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Characterization and discrimination of polysaccharides from different species of *Cordyceps* using saccharide mapping based on PACE and HPTLC

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ABSTRACT

Polysaccharides from seven species of natural and cultured *Cordyceps* were firstly investigated and compared using saccharide mapping, partially acidic/enzymatic (α-amylase, β-glucanase and pectinase) digestion followed with polysaccharide analysis by using carbohydrate gel electrophoresis (PACE) and high performance thin layer chromatography (HPTLC) analysis, respectively, to obtain the comprehensive profiles of hydrolysates of the polysaccharides and their characters. The results showed that 1,4-α-D-glucosidic, 1,4-β-D-glucosidic and 1,4-α-D-galactosidic linkages were existed in natural and cultured *Cordyceps sinensis*, cultured *Cordyceps militaris*, natural *Cordyceps gracilis* and *Cordyceps ciecadae*. The similarity of polysaccharides from cultured *C. militaris* to natural *C. sinensis* was relatively high, which might contribute to the rational use of *C. militaris*. Moreover, different species of natural and cultured *Cordyceps* can be differentiated based on the saccharide mapping, which is helpful to well understand the structural characters of polysaccharides from different species of *Cordyceps* and to improve the quality control of polysaccharides in natural and cultured *Cordyceps*.

1. Introduction

Cordyceps, one of the well-known tonic foods and traditional Chinese medicines, is a composite consisting of the stromata of the fungus, *Cordyceps sinensis* (Berk.) Sacc. parasitized on the larva and the dead caterpillar. So far, approximately 90 species of *Cordyceps* have been found in China (Zhong et al., 2009). However, only *C. sinensis* is officially recorded in Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010). Sure, *Cordyceps* is commonly used in China for prevention and treatment of a variety of diseases (Zhu, Halpern, & Jones, 1998a, 1998b). Indeed, polysaccharides are considered as the main bioactive components of *Cordyceps* (Paterson, 2008; Shashidhar, Giridhar, Udaya Sankar, & Manohar, 2013; Zhong et al., 2009; Zhou, Gong, Su, Lin, & Tang, 2009), which are responsible for lots of bioactivities of *Cordyceps*, such as anti-tumor, immunomodulatory, anti-oxidation, hypoglycemic, and hypolipidemic activities (Shashidhar et al., 2013; Zhong et al., 2009). Up to date, only few works have been performed for comparison of polysaccharides in natural and cultured *C. sinensis* (Guan, Yang, & Li, 2010; Guan, Zhao, Feng, Hu, & Li, 2011) due to the structural complicity of polysaccharides and rare materials. Therefore, comparison and characterization of polysaccharides from various different species of *Cordyceps* are very important for improving the quality control of natural and cultured *Cordyceps*.

Saccharide mapping based on enzymatic hydrolysis followed by high-performance size-exclusion chromatography (HPSEC) analysis has been used for comparison of polysaccharides from natural and cultured *C. sinensis* (Guan et al., 2011). However, HPSEC is difficult to simultaneously separate both polysaccharides and their hydrolysates. In addition, the sensitivity of evaporative light scattering detector (ELSD) and refractive index detection (RID), which are widely used for detection of saccharides without UV absorbance, is poor (Li, Wu, Lv, & Zhao, 2013). Actually, the polysaccharide analysis by using carbohydrate gel electrophoresis (PACE) analysis has been employed for analysis of polysaccharide from *Ganoderma* spp. in our previous studies (Wu, Xie, Hu, Zhao, & Li, 2013), which has been approved as the high sensitive, high resolution and high throughput method for analysis of enzymatic

Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DP, degree of polymerization; ELSD, evaporative light scattering detector; HPTLC, high-performance thin-layer chromatography; HPSEC, high-performance size-exclusion chromatography; PACE, polysaccharide analysis by using carbohydrate gel electrophoresis; RID, refractive index detection; SMC, simulative mean chromatogram; TFA, trifluoroacetic acid.

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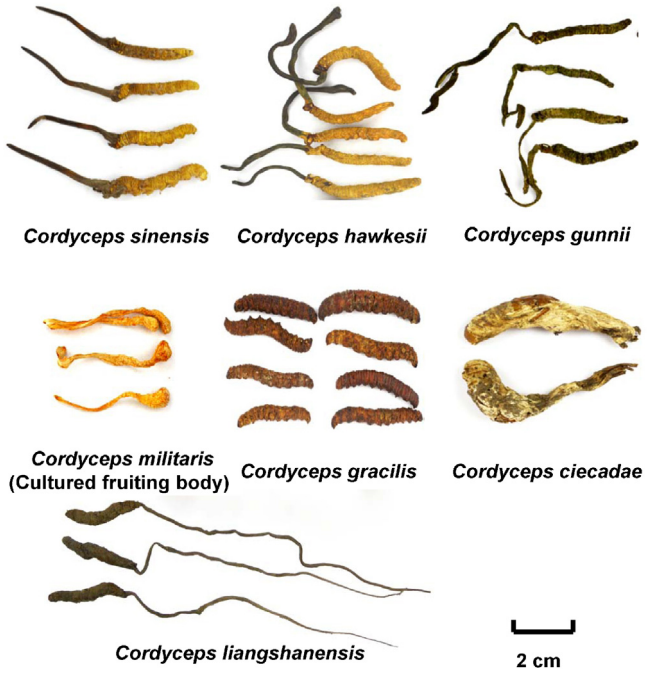

Fig. 1. The typical samples of natural and cultured *Cordyceps*.

hydrolysates containing oligosaccharides. However, the resolution of PACE for analysis of different monosaccharides is poor due to their 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) derivatives (Goubet, Jackson, Deery, & Dupree, 2002). Actually, high-performance thin-layer chromatography (HPTLC) is available for separation of different types of monosaccharides (Yang, Guan, Zhang, & Li, 2010), which has been used for analysis of the acid hydrolysates of polysaccharides from two species of *Ganoderma* (Xie et al., 2012). Therefore, the combination of HPTLC and PACE is a good choice for completely understanding comprehensive profile of enzymatic and partial acid hydrolysates of polysaccharides. In this study, polysaccharides from different species of *Cordyceps*, including *C. sinensis*, *Cordyceps militaris*, *Cordyceps gunnii*, *Cordyceps liangshanensis*, *Cordyceps gracilis*, *Cordyceps hawkesii* and *Cordyceps ciecadae* were characterized and compared using saccharide mapping based on HPTLC and PACE analysis, which were helpful to well understand the structural characters of polysaccharides in different species of *Cordyceps* and to improve their quality control of polysaccharides.

2. Materials and methods

2.1. Materials and chemicals

Seven batches (NC1–NC7) of natural *C. sinensis* (NC) and *C. gunnii*, *C. liangshanensis*, *C. gracilis*, *C. hawkesii* and *C. ciecadae* were obtained from different places of China. Nine batches (CM1–CM9) of cultured *C. militaris* (CM) and ten samples (CC1–CC10) of cultured *C. sinensis* (CC) were collected from different manufacturers in China. Other cultured *C. sinensis* samples (CC11 and CC12) were produced in our laboratory (Table 1). Identities of these natural *Cordyceps* spp. were confirmed by Professor Shao-Ping Li, University of Macau, Macau SAR, China. Their characteristic information (Fig. 1) was in accordance with the previous reports (National Institutes for Food and Drug Control & Guangdong Institute for Food and Drug Control, 2011). Species of the cultured *Cordyceps* were certified by State Food and Drug Administration of China or manufacturer. The voucher specimens were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macao, China.

D-Glucose, D-galactose, D-galacturonic acid, soluble starch, cellulose, dextran 2000, dextranase (EC 3.2.1.11), α-amylase (EC 3.2.1.1), β-D-glucanase (EC 3.2.1.6) and cellulase (EC 3.2.1.4) were purchased from Sigma (St. Louis, MO, USA). Laminaribiose (95%), laminaritetraose (95%), laminarihexaose (95%), pectic galactan, oat glucan, pectinase (EC 3.2.1.15) and isoamylase (EC 3.2.1.68) were purchased from Megazyme (Wicklow, Ireland). ANTS was purchased from Tokyo Chemical Industry (Tokyo, Japan). Silica gel 60 TLC plates were obtained from Merk (Merk, Darmstadt, Germany). Polyacrylamide containing a ratio of acrylamide/N,N-methylenebisacrylamide (19:1, w/w) was obtained from Bio-Rad (Hercules, CA, USA). Deionized water was prepared by a Millipore Milli-Q Plus system (Millipore, Bedford, MA, USA). All the other reagents were of analytical grade.

2.2. Preparation of polysaccharides by pressurized liquid extraction (PLE)

The samples were carefully cleaned using a small dry brush, then dried at 40 °C for 24 h, and pulverized via grinding. PLE was performed on a Dionex ASE 200 system (Dionex Corp., Sunnyvale, CA, USA). The powders of sample materials (0.5 g) were mixed with diatomaceous earth in a proportion of 1:1 and placed into an 11 mL of stainless steel extraction cell, then extracted with water under 100 °C for 5 min of static time for one cycle with pressure at 1.034×10^4 kPa. The extract (~20 mL) purged out by nitrogen was transferred into a 25 mL volumetric flask, which was made up to its volume with water. Then the aqueous extract (20 mL) was evaporated to 5 mL of solution under vacuum. Subsequently, ethanol (95%, w/v) was added to the final concentration of 80% (v/v) for precipitation of crude polysaccharides. After standing for 12 h at 4 °C, centrifugation (4500 × *g* for 15 min) was performed. The precipitate was dried on water bath (80 °C), and then redissolved in 5 mL of hot water (80 °C). After centrifugation (4500 × *g* for 15 min), the supernatant was transferred to an ultracentrifugal filter (molecular weight cutoff: 3 kDa, Millipore, Billerica, MA, USA), and then the low molecular weight compounds (Mw < 3 kDa) were removed by centrifugation (4000 × *g*, 20 min, 25 °C). Then, deionized water was added into the supernatant to a total volume of 5 mL. The content of sugar in crude polysaccharides was determined using phenol-sulfuric acid assay with glucose as reference standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Finally, the crude polysaccharides, which were prepared in duplicates, were obtained for further analysis.

2.3. Partial acid hydrolysis of polysaccharides

The crude polysaccharide solutions (~0.5 mg/200 μL) were treated with trifluoroacetic acid (TFA) at a final concentration of 0.5 mol/L in a total volume of 300 μL. The suspensions were incubated at 80 °C for 5 h. After hydrolysis, the hydrolysates were washed with methanol and evaporated to dryness with a nitrogen evaporator at 35 °C for three times to remove the residue of TFA. The dried products were stored in 4 °C before derivatization with ANTS for PACE analysis, and redissolved in 100 μL of methanol for HPTLC analysis, respectively.

2.4. Enzymatic digestion of polysaccharides

Polysaccharide solutions (~0.5 mg/200 μL) were mixed with certain enzyme (the final concentration of dextranase, β-D-glucanase, isoamylase, α-amylase, cellulase and pectinase were 2 U/mL, 2 U/mL, 20 U/mL, 20 U/mL, 20 U/mL and 20 U/mL, respectively) in a total volume of 300 μL and digested overnight (12 h) at 40 °C. Then the mixtures were heated at 80 °C for 20 min to denature the enzymes. After centrifugation (10,000 × *g*) at room

Table 1
Summary of the investigated samples.

No.	Code	Species	Sample type	Source
1	NC1	*Cordyceps sinensis*	Natural *Cordyceps*	Qinghai Province
2	NC2	*C. sinensis*	Natural *Cordyceps*	Qinghai Province
3	NC3	*C. sinensis*	Natural *Cordyceps*	Sichuan Province
4	NC4	*C. sinensis*	Natural *Cordyceps*	Sichuan Province
5	NC5	*C. sinensis*	Natural *Cordyceps*	Yunnan Province
6	NC6	*C. sinensis*	Natural *Cordyceps*	Qinghai Province
7	NC7	*C. sinensis*	Natural *Cordyceps*	Tibet
8	AC1	*Cordyceps gunnii*	Natural *Cordyceps*	Anhui Province
9	AC2	*Cordyceps liangshanensis*	Natural *Cordyceps*	Sichuan Province
10	AC3	*Cordyceps hawkesii*	Natural *Cordyceps*	Sichaun Province
11	AC4	*C. liangshanensis*	Natural *Cordyceps*	Sichuan Province
12	AC5	*Cordyceps gracilis*	Natural *Cordyceps*	Xinjiang
13	AC6	*C. hawkesii*	Natural *Cordyceps*	Gansu Province
14	AC7	*Cordyceps ciecadae*	Natural *Cordyceps*	Sichuan Province
15	AC8	*Cordyceps gunnii*	Natural *Cordyceps*	Shanxi Province
16	CM1	Cultural *Cordyceps militaris*	Fruiting body	Healthfirst, Guangdong
17	CM2	Cultural *C. militaris*	Fruiting body	Quanxin, Shanghai
18	CM3	Cultural *C. militaris*	Fruiting body	AoHanXinhui, Neimenggu
19	CM4	Cultural *C. militaris*	Fruiting body	Hongyu, Jiangsu
20	CM5	Cultural *C. militaris*	Fruiting body	Yatai, Liaoning
21	CM6	Cultural *C. militaris*	Fruiting body	Lanshi, Liaoning
22	CM7	Cultural *C. militaris*	Fruiting body	Xinhuikangyi, Guangdong
23	CM8	Cultural *C. militaris*	Fruiting body	Dingsheng, Liaoning
24	CM9	Cultural *C. militaris*	Fruiting body	Jiafeng, Heilongjiang
25	CC1	Cultural *Cordyceps sinensis*	Mycelia	Qinghai University, Xining
26	CC2	Cultural *C. sinensis*	Mycelia	Huadong, Zhejiang
27	CC3	Cultural *C. sinensis*	Mycelia	Anhui Agricultural University, Hefei
28	CC4	Cultural *C. sinensis*	Mycelia	Jolly, Zhejiang
29	CC5	Cultural *C. sinensis*	Mycelia	Jolly, Zhejiang
30	CC6	Cultural *C. sinensis*	Mycelia	Jolly, Zhejiang
31	CC7	Cultural *C. sinensis*	Mycelia	Ji Min Ke Xin, Jiangxi
32	CC8	Cultural *C. sinensis*	Mycelia	Ji Min Ke Xin, Jiangxi
33	CC9	Cultural *C. sinensis*	Mycelia	Jolly, Zhejiang
34	CC10	Cultural *C. sinensis*	Mycelia	Huadong, Zhejiang
35	CC11	Cultural *C. sinensis*	Mycelia	Lab cultured
36	CC12	Cultural *C. sinensis*	Mycelia	Lab cultured

temperature for 20 min (CT15RE, Hitachi Koki Co., Ltd. Tokyo, Japan), the supernatants were evaporated to dryness with a nitrogen evaporator at 35 °C and then were used for derivatization for PACE analysis, and redissolved in 100 μL of methanol for HPTLC analysis, respectively. Polysaccharide solution without enzymes, treated as described above, was used as blank control. The polysaccharide standards, including pectic galactan, oat glucan, starch, cellulose and dextran (5 mg/mL, 100 μL), were treated with the corresponding enzymes, respectively, under the above conditions. The enzymatic hydrolysates were used as markers for PACE and HPTLC.

2.5. Derivatization with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS)

The derivatization was carried out according to the previously reported method (Wu et al., 2013). Briefly, ANTS was prepared in acetic acid/water (3:17, v/v) at a final concentration of 0.1 mol/L. $NaCNBH_3$ (1 mol/L) was solubilized in dimethyl sulfoxide for ANTS derivatization. To each dry sample and sugar standards, 50 μL of ANTS solution and 50 μL of $NaCNBH_3$ solution were added. The reagents were mixed, centrifuged and incubated at 37 °C for 17 h. Then the solution was evaporated to dryness with a nitrogen evaporator at 35 °C, and the derivatized sugars were resuspended in 300 μL of urea (6 mol/L) solution and stored at −20 °C before use.

2.6. PACE analysis

PACE was performed according to our previously reported method (Wu et al., 2013). In brief, all the samples (1–3 μL depending of the sugar concentration) were separated using a vertical slab gel electrophoresis apparatus, Mini-Protean Tetra System (Bio-Rad, Hercules, CA, USA), with 10 cm plates, 1.0-mm spacer, and well of width 0.3 cm. Electrophoresis of 30% (w/v) polyacrylamide in the resolving gel with a stacking gel of 8% (w/v) polyacrylamide was used for separation of partial acid and enzymatic hydrolysates. The electrophoresis buffer was 0.1 mol/L Tris-boric (pH 8.2). The samples were firstly electrophoresed at 200 V for 20 min and then at 700 V for 45 min, to move bromophenol blue (migration indicator) to the designed level. All runs were performed at least two times independently. Gels were imaged using an InGenius LHR CCD camera system (Syngene, Cambridge, UK) under UV 365 nm.

2.7. HPTLC analysis

All the samples (5–10 μL) were applied on a 20 cm × 10 cm silica gel plate (Merck, Darmstadt, Germany) with an AS30 HPTLC Applicator (Desaga GmbH, Germany). The bands were 6 mm wide, 10 mm distance, and 10 mm from the bottom edge. Then all the plates were firstly developed to a distance of 90 mm with 1-butanol/isopropanol/acedic acid/water, 7:5:1:2 (v/v/v/v) as mobile phase at room temperature. Then the plate was dried in a stream of cool air and was placed in the same chamber to develop a distance of 95 mm with the same mobile phase. Finally, the developed plates were colorized with aniline-diphenylamine-phosphoric acid solution and heated at 105 °C for 5 min on a YOKO-XR plate heater (Wuhan YOKO technology Ltd., China) and covered with transparent glass and photographed.

2.8. Method validation

The method repeatability, the sample stability and the limit of detection of both PACE and HPTLC were investigated,

Table 2
Limit of detection (LOD) for the compounds investigated in HPTLC and PACE method.

Analyte	LOD for HPTLC (ng)	LOD for PACE (ng)
Glucose	40.0	4.5
Laminaribiose (DP2)	80.0	8.5
Laminarihexaose (DP6)	400.0	24.7

respectively. In brief, for investigation of the method repeatability of both PACE and HPTLC, the selected samples were prepared thrice, electrophoresed and developed twice independently under the same conditions, respectively. Moreover, for investigation of the sample stability for both PACE and HPTLC, the partial acid hydrolysates of polysaccharides from natural *C. sinensis* were prepared, and stored at −20 °C for PACE and 4 °C for HPTLC on successive day 1–5, respectively. Finally, the mixed standards of glucose, laminaribiose (DP2) and laminarihexaose (DP6) were used for determination of the limit of detection of PACE and HPTLC, respectively, and the limit of detection was calculated based on the visual bands of the lowest concentration of mixed standards.

2.9. Data analysis

The optical densities of bands in electronic images and digital scanning profiles of PACE analysis were generated and analyzed using Quantity-One software (version 4.6.2, Bio-Rad, Hercules, USA). The similarities of the tested samples, as well as the simulative mean chromatogram were calculated and generated using the professional software named "Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine" (Matlab version, Ver1.315, developed by the Research Center of Modernization of Chinese Herbal Medicine, Central South University and the Hong Kong Polytechnic University).

3. Results and discussion

3.1. Method validation of PACE and HPTLC

HPTLC (Xie et al., 2012) and PACE (Wu et al., 2013) are good methods for analysis of monosaccharides and oligosaccharides derived from polysaccharides, respectively, and both of them are simple and high throughput. Fig. 2 shows the method repeatability, the sample stability, and the limit of detection (LOD) for the methods of PACE and HPTLC. The results indicated that the method repeatability of both PACE and HPTLC was good. Moreover, the results also showed that the samples for PACE analysis stored at −20 °C, and the samples for HPTLC analysis stored at 4 °C were stable. Actually, the samples stability for PACE method could be stable for at least 6 months at −20 °C (Goubet, Dupree, & Johansen, 2011). The LOD for PACE and HPTLC methods are summarized in Table 2. The sensitivity of PACE was much higher than that of HPTLC, though the sensitivity of HPTLC was much higher than that of ELSD and RID, the commonly used detectors for compouds without UV absorbance (Morlock & Sabir, 2011). In addition, the resolution of PACE for separation of oligosaccharides was much higher than that of HPTLC (Fig. 2A and B).

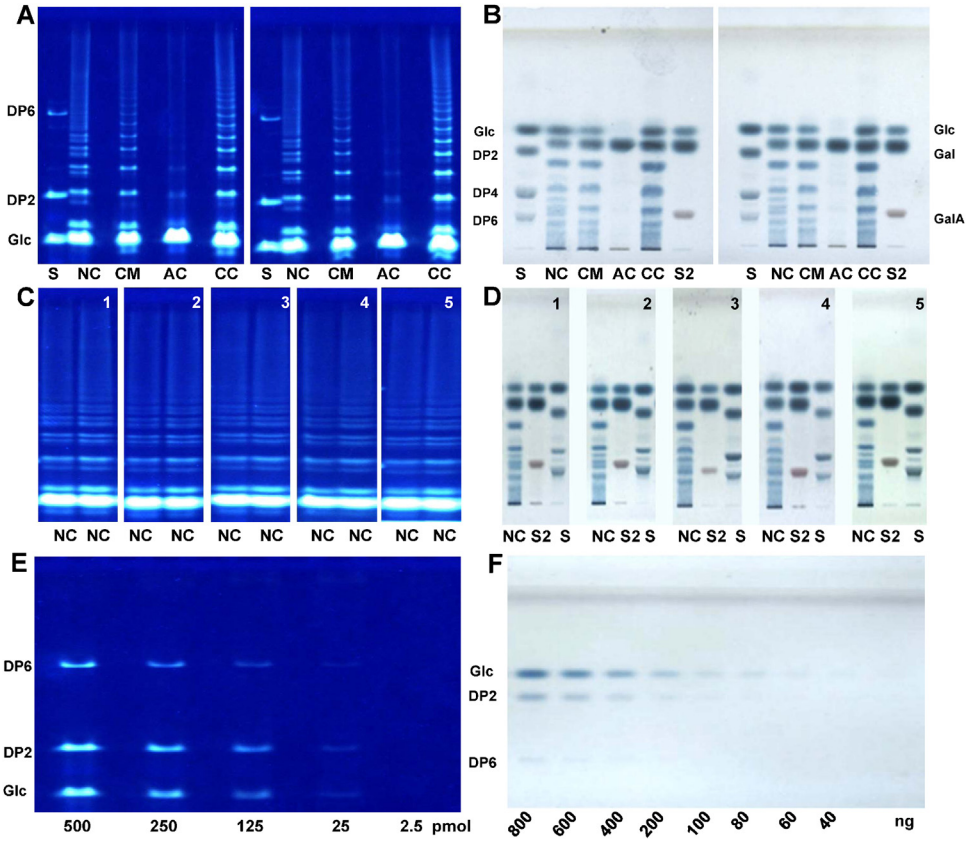

Fig. 2. Repeatability (A and B), sample stability (C and D), and limit of detection (E and F) of PACE and HPTLC. **S**, Glc, laminaribiose (DP2), laminariteetraose (DP4) and laminarihexaose (DP6); **S2**, Glc, Gal and GalA; **NC**, **AC**, **CM** and **CC**, partial acid hydrolysates of polysaccharides from same sample of natural *Cordyceps sinensis*, *Cordyceps gunnii*, *Cordyceps militaris*, and cultured *C. sinensis*, respectively; **C** and **D**, samples stored for 1, 2, 3, 4 and 5 days at −20 °C for PACE analysis and 4 °C for HPTLC analysis, respectively; **E** and **F**, PACE and HPTLC profiles of **S** with series concentrations.

3.2. Partial acid hydrolysates profiles of polysaccharide from natural and cultured Cordyceps spp.

Gas chromatography–mass spectrometry (GC–MS) based on the complete acid hydrolysates of polysaccharides were developed for evaluation of polysaccharides from natural and cultured *Cordyceps* spp. (Guan et al., 2010). However, the compositional monosaccharides of natural and cultured *Cordyceps* spp. were the same, and the ratio of monosaccharides obtained may be not correct due to the degradation of monosaccharides under strong acid conditions. Moreover, GC–MS is usually not suitable for analysis of oligosaccharides, and is time consuming for analysis of a large scale of samples. In this study, partial acid hydrolysates of polysaccharides, under mild acid hydrolysis, from natural and cultured *Cordyceps* were investigated using PACE and HPTLC analysis, respectively. The partial acid hydrolysates of polysaccharides from natural *C. sinensis* were greatly similar in both PACE and HPTLC fingerprints (Fig. 3), which were obviously different from those of other species of *Cordyceps*, including *C. gunnii* (AC1 and AC8), *C. liangshanensis* (AC2 and AC4), and *C. hawkesii* (AC3 and AC6), cultured *C. militaris* (CM6 and CM7) and cultured *C. sinensis* (CC3, CC5, CC6, CC9 and CC11). It is interesting that both PACE and HPTLC profiles of some samples, such as *C. gracilis* (AC5) and *C. ciecadae* (AC7), cultured *C. militaris* from different manufacturers including CM1, CM2, CM3, CM4, CM5, CM8 and CM9, as well as cultured *C. sinensis* from different manufacturers including CC1, CC2, CC4, CC7, CC8, CC10 and CC12 were similar although the intensity of some bands was variant, which could be differentiated from natural *C. sinensis* (Fig. 3).

In addition, more bands were detected by using PACE compared to those of HPTLC, which meant that more information for structures of the polysaccharides was obtained. Therefore, the similarities of each sample were evaluated based on the PACE fingerprints according to our previous reported method (Wu et al., 2013). In brief, the chromatogram of each sample was obtained based on the gel image using Quantity-One software (Fig. 3B). The similarities of their entire chromatographic patterns were evaluated by using "Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine". The average correlation coefficient of each chromatogram of natural *C. sinensis* to their simulative mean chromatogram (SMC-NC-H) was 0.963 ± 0.017 ($n = 7$; Table 3), which supported the acidic hydrolysate fingerprints of polysaccharides from natural *C. sinensis* were pretty similar. Finally, a representative sample (NC6) was selected as reference for comparison with other species of natural and cultured *Cordyceps*. The correlation coefficient of individual chromatogram of other species of natural *Cordyceps*, cultured *C. sinensis* and cultured *C. militaris* to the chromatogram of the selected natural *C. sinensis* (NC6-H) were summarized in Table 4. The results showed that *C. gracilis* (AC5) and *C. ciecadae* (AC7), cultured *C. militaris* (CM1–CM9, except CM6 and CM7), and cultured *C. sinensis* (CC10 and CC12) had relatively high similarity to natural *C. sinensis*, but the intensity of some bands was different (Fig. 3B). The cultured *C. militaris* (except CM6 and CM7) produced by the different manufactures had high similarity, which might mainly attribute to their same species and the similar culture conditions. Actually, the species of *Cordyceps* and the culture conditions had significant effects on the formation of polysaccharides (Kim & Yun, 2005; Paterson, 2008; Wu et al., 2012). Moreover, the similarity among cultured *C. sinensis* produced by the different manufactures was greatly variant, which might due to the different strains isolated from natural *C. sinensis* and the

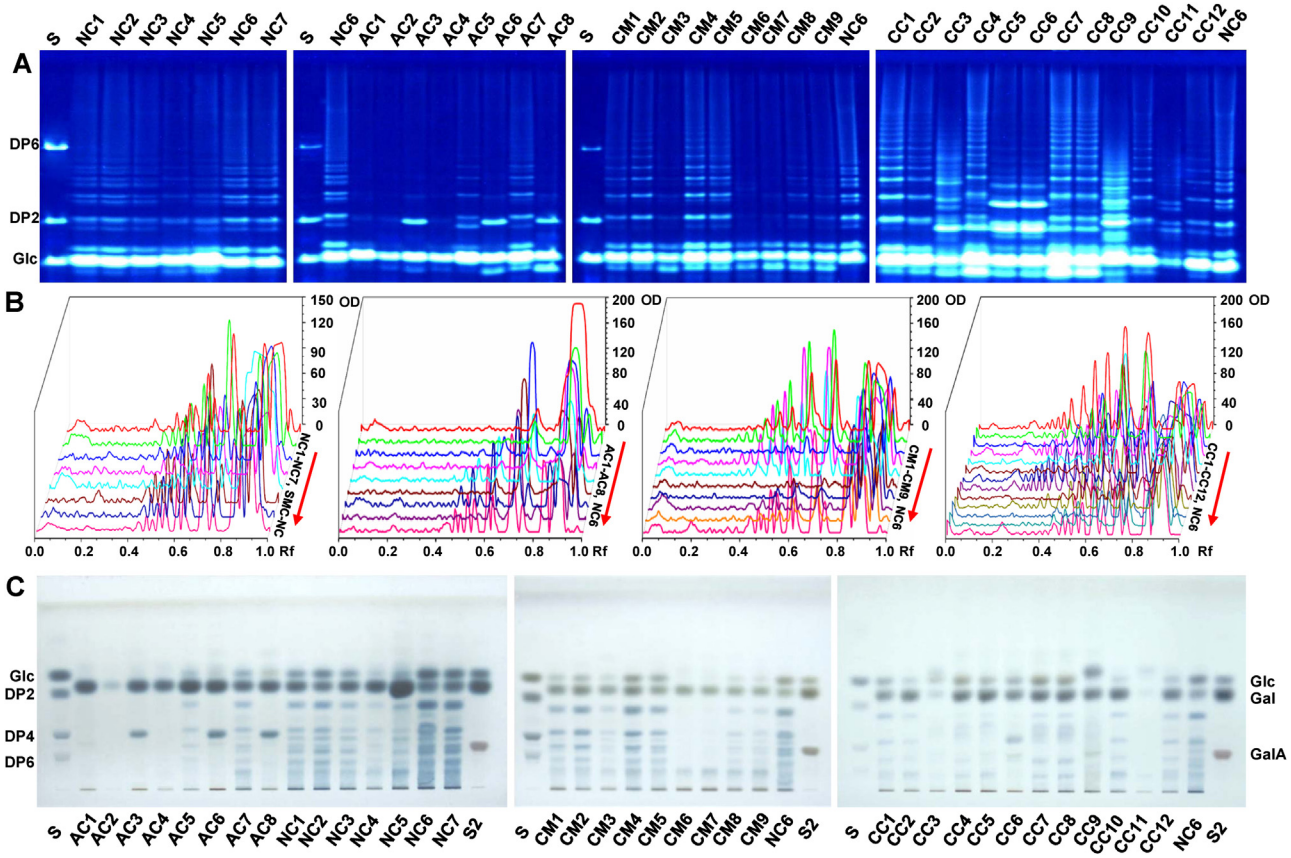

Fig. 3. PACE fingerprints (A) and chromatograms (B) as well as HPTLC profiles (C) of partial acid hydrolysates of polysaccharides from natural and cultured *Cordyceps*. **S**, Glc, laminaribiose (DP2), laminariteetraose (DP4) and laminarihexaose (DP6); **S2**, Glc, Gal and GalA; **SMC-NC**, the simulative mean chromatograms of partial acid hydrolysates of polysaccharides from natural *Cordyceps sinensis*. The sample codes were the same as in Table 1.

Table 3
The correlation coefficient of each natural *C. sinensis* to their simulative mean chromatogram.

	Samples						
	NC1	NC2	NC3	NC4	NC5	NC6	NC7
Partial acid hydrolysates							
SMC-NC-H	0.9891	0.9691	0.9590	0.9352	0.9463	0.9668	0.9740
α-Amylase digestions							
SMC-NC-A	0.9519	0.9609	0.9479	0.9128	0.9297	0.9681	0.9849
β-Glucanase digestions							
SMC-NC-G	0.9909	0.9815	0.9753	0.9330	0.9454	0.9517	0.9831
Pectinase digestions							
SMC-NC-P	0.9965	0.9980	0.9986	0.9770	0.9995	0.9919	0.9642

The sample codes were the same as in Table 1.

fermentation conditions (Zhong et al., 2009). However, partial acid hydrolysis is non-specific. Therefore, enzymatic hydrolysis based on chemical structural characters of polysaccharides was further investigated.

3.3. Enzymatic fingerprints of polysaccharides

3.3.1. Selection of endoglycosidases for digestion of polysaccharides in C. sinensis

Previous studies have shown that polysaccharides from natural and cultured *Cordyceps* usually consist of galactose, glucose and mannose (Guan et al., 2010; Li et al., 2003), and the major linkages in the bioactive polysaccharide are 1,3(4)-β-D-Glc*p*, 1,4(6)-α-D-Glc*p*, 1,2(6)-α-D-Man*p*, 1,4(6)-β-D-Man*p*, 1,4(6)-α-D-Gal*p* (Wang et al., 2011; Wu, Sun, & Pan, 2005; Wu, Sun, & Pan, 2006; Yu et al., 2007; Yu et al., 2009). Indeed, α-amylase, β-glucanase, pectinase, dextranase, β-mannanase, cellulase and isoamylase had obvious effects on polysaccharides from natural *C. sinensis*, but specific characteristics were not found due to poor resolution of HPSEC for both polysaccharides and their hydrolysates (Guan et al., 2011). Therefore, the enzymatic digestions of α-amylase, β-glucanase, pectinase, dextranase, cellulase and isoamylase on polysaccharides were analyzed by using PACE, respectively. Fig. 4 showed that the polysaccharides from natural and cultured *C. sinensis* exhibited positive response to the selected enzymes, which were in accordance with our previous reports (Guan et al., 2011). Especially, polysaccharides in natural and cultured *C. sinensis* could be differentiated based on the α-amylase, β-glucanase and pectinase digested polysaccharides fingerprints, and the chemical characteristics of the hydrolysates were distinctive. Furthermore, these enzymes might digest the backbone of the bioactive polysaccharides in *C. sinensis* (Wu et al., 2005; Zhong et al., 2009; Zhou et al., 2009). Therefore, α-amylase, β-glucanase and pectinase were selected for establishment of the enzymatic hydrolysate fingerprints of polysaccharides in natural and cultured *Cordyceps*.

Table 4
The correlation coefficient of each tested sample to the chromatogram of natural *Cordyceps sinensis*.

Samples	The chromatograms of partial acid and enzymatic hydrolysates			
	NC6-H	NC6-A	NC6-G	NC6-P
Adulterants				
AC1	0.6127	0.1582	0.0240	0.9650
AC2	0.5725	0.0235	0.0969	0.9736
AC3	0.5656	0.0552	0.6114	0.9689
AC4	0.6335	0.0137	0.0609	0.9752
AC5	0.8078	0.8848	0.8752	0.9805
AC6	0.5238	0.0357	0.6548	0.9809
AC7	0.8540	0.8091	0.8932	0.9888
AC8	0.4990	0.0441	0.6954	0.9611
Cultural Cordycpes militaris				
CM1	0.8622	0.8961	0.9541	0.9700
CM2	0.8780	0.9105	0.9390	0.9781
CM3	0.7110	0.9024	0.9639	0.9860
CM4	0.8346	0.9312	0.9103	0.9742
CM5	0.8539	0.9108	0.9757	0.9640
CM6	0.5483	0.5796	0.6388	0.9626
CM7	0.5683	0.7585	0.6760	0.9431
CM8	0.7847	0.8804	0.8973	0.9699
CM9	0.7747	0.8589	0.8423	0.9803
Cultural Cordycpes sinensis				
CC1	0.7004	0.8778	0.6843	0.9473
CC2	0.7390	0.7277	0.8303	0.9827
CC3	0.6144	0.5917	0.6714	0.8844
CC4	0.6924	0.6902	0.7397	0.8275
CC5	0.3841	0.6388	0.7417	0.8344
CC6	0.3607	0.6753	0.6540	0.3576
CC7	0.6833	0.7859	0.6308	0.8720
CC8	0.6956	0.7214	0.6911	0.8699
CC9	0.4616	0.0415	0.0779	0.9283
CC10	0.8624	0.7648	0.8734	0.9616
CC11	0.7449	0.0214	0.0532	0.9254
CC12	0.8805	0.7959	0.8490	0.9320

The sample codes were the same as in Table 1.

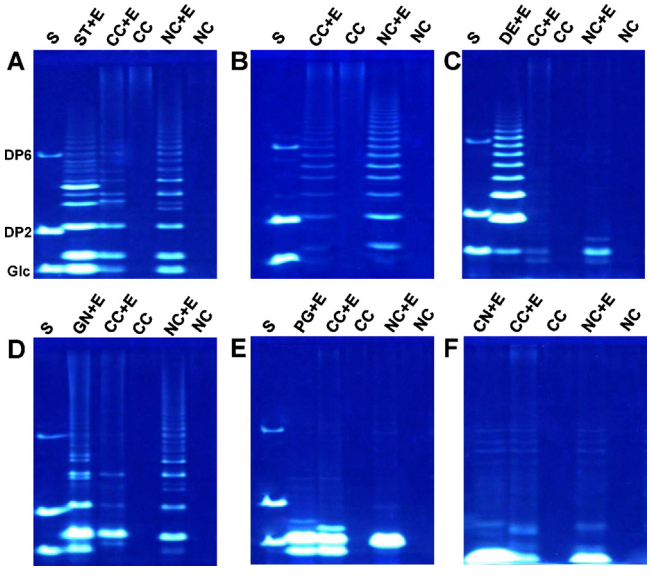

Fig. 4. Selection of response characteristic enzymes for polysaccharides from natural and cultured *C. sinensis*. **S**, Glc, laminaribiose (DP2), and laminarihexaose (DP6); **NC** + **E** and **CC** + **E**, enzymatic hydrolysis of polysaccharides from natural and cultured *C. sinensis*, respectively; **NC** and **CC**, polysaccharides without enzymes; **A**, **B**, **C**, **D**, **E** and **F**, α-amylase, isoamylase, dextranase, β-D-glucanase, pectinase and cellulase, respectively; **ST**, **DE**, **GN**, **PG** and **CN**, the polysaccharide standards, starch, dextran, oat glucan, pectic galactan, and cellulose digested with the corresponding enzyme used as positive control, respectively.

3.3.2. α-Amylase digested polysaccharides fingerprints of natural C. sinensis

Saccharide mapping with enzymatic digestion is a specific and mild hydrolysis approach with high selectivity, which has been used for discrimination and characterization of polysaccharides from traditional Chinese medicines in our group (Guan & Li, 2010; Wu et al., 2013; Xu, Guan, Chen, Zhao, & Li, 2011). Previously, saccharide mapping based on HPSEC coupled with ELSD has been performed for comparison of polysaccharides from natural and cultured *C. sinensis* (Guan et al., 2011). However, HPSEC is difficult to simultaneously separate both polysaccharides and their hydrolysates. In addition, the sensitivity of ELSD is relatively poor. Therefore, both HPTLC and PACE were performed for analysis of the specific characteristics of enzymatic digestions. PACE and HPTLC fingerprints of α-amylase digested polysaccharides from natural *C. sinensis* are shown in Fig. 5. Their specific characteristics of enzymatic digestions of polysaccharides in natural *C. sinensis* collected from different regions of China, detected by both HPTLC and PACE, were similar. The results showed that polysaccharides in natural *C. sinensis* could be completely digested to produce the mainly low molecular weight sugars, with degree of polymerization (DP) between 1 and 6, which were not existed in samples before enzymatic hydrolysis (data unshown). The results suggested the main structure of polysaccharides from natural *C. sinensis* contained 1,4-α-glucosidic linkage, which were in accordance with the previous report (Zhou et al., 2009). Especially, more bands were detected by using PACE (13 bands, Fig. 5A) than HPTLC (7 bands, Fig. 5C), which suggested that the resolution of PACE for separation of oligosaccharides was much higher than that of HPTLC. Therefore, more information on the structure of polysaccharides could be obtained by using PACE. The similarities of each sample were calculated based on the chromatograms of PACE fingerprints (Fig. 5B). The correlation coefficient of individual chromatogram of natural *C. sinensis* to their simulative mean chromatogram (SMC-NC-A) was summarized in Table 3. Meanwhile, the average correlation coefficient of each chromatogram to SMC-NC-A was 0.954 ± 0.024 ($n = 7$), which supported the enzymatic fingerprints of polysaccharides from natural *C. sinensis* were pretty similar. Finally, a representative sample (NC6) was selected as reference for comparison with other species of natural and cultured *Cordyceps*.

3.3.3. β-Glucanase digested polysaccharides fingerprints of natural C. sinensis

PACE and HPTLC fingerprints of β-glucanase digested polysaccharides from natural *C. sinensis* are shown in Fig. 6. The specific characteristics of enzymatic digestions detected by both HPTLC and PACE were similar to the results of α-amylase digested polysaccharides. The results showed that the mainly small sugars of the hydrolysates were saccharides with DP from 1 to more than 6 (Fig. 6). β-D-Glucanase can act 1,4 or 1,3 bonds on the 1,3(4)-β-D-glucan. Meanwhile, our previous studies showed that 1,3-β-D-glucanase had no effect on the polysaccharide from natural *C. sinensis* (Guan et al., 2011). Therefore, the results suggested the main structure of polysaccharides from natural *C. sinensis* also contained 1,4-β-glucosidic linkages. Furthermore, both HPTLC and PACE fingerprints suggested that polysaccharides from natural *C. sinensis* collected from different regions of China were similar to those of α-amylase digested polysaccharides. As shown in Fig. 6A and C, more bands were detected by using PACE (16 bands) than HPTLC (5 bands). Therefore, the correlation coefficient of individual chromatogram of natural *C. sinensis* to their simulative mean chromatogram (SMC-NC-G) based on the chromatograms of PACE

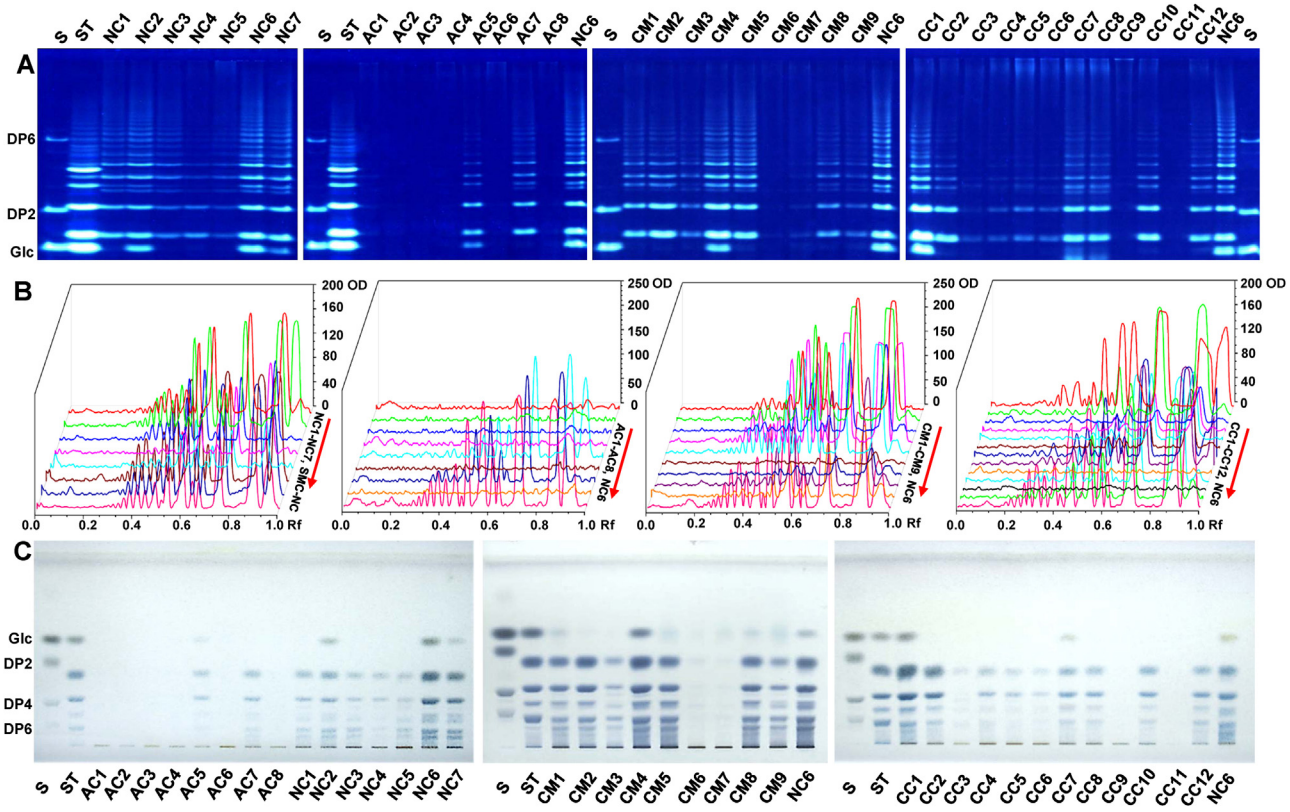

Fig. 5. PACE fingerprints (A) and chromatograms (B) as well as HPTLC profiles (C) of α-amylase digested polysaccharides from natural and cultured *Cordyceps*. **S**, Glc, laminaribiose (DP2), laminariteetraose (DP4) and laminarihexaose (DP6); **ST**, α-amylase digested starch used as positive control; **SMC-NC**, the simulative mean chromatograms of the α-amylase digested polysaccharides from natural *Cordyceps sinensis*. The sample codes were the same as in Table 1.

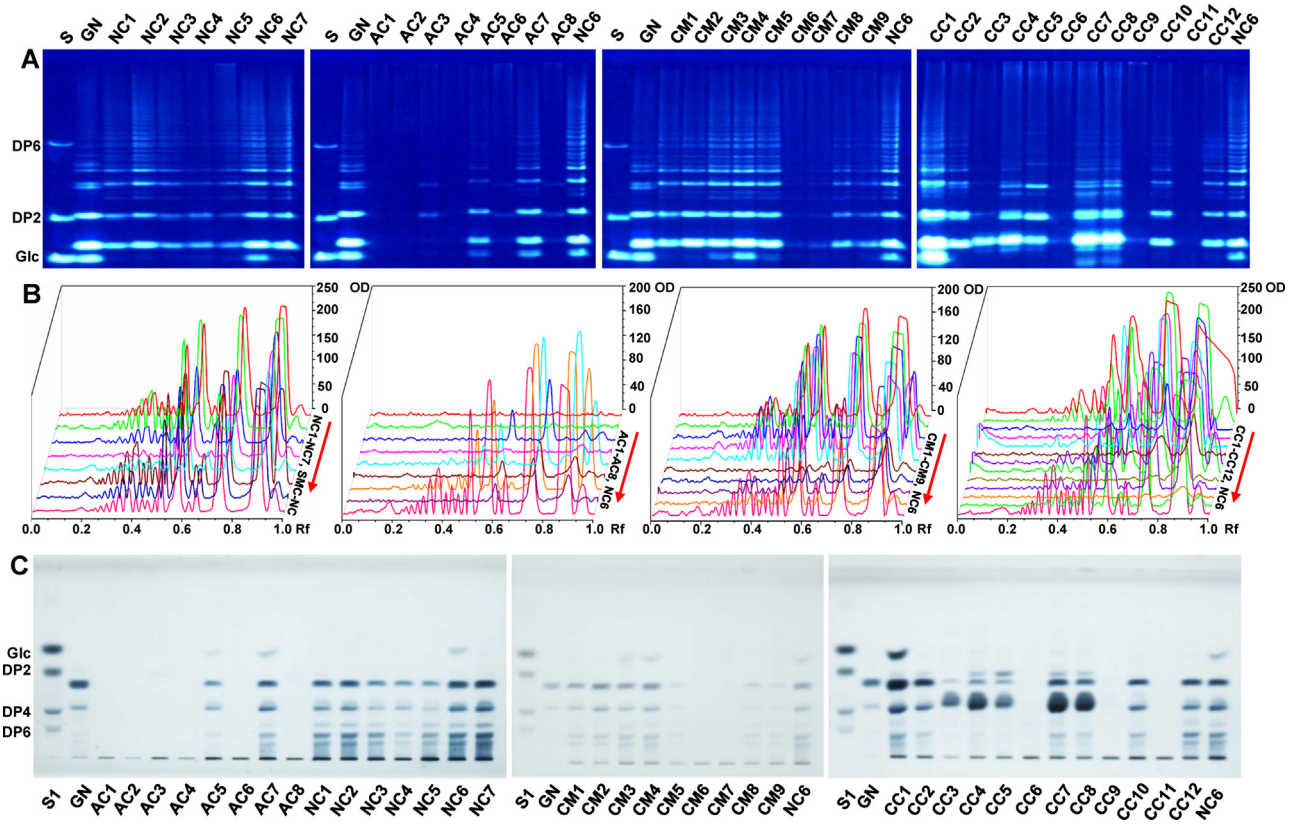

Fig. 6. PACE fingerprints (A) and chromatograms (B) as well as HPTLC profiles (C) of β-glucanase digested polysaccharides from natural and cultured *Cordyceps*. **S**, Glc, laminaribiose (DP2), laminariteetraose (DP4) and laminarihexaose (DP6); **GN**, β-glucanase digested oat glucan used as positive control; **SMC-NC**, the simulative mean chromatograms of the β-glucanase digested polysaccharides from natural *Cordyceps sinensis*. The sample codes were the same as in Table 1.

fingerprints was evaluated and summarized in Table 3, and the average correlation coefficient of each chromatogram to SMC-NC-G was 0.960 ± 0.030 (n = 7). Finally, a representative sample (NC6) was also selected as reference for comparison with other species of natural and cultured *Cordyceps*.

3.3.4. Pectinase digested polysaccharides fingerprints of natural C. sinensis

PACE and HPTLC fingerprints of pectinase digested polysaccharides from natural *C. sinensis* are shown in Fig. 7. The results showed that all tested samples of natural *C. sinensis* could be hydrolyzed by pectinase, which supported our previous report (Guan et al., 2011). Pectinase can act on the 1,4-α-D-galactosidic and 1,4-α-D-galactosiduronic linkages of pectic galactan and polygalacturonan, respectively. Previous studies have shown that polysaccharides from natural *C. sinensis* usually consist of galactose, glucose and mannose (Guan et al., 2010). HPTLC fingerprints showed that the enzymatic hydrolysates with pectinase were galactose and glucose (Fig. 7C), which were in accordance with our previous report (Guan et al., 2011). Therefore, the results suggested that polysaccharides from natural *C. sinensis* richly contained 1,4-α-D-galactosidic linkages. Furthermore, both HPTLC and PACE fingerprints suggested that polysaccharides from natural *C. sinensis* collected from different regions of China were similar to those of α-amylase and β-glucanase digested polysaccharides, and the average correlation coefficient of individual chromatogram, obtained based on the chromatograms of PACE fingerprints (Fig. 7B), of natural *C. sinensis* to their simulative mean chromatogram (SMC-NC-P) was 0.993 ± 0.008 (n = 7; Table 3). Moreover, as shown in Fig. 7C, galactose and glucose in the hydrolysates could be efficiently separated by using HPTLC analysis, however, which was not available for PACE analysis (Fig. 7A). The results indicated that the resolution for separation of monosaccharides by using HPTLC was much better than that of PACE. Therefore, the combination of HPTLC and PACE analysis was performed for comparison of polysaccharides from natural and cultured *Cordyceps*.

3.3.5. Comparison of polysaccharides from natural and cultured Cordyceps spp.

HPSEC coupled with ELSD analysis showed that the enzymatic response of polysaccharides from natural *C. sinensis* (four batches), cultured *C. sinensis* (two batches), and cultured *C. militaris* (two batches) were similar (Guan et al., 2011). However, enzymatic digestions followed by PACE and HPTLC analysis showed the difference among various species of *Cordyceps*, especially the adulterants (AC), were obvious. The similarity among cultured *C. militaris* and *C. sinensis* produced in different manufactures in China were also found (Figs. 5–7). Briefly, polysaccharides from the adulterants including *C. gunnii* (AC1 and AC8), *C. liangshanensis* (AC2 and AC4), and *C. hawkesii* (AC3 and AC6) could not be hydrolyzed by α-amylase (Fig. 5), meanwhile AC1, AC2 and AC4 also could not be digested by β-D-glucanase (Fig. 6), although their pectinase fingerprints were pretty similar (Fig. 7). Therefore, they could be directly differentiated from natural *C. sinensis* based on enzymatic fingerprints of α-amylase and β-D-glucanase. It is interesting that profile of α-amylase, β-D-glucanase and pectinase digested polysaccharides from AC5 and AC7 were similar with those of natural *C. sinensis*. In addition, polysaccharides from commercial *C. militaris* (CM1–CM9) produced by different manufactures in China could be digested by α-amylase, β-D-glucanase and pectinase, respectively. The three types of fingerprints of polysaccharides from commercial *C. militaris* (CM1, CM2, CM3, CM4, CM5, CM8 and CM9) were similar

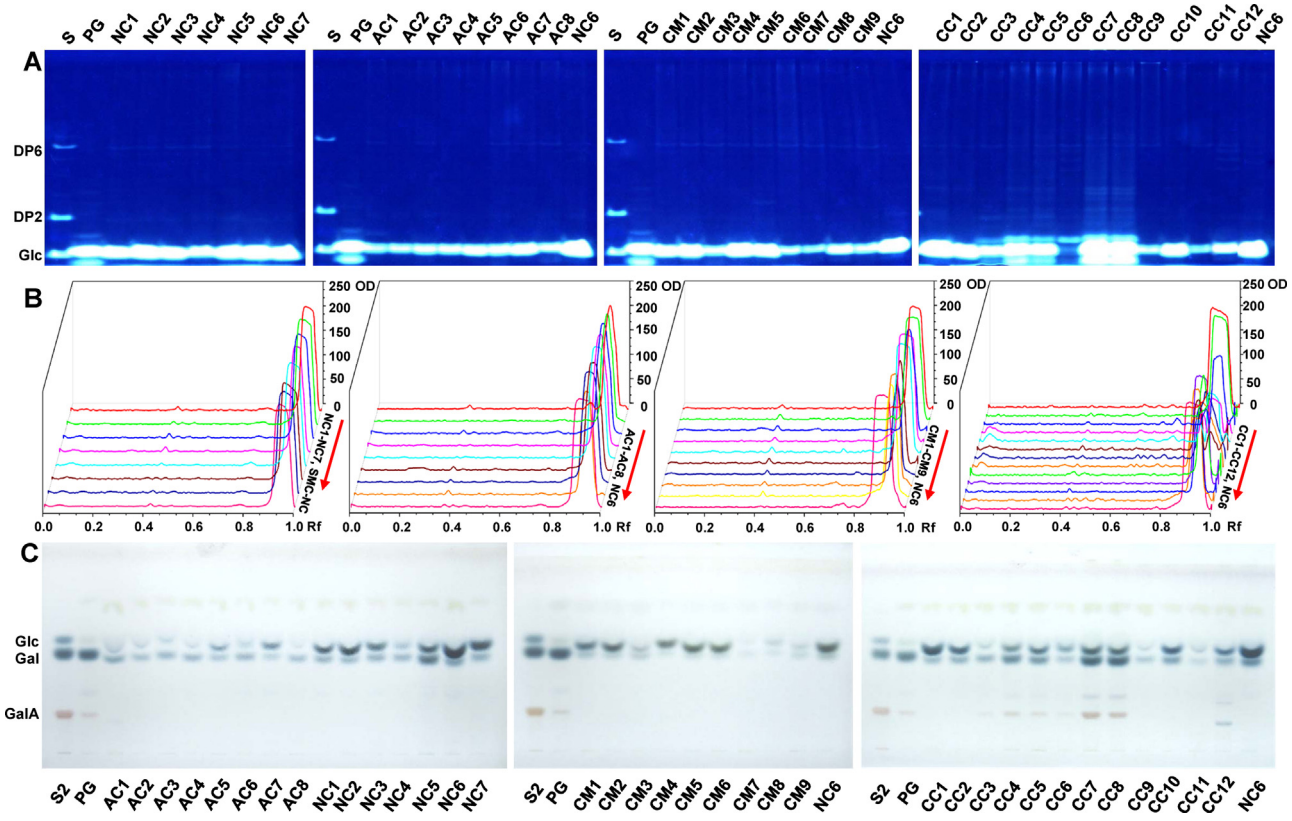

Fig. 7. PACE fingerprints (A) and chromatograms (B) as well as HPTLC profiles (C) of pectinase digested polysaccharides from natural and cultured *Cordyceps*. **S**, Glc, laminaribiose (DP2) and laminarihexaose (DP6); **S2**, Glc, Gal and GalA; **PG**, pectinase digested pectic galactan used as positive control; **SMC-NC**, the simulative mean chromatograms of the pectinase digested polysaccharides from natural *Cordyceps sinensis*. The sample codes were the same as in Table 1.

with those of natural *C. sinensis* (Figs. 5–7). However, both HPTLC and PACE fingerprints of α-amylase and β-D-glucanase digested polysaccharides of CM6 and CM7 were obviously different from those of natural *C. sinensis*, which might attribute to the different culture conditions could induce various polysaccharide formation (Wu et al., 2012; Yue, Ye, Zhou, Sun, & Lin, 2013). Furthermore, polysaccharides from cultured *C. sinensis* (CC9 and CC11) could not be hydrolyzed by α-amylase (Fig. 5), meanwhile CC11 also could not be digested by β-D-glucanase (Fig. 6), which could be directly discriminated from natural *C. sinensis* based on enzymatic fingerprints of α-amylase and β-D-glucanase. Moreover, PACE fingerprints of pectinase digested polysaccharides from CC3, CC4, CC5, CC6, CC7 and CC8 were obviously different from those of natural *C. sinensis* (Fig. 7A). GalA was detected in polysaccharides in CC by using HPTLC analysis (Fig. 7C), which was not existed in natural *C. sinensis*. The results were in accordance with our previous report that GalA was existed in cultured *C. sinensis* (Guan et al., 2011). Therefore, the commercial products, including CC3, CC4, CC5, CC6, CC7 and CC8, could be discriminated from natural *C. sinensis* based on the HPTLC fingerprints of pectinase. Especially, both HPTLC and PACE fingerprints of α-amylase and β-D-glucanase digested polysaccharides from CC1, CC2, CC7, CC8, CC10 and CC12 were similar, but their fingerprints were different from those of natural *C. sinensis*.

The correlation coefficient of individual chromatogram of cultured *C. militaris*, cultured *C. sinensis* and adulterants to the chromatogram of the representative natural *C. sinensis* (NC6) were summarized in Table 4. The results showed that the commercial products, including cultured *C. militaris* (CM1–CM9, except CM6 and CM7), cultured *C. sinensis* (CC2, CC10 and CC12) had relatively high similarity to natural *C. sinensis* although the intensity of several bands was variant (Figs. 5B, 6B and 7B), which is helpful for rational use of the commercial *Cordyceps* products, and beneficial to improve the quality control of cultured and natural *Cordyceps*. Especially, it is interesting that the different species of *Cordyceps* (*C. gracilis* and *C. ciecadae*) also had relatively high similarity to natural *C. sinensis*, which may be helpful to develop *Cordyceps* polysaccharides as substitute of polysaccharides from natural *C. sinensis*. Furthermore, the great difference among the fingerprints of polysaccharides from cultured *C. sinensis* might attribute to the different strains isolated from natural *C. sinensis* (Yue et al., 2013).

4. Conclusions

Saccharide mapping based on PACE and HPTLC analysis, are good methods with high repeatability, stability, sensitivity and throughput for analysis of polysaccharides from natural and cultured *Cordyceps* spp., which is helpful to improve their quality control of polysaccharides, and beneficial for rational usage of commercial *Cordyceps* products, as well as the development of cultured *Cordyceps* as a source of polysaccharides in natural *C. sinensis*.

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