of grafted piperic acid and the proton signal ($\delta = 5.18$ ppm) of starch backbone in the ^{1}H NMR spectrum of SPE.

2.2. Preparation of piperine

Piper longum L. fruit powder (1000 g) was extracted with 95% ethanol(1000 ml) under refluxed for 4 h for three times. The supernatant collected by centrifugation at 3600 r/h was dried in a vacuum and designated as a crud piperine. The crude piperine was further purified by recrystallized in ethanol, 10.05 g of powdery, yellow piperine (mp: 129–130 °C, retention time: 2.250 min with analysis column of symmetry C_{18} in eluent of 80% methanol/30% distilled water) was obtained.

2.3. Perparation of piperic acid

Piperine (10 g) was dissolved in 300 ml of anhydrous ethanol containing 20% KOH (wt%) in a 500 ml reaction flask equipped with a reflux condenser. The mixture was heated at reflux with stirring for 10 h to give the precipitate of potassium piperate. After filtration, the precipitate of potassium piperate was washed three times with anhydrous ethanol. The precipitate was dissolved in distilled water, and then purified by precipitating piperic acid in water by adding HCl solution (0.1 M). The yellow precipitate of piperic acid was filtrated, washed with distilled water (200 ml) three times to produce 8.66 g of powdery yellow piperic acid (yield 86.6%, mp: 206–208 °C, retention time: 0.938 min with analysis column of Symmetry C_{18} in eluent of 70% methanol/30% distilled water) by freeze-drying from water.

2.4. Synthesis of amphiphilic starch piperinic ester

To synthesize starch piperinic ester, a two-step reaction was carried out according to the method reported in the

literature (Ruxandra, Jaqueline, & Patrick, 2002). 1.090 g (5 mmol) of piperic acid and 0.907 g of carbonyldiimidazole (5.5 mmol) were dissolved in 6 ml of anhydrous DMSO in a 25 ml round-bottomed reaction flask equipped with a reflux condenser and connected to the N_2 line. The flask was heated at 70 °C under N_2, CO_2 evolution was observed. The reaction was not stopped until CO_2 was no longer released in 4 h, and the activated polymer formed. Then 1.238 g of water soluble starch was dissolved in 6 ml of anhydrous DMSO and added. The mixture was stirred for 24 h at 80 °C under N_2. The reaction mixture was dropped into 50 ml of hot anhydrous acetone to give the precipitated polymer, which was isolated by centrifugation and washed twice by ethanol. The precipitate was dissolved in DMSO completely, and dialyzed for 24 h against DMSO and then for 48 h against distilled water using a dialysis membrane (MWCO = 2000–3000 g/mol). 1.36 g of slight yellow, powdery starch piperinic ester was obtained by freeze-drying from water.

2.5. Characterization of starch piperinic ester

The structures of the starch piperinic esters were determined by ^{1}H NMR, ^{13}C NMR and FTIR. The Fourier transform infrared (FTIR) spectrum of starch piperinic ester was recorded using Bio-Rad FTS 6000 spectrometer at room temperature using KBr pallet. ^{13}C NMR and ^{1}H NMR spectra were recorded on a Bruker AV 400 MHz in DMSO-d_6 at 25 °C, Chemical shifts were given in parts per million (ppm) from tetramethylsilane. The degree substitution (%) of piperic acid group was calculated from the peak intensities of ethylene proton signal (6.042 ppm) of grafted piperic acid and that of the ethylene proton signal (5.490 ppm) of starch backbone in the ^{1}H NMR spectrum of starch piperinic ester.

2.6. Anti-hyperlipidemia activity assay

2.6.1. Animals and experimental design

Male wistar rats (SPF grade, Research center for laboratory animals science, Beijing, China), weighed 180.5 ± 5.3 g, were used for the evaluation of anti-hyperlipidemia activity of the polymer (starch piperinic ester with DS = 42.3%). The rats were housed in stainless steel cages under ambient temperature of 21 ± 5 °C and 40–70% relative humidity and with 12 h light/dark schedule. The rats were fed with standardized laboratory chow for acclimation period of days. The experimental animals were fed with standardized laboratory chow for an acclimation period of 4–5 days. Then, the wistar rats were weighed and randomly divided into five groups (normal group, control group, positive control group and treated group) so that the mean values and deviations of weights were similar for individual groups. Simvastatin, derived from fungal fermentation for treating patient with hyperlipidemia was chosen as a positive drug in this study.

After acclimation period, the normal group was fed with standardized laboratory chow, meanwhile the other groups were fed with a cholesterol-rich diet (Cholesterol 3%, Sodium cholate 0.5% and Lard 10%). Animals had free access to water and food. Each sample was formulated in Tween 80 (0.5% in water) suspension after which was underwent triturating in a mortar and pestle. Positive control and treated groups were orally administered daily with 4 ml/kg B.W. of the Tween 80 suspension containing samples. Control and normal animals received the same amount of Tween 80 (0.5% in water). At the end of the 15 day experiments, rats were fasted for 12 h. Blood samples of rats were taken from cervical arteries after anesthetized with diethyl ether. After coagulation under ambient temperature, the blood samples were centrifuged with TGL-16 centrifuge (Shanghai, China) at 3000 rpm for 15 min at 4 °C, serums were separated and frozen at −20 °C until analysis with Proton Evolution Random Analyzer.

2.6.2. Biochemical estimation of serum lipid parameters

The following six parameters were measured by means of a commercial kits (Zhong Sheng Bei Kong Biotech., Beijing, China) with proton evolution random analyzer (Shanghai, China). Serum total cholesterol (TC), triglyceride (TG) were measured enzymatically. High density lipoprotein cholesterol (HDL-C) was measured after PTA-Mg^{2+} selective precipitation of both low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein (VLDL). The LDL-C value was obtained by subtracting cholesterol, which was measured enzymatically after PVS selective precipitation of LDL-C, from TC.

2.6.3. Statistical analysis

All results were expressed as means ± SEM. Statistical significance of difference between groups was tested by one-way analysis of variance (ANOVA) using the stat graphic computer program. In all statistical analyses, $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Synthesis of starch piperinic ester

Starch piperinic esters were prepared by a synthetic route as illustrated in Fig. 1. In the first step, piperic acid was obtained by hydrolyzing natural piperine. In the second step, *N*,*N*′-carbonyldiimidazole (CDI) in excess was used for conversion of carboxylic groups of piperic acid into a imidazolide-activated piperic acid. In the third step, starch dissolved in DMSO was added to the no-isolated mixture containing imidazole. The esterification was achieved by homogeneous reaction of the starch with a piperic acid imidazolide which was prepared in situ by conversion of piperic acid with *N*,*N*-carbonyldiimidazole (CDI).

For the coupling reaction of piperic acid and starch, different strategies were tried. For example, the carboxylic

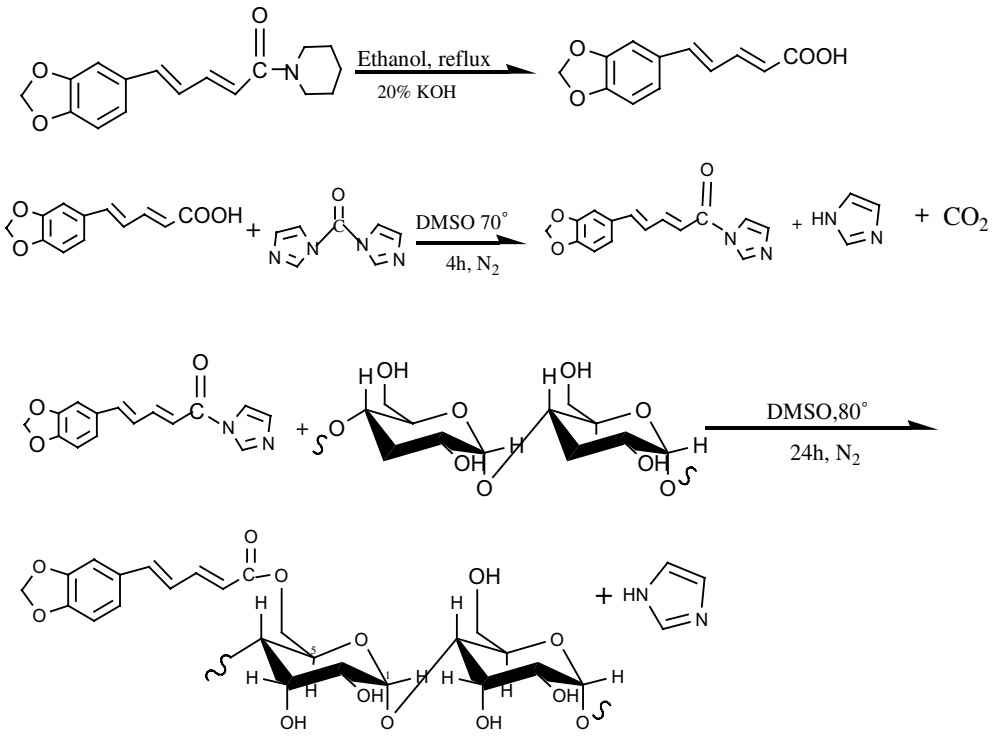

Fig. 1. Synthetic route of starch piperinic ester.

group of piperic acid can be activated with DCC/DMAP, but coupling with polysaccharide needed several days and the yield did not exceed 40% in our case. In contrast to DCC/DMAP, the activation of piperic acid with *N*,*N*′-carbonyldiimidazole (CDI) was more efficient and convenient at the mild reaction condition. The activated carboxylic acid can react with the hydroxyl group of polysaccharide to produce starch esters in high yield (up to 98.85%), and the by products (imidazole) were nontoxic and completely removable. During conversion with CDI, only carbon dioxide and imidazole are formed. Thus, pure starch piperinic ester with high degree of the substitution was obtained simply by precipitation in ethanol and washing with ethanol.

It is obvious that the *N*,*N*′-carbonyldiimidazole (CDI) in excess was used for conversion of carboxylic groups of piperic acid into an imidazolide-activated piperic acid. The result show that the degree of the substitution (DS) of the starch piperinic esters was up to 57.9% in presence of suitable CDI as catalyst in DMSO. The details of reaction were shown in Table 1. The activation of carboxylic group has more advantages, such as in short reaction time, in good yield, when CDI is used as acylating agent. The results demonstrated that CDI is a quite effective agent for the activation of piperic acid and coupling reaction with starch, and DMSO is the best solvent for the

Table 1
Reagents and results for the esterification of water soluble starch with piperic acid in the presence of CDI in DMSO for 24 h

Reagent (mmol)			Product (SPE)	
Piperic acid	Starch[a]	CDI	DS[b] (mol %)	Yield[c] (%)
1.201	5.556	1.440	15.1	97.6
2.401	5.556	2.440	42.3	98.3
3.601	5.556	4.320	57.9	90.9

[a] Molar was mol of repeating unit of D-glucose (GLU) in starch.
[b] Degree of substitution determined by ^{1}H NMR of products.
[c] Yield of starch piperinic ester calculated in ration of starch of reagent.

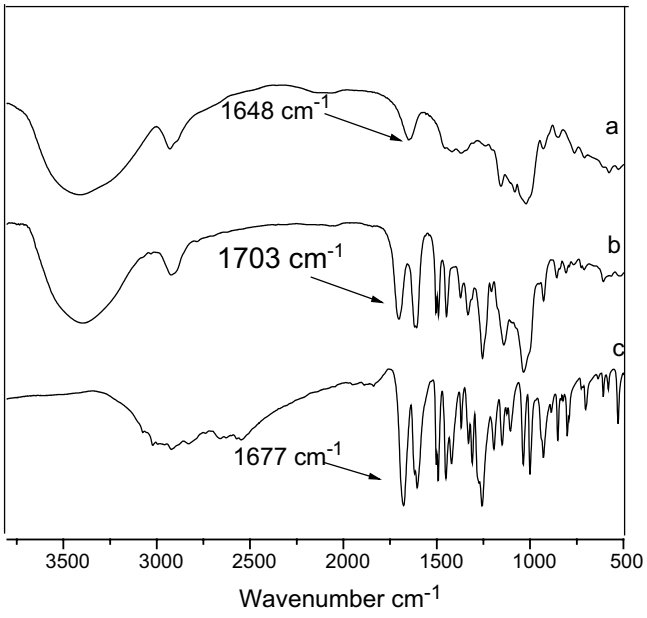

Fig. 2. FTIR spectra of starch (a), SPE (b) and piperic acid (c).

coupling reaction between piperic acid and polysaccharide.

3.2. Characterization of starch piperinic ester (SPE)

We found that starch piperinic ester (SPE) had good solubility only in DMSO, although starch (Mn = 12,000 D) was soluble in both H_2O and DMSO, and piperic acid was soluble in DMSO, ethanol and acetone. The structure of the starch piperinic ester was analyzed by ^1H NMR, ^{13}C NMR and FTIR. The degree of substitution (DS) was calculated from the ratio of the integral peak H_{18} ($\delta = 6.046$) of piperic acid and H_1 ($\delta = 5.490$) of starch backbone in the ^1H NMR spectrum of SPE. The result shows that the

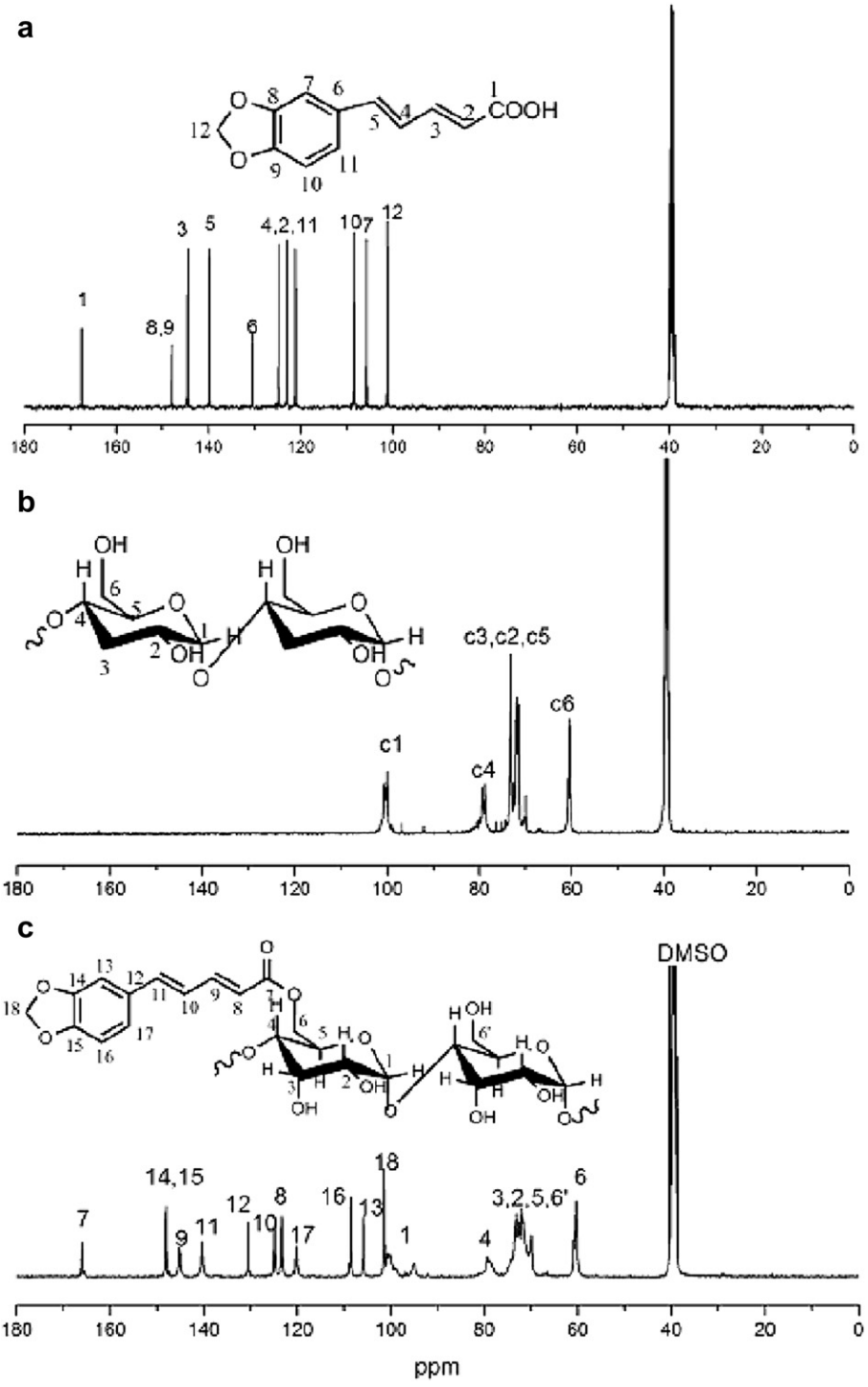

Fig. 3. The ^{13}C NMR Spectra of piperic acid, water soluble starch and SPE with DMSO-d_6 as solvent at 25 °C. (a) piperic acid, (b) starch (Mn = 12,000 D), (c) SPE.

Table 2
Effects of oral administration of piperic acid and starch piperinic ester on lipid profiles in rats supplemented with a cholesterol-rich diets after 15 days[a]

Group	HDL-C (mmol/L)	TC (mmol/L)	TG (mmol/L)	LDL-C (mmol/L)
Normal	$2.06 \pm 0.27^{*}$	$0.74 \pm 0.13^{*}$	$1.78 \pm 0.97^{*}$	$0.31 \pm 0.12^{*}$
Control	15.35 ± 3.72	1.38 ± 0.47	4.20 ± 0.81	0.13 ± 0.03
Simvastatin	$7.86 \pm 1.41^{*}$	0.95 ± 0.19	$1.57 \pm 0.27^{*}$	$0.33 \pm 0.12^{*}$
PA	$8.58 \pm 0.68^{*}$	$0.92 \pm 0.13^{*}$	$2.23 \pm 1.19^{*}$	0.22 ± 0.21
SPE	$7.43 \pm 0.95^{*}$	$0.68 \pm 0.13^{*}$	$1.83 \pm 0.19^{*}$	$0.28 \pm 0.03^{*}$

TC, total cholesterol. TG, triglyceride.
HDL-C, high density lipoprotein cholesterol.
LDL-C, low density lipoprotein cholesterol.
PA, piperic acid. SPE, starch piperinic ester with DS = 42.3%.
[a] Values were means ± SEM ($n = 10$).
* $p < 0.05$ significantly different from control.

degree of the substitution (DS) of the starch piperinic esters is up to 60%, according to the calculation of ^{1}H NMR.

The IR spectra of starch (a), SPE (b) and piperic acid (c) are shown in Fig. 2. Comparing the spectrum of SPE (b) with the starch (a), new strong absorptions emerge at 1703 and 1255 cm^{-1}, assigned to carbonyl(C=O) of ester and the vibration peak of ester group (CO–O), respectively. Meanwhile, two new sharp peaks appear at 1608 and 1504 cm^{-1} for the backbone vibrations of benzene ring. The vibration peak of carbonyl (=CO) for carboxylic function is at 1677 cm^{-1} in the spectra of piperic acid (c), and at 1703 cm^{-1} in the spectra of SPE (b). A red shift from 1677 to 1703 cm^{-1} was observed. The result indicated the formation of ester groups (–COO–) in the starch piperinic ester.

The chemical structure of starch piprinic ester was confirmed by ^{13}C NMR. ^{13}C NMR spectra of piperic acid, starch and starch piperinic ester in DMSO are shown in Fig. 3.

As shown in Fig. 2(c), the signals of the starch backbone chain obviously, indicate that the structure of the original polysaccharide remained; new peaks due to piperic acid are all detectable, as evidence of the formation of starch piperinic ester in agreement with the above-mentioned FT-IR results. The resonance signal at 166 ppm was ascribed to carbonyl carbon of starch piperinic ester. All the evidences from the spectra of FTIR and NMR indicate that starch piprinic ester was synthesized successfully.

3.3. Anti-hyperlipidemia activity assay

The parameters of rat serum were analyzed by statistical method to detect any significant differences among groups ($P < 0.05$). The anti-hyperlipidemia activities of samples assessed by comparing the serum total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), triglyceride (TG) in the administration groups with those of the control group. The statistical analysis results are shown in Table 2. Piperic acid and starch piperinic ester groups show similar trend on meditating serum TC, TG, LDL-C and HDL-C levels comparing to the control group. The starch piperinic ester group significantly reduced TC (−51.66%), TG (−50.72%) and LDL-C (−56.43%), and increased HDL-C (+115.38%) in the serum. These values exhibit there are significant differences accrued between the serum parameters of treating group fed with samples and that of the control group. Whereas piperic acid group reduced the serum TC level (−44.10%), TG level (−33.33%) and LDL-C level (−46.90%), and increased HDL-C level (+69.23%). In addition, there is no significant difference of HDL-C level between piperic acid and that of the control group. The results demonstrated that starch piperinic ester has high anti-hyperlipidemia activity.

4. Conclusions

A new kind of starch piperinic ester has been synthesized successfully for the first time. In this paper, we report one efficient way for synthesis of new grafted copolymers, starch piperinic esters. The degree of the substitution (DS) of the starch piperinic esters was determined by the ratio H ($\delta = 6.042$ ppm) of grafted piperic acid and the ethylene proton signal ($\delta = 5.490$ ppm) of starch backbone in the ^{1}H NMR spectra of SPE. With the aim to further use of these materials for potential applications of anti-hyperlipidemic pro-drug, grafting was achieved through ester bridges to ensure a good biodegradability. The pharmacological experiment demonstrates that starch piperinic ester have good anti-hyperlipidemic activity. The research provide a promising strategy to hunt for novel polymers possessing efficient anti-hyperlipidemic activity by chemical modification of starch or other polysaccharides.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 20064001) and The Science Foundation of Inner Mongolia Autonomous Region Government.

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