Capillary Zone Electrophoresis with Amperometric Detection for Composition Analysis of Laminarin

WANG, Qing-Jiang(王清江)[a] DING, Fei(丁飞)[a] LI, Hui(李辉)[b] HE, Pin-Gang(何品刚)[a] FANG, Yu-Zhi(方禹之)*,[a]

[a] *Department of Chemistry, East China Normal University, Shanghai 200062, China*
[b] *School of Chemistry and Chemical Engineering, Shanghai Jiaotong University, Shanghai 200240, China*

The composition of laminarin was firstly determined by analyzing its hydrolysis monosaccharides with capillary zone electrophoresis-amperometric detection (CZE-AD). Under the selected optimum conditions, fucose, galactose, glucose, mannose and xylose, which are hydrolysis products of laminarin, could be perfectly separated within 20 min and showed significant current responses at copper electrodes. The linear ranges of fucose, galactose and glucose were from 1.0×10^{-6} to 2.0×10^{-4} mol·L^{-1}, those of mannose and xylose were from 5.0×10^{-6} to 2.0×10^{-4} mol·L^{-1}, and their detection limits were at 10^{-7} mol·L^{-1} level ($S/N=3$). The molar ratio of fucose, galactose, glucose, mannose and xylose in laminarin was 10.5:2.8:1.0:7.3:3.4 and the purity of this polysaccharide leached by the introduced leaching method was 95.7%. Compared to usual UV-vis and other spectrometric methods, analyzing polysaccharide by this method has some merits of quickness, low-volume sampling, simple instrumentation, high sensitivity and high reproducibility.

Keywords laminarin, capillary zone electrophoresis, amperometric detection

Introduction

In recent decades, many kinds of plant polysaccharides have been extensively studied for their potential value in immunology, especially in cancer therapeusis. Laminarin, one kind of heteropolysaccharides isolated from *Laminaria japonica*, was found to be one of the inhibitors of basic fabric cell-generation factor (bFGF) and depress the formation of tube structure of endothelial cells, which further depress the activation of rats' cancer cells.[1] It was also found that laminarin sulfate could inhibit extracellular matrix (ECM) degradation by mammalian heparinase and prevent experimental autoimmune encephalomyelitis (EAE).[2]

Since polysaccharides are very large molecules without active chemico-analytic characters and usually combined with proteins, pigments and other substances, their analysis is relatively difficult. Many analytical methods, such as NMR,[3,4] TLC,[5] GCC,[6] UV,[7] GC[8] and HPLC,[9] have been studied and applied in the structural and quantitative analysis of polysaccharides. However, most above methods are suffered from complicated instruments and expensive reagents, while others can only be used in qualitative or approximately quantitative analysis.

Recently, capillary electrophoresis (CE) has been introduced into the analysis of monosaccharides and simple polysaccharides. Because polysaccharides can be hydrolyzed into one or some kinds of monosaccharides with definite ratios, their compositions, metabolic kinetics in bodies and purity in medicines could be determined by analyzing the types and contents of their hydrolyzed monosaccharides after CE separations.[10-12] Currently, UV-vis and laser-induced fluorescence (LIF) are used in combination with CE. Because there is no chromophoric group in saccharide molecules, extra procedures of pre- or post-column derivations are required for the detection of monosaccharides after CE separations when UV-vis absorption or LIF detection is used.[13] However, detecting monosaccharides by using electrochemical method (ED) can overcome above disadvantage, because the electroactive hydroxyl groups in carbohydrates can be catalytically oxidized on the surface of copper or other metallic electrodes. Furthermore, the electrochemical detection possesses higher sensitivity than UV-vis and simple instrument than LIF.[14-17]

In this paper, the CZE-AD method was firstly applied to study the composition of laminarin by analyzing its hydrolyzed monosaccharides. Experiments showed that the molar ratio of fucose, galactose, glucose, mannose and xylose in laminarin was 10.5:2.8:1.0:7.3:3.4 and the purity of this polysaccharide leached by the introduced leaching method was 95.7%. The results also showed that this method was of quickness, low-volume sampling, simple instrument and operation, high sensitivity and high reproducibility.

* E-mail: yuzhi@online.sh.cn
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Experimental

Apparatus

CZE-AD system was laboratory-built.[18,19] CZE was driven by a high-voltage supplier (± 30 kV, Shanghai Institute of Nuclear Research, China). Separations were performed in a fused silica capillary (Hebei Yongnian Laser-fiber factory, China) with 25 μm i.d., 360 μm o.d. and 50 cm long. Potential control and current output were employed by a BAS LC-3D amperometric detector (Bioanalytical System, West Lafayette, IN, USA). Electropherograms were recorded by a chart recorder (Model XWT-204, Shanghai Dahua Instrument Factory, China). Electrochemical experiments were carried out on a CHI 630 electrochemical analyzer (CHI Instruments, USA). A three-electrode system was used in both electrochemistry and detection experiments, which consisted of a disk-shaped copper working electrode (Φ120 μm), a saturated calomel reference electrode (SCE) and a platinum wire counter electrode.

Reagents

All reagents were of analytical-reagent grade. Fucose, galactose, glucose, mannose and xylose were purchased from Shanghai Chemical Reagents Company, China National Medicines (Group). Their stock solutions with a concentration of 1.0×10^{-2} mol·L^{-1} were prepared with doubly distilled water and diluted to needed concentrations in CE experiments.

Laminaria japonica was purchased from Chinese Traditional Medicine Department of Shanghai Medicine Company.

Preparation of working electrode

This copper electrode was prepared according to the Ref. 16. Prior to use, the surface of the copper electrode was polished with emery paper and alumina powder respectively, then it was sonicated in doubly distilled water for 3 min to get enough cleanness.

Before experiments, the three-electrode system was fixed in corresponding positions of the electrochemical cell and the copper disk electrode was carefully adjusted to make an effective injection to the off-side of the capillary by the three-dimension positioner.

CZE *operation*

Before CZE separations, all used solutions were filtered through 0.45 μm polypropylene acrodisc syringe filter and sonicated for 5 min to remove bubbles. The capillary was sequentially rinsed with 1.0 mol·L^{-1} hydrochloric acid, doubly distilled water and 1.0 mol·L^{-1} sodium hydroxide 3 min for each and running buffer till the current inside of the capillary reached stable state. This was important to get a reproducible EOF.

The optimal conditions of this experiment were 12 kV as separation voltage, 0.060 mol·L^{-1} NaOH as buffer solution, 7 s as sampling time and 0.60 V as detection potential.

Sample analysis

An accurate weight of *Laminaria japonica* was firstly refluxed with acetone and 1∶1 (V∶V) ethanol-ether, respectively, under a boiled-water bath for 2 h to remove pigments. Then, the residue was leached by stirring in water at weak boiling state 3 times each for 5 h. The leaching solution from above operation was concentrated and precipitated by adding nonaqueous ethanol. The precipitate was separated from above solution by centrifugalization, dialyzed and purged with 3∶1 (V∶V) chloroform-isopentanol to remove hetero-proteins. After frozen and dried, the higher pure laminarin was obtained.

0.1 g of laminarin was refluxed with 2.5 mol·L^{-1} sulfuric acid for 10 h under a boiled-water bath and the protection of nitrogen. The filtered liquid from above operation was a mixture of monosaccharides from the hydrolyzation of this polysaccharide, and was diluted into 250 mL and further diluted 100 times for CE analysis.

Results and discussion

Selection of working electrode and detection potential

The usually used carbon electrodes in CZE-AD system are not suitable to determine carbohydrates because carbohydrates have bigger overpotentials at them. However, carbohydrates can express stronger current responses at copper electrodes under a basic environment. So the copper electrode was selected as working electrode in this experiment.

The hydrodynamic voltammograms (HDVs) of fucose (1.0×10^{-5} mol·L^{-1}), galactose (1.0×10^{-5} mol·L^{-1}), glucose (1.0×10^{-5} mol·L^{-1}), mannose (2.5×10^{-5} mol·L^{-1}) and xylose (2.5×10^{-5} mol·L^{-1}) in 0.060 mol·L^{-1} NaOH solution are shown in Fig. 1, which were obtained by monitoring their current responses after CE separations at different applied potentials. It was found that the current responses of both the monosaccharides and the blank solution increased with the increase of the applied potential. In order to get higher sensitivity and the best signal-to-noise ratio, 0.60 V was selected as the detection potential in this experiment.

Separation conditions

Under strong basic environments, carbohydrates are negatively charged by ionizing their hydrogen ions in hydroxyl groups and different carbohydrates have different charge-mass ratios, so they can be separated by CZE. In this experiment, sodium hydroxide was chosen as buffer

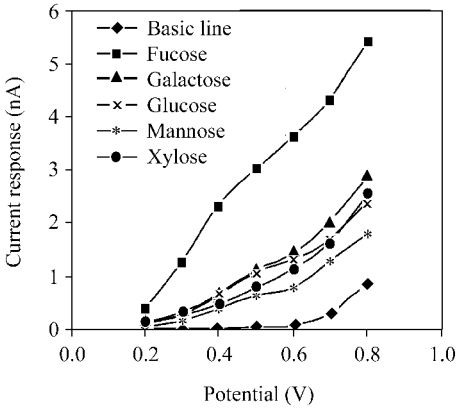

Fig. 1 Hydrodynamic voltammograms of five standard monosaccharides in CZE under different detection voltages from +0.2 to +0.8 V. Other conditions as the optimum.

solution. The migration time of fucose, galactose, glucose, mannose and xylose at different concentrations of NaOH buffers is shown in Fig. 2, where other conditions were the same as the optimum conditions. The results showed that the separation efficiency of the four monosaccharides was sensitive to NaOH concentration, because they had similar dissociation constants and molecular weights. When the concentration of NaOH was less than 0.030 mol·L^{-1}, the current peaks of galactose, glucose and arabinose were partly overlapped while other current peaks were perfectly separated. When the concentration of NaOH was more than 0.050 mol·L^{-1}, satisfactory resolution was obtained, but the migration time was prolonged and the current responses decreased. To get the best separation effect and current responses, 0.060 mol·L^{-1} NaOH was chosen for achieving the best separation.

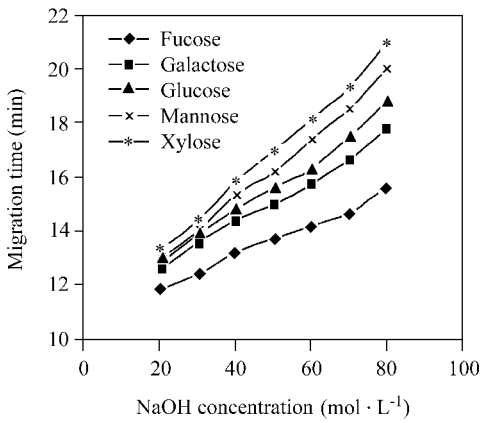

Fig. 2 Effects of running buffer concentration on migration time. NaOH concentration was changed from 0.020 to 0.080 mol·L^{-1} and other conditions were the optimum.

Fig. 3 is the migration time of the five monosaccharides under the separation voltage range of 6—20 kV and other conditions as the optimum. When the separation voltage was less than 9 kV, the five monosaccharides were separated perfectly, but the peaks were somewhat wide and the migration time was too long. The migration time of the analytes was significant shortened and their corresponding current peaks were sharpened when the separation voltage was increased. However, if the separation voltage was more than 15 kV, the current peaks of glucose, arabinose and rhamnose were somewhat widen owing to the more Joule heat produced by the higher current inside of the capillary. For the comprehensive consideration of the migration time and separation efficiency, 12 kV was selected as separation voltage and all the five monosaccharides could be perfectly separated within 20 min under this condition.

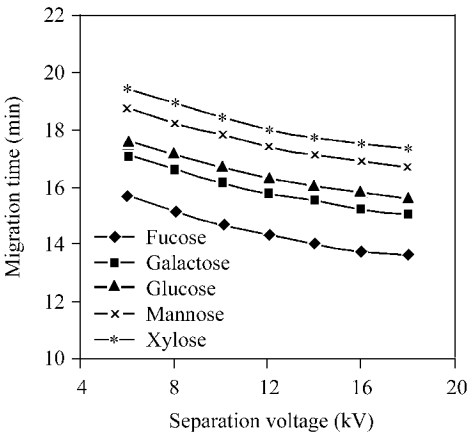

Fig. 3 Effects of separation voltage on migration time. Separation voltage was changed from 6 to 18 kV and other conditions as the optimum.

The electrokinetic sampling time changed from 4 to 16 s was tested with the other conditions as the optimum. It was found that when the sampling time was changed from 5 to 12 s, the peak currents were increased correspondingly. However, the current peaks of the analytes were obviously broadened if the sampling time was more than 12 s. 7 s was selected as sampling time in this experiment and satisfactory results were obtained under this condition.

The electropherograms of standard monosaccharides under the above optimum conditions of CZE-AD is shown as Fig. 4.

Linearity, reproducibility and detection limits

A series of standard solutions of the five monosaccharides with a concentration range from 1.0×10^{-6} to 5.0×10^{-4} mol·L^{-1} were analyzed under the optimum conditions and the results are shown in Table 1. The linear ranges of fucose, galactose and glucose were from 1.0×10^{-6} to 2.0×10^{-4} mol·L^{-1}, those of mannose and xylose were from 5.0×10^{-6} to 2.0×10^{-4} mol·L^{-1}, and the detection limits of most of five monosaccharides were at 10^{-7} mol·L^{-1} level ($S/N=3$), which showed that this method was very sensitive. Table 2 is the relatively standard deviations (RSD) of both the migration time and peak currents of the analytes with a concentration when the analysis was repeated for 6 times under the same conditions. All the RSDs

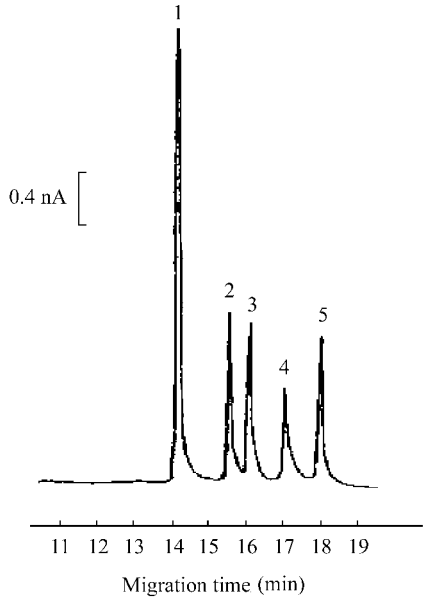

Fig. 4 Electropherograms of standard monosaccharides solution under the optimum conditions of CZE-AD. (1) Fucose (1.0×10^{-5} mol·L^{-1}), (2) galactose (1.0×10^{-5} mol·L^{-1}), (3) glucose (1.0×10^{-5} mol·L^{-1}), (4) mannose (2.5×10^{-5} mol·L^{-1}), (5) xylose (2.5×10^{-5} mol·L^{-1}).

were less than 4%, which demonstrated that this method was of good reproducibility.

Sample analysis

This method was used to analyze the hydrolyzates of laminarin under the selected optimum conditions and the electropherograms were obtained as Fig. 5. After 6 times of repetition, the molar ratio of fucose, galactose, glucose, mannose and xylose, which are the constituents of laminarin, was calculated as 10.5∶2.8∶1.0∶7.3∶3.4. The purity of the laminarin leached according to the procedure in section 2.4 was calculated as 95.7%. All above results are shown in Table 3.

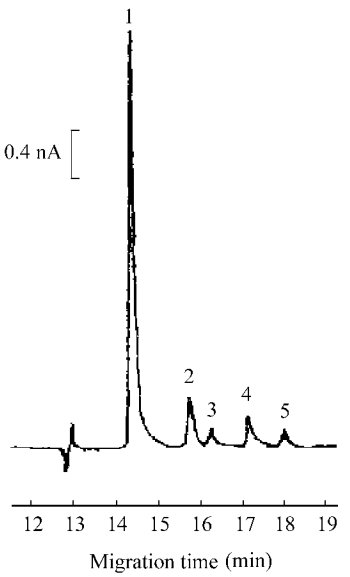

Fig. 5 Electropherograms of hydrolysis products from laminarin under the optimum conditions of CZE-AD. (1) Fucose, (2) glucose, (3) arabinose, (4) rhamnose, (5) xylose.

Table 1 Regression equation and detection limit[a]

Analyte	Regression equation I (nA); c (mol·L^{-1})	R^2	Linear range (mol·L^{-1})	Detection limit (mol·L^{-1})
Fucose	$I = 3.56\times10^5 c + 0.09$	0.9991	1×10^{-6}—2×10^{-4}	8.2×10^{-8}
Galactose	$I = 1.39\times10^5 c + 0.05$	0.9993	1×10^{-6}—2×10^{-4}	2.2×10^{-7}
Glucose	$I = 1.31\times10^5 c + 0.04$	0.9977	1×10^{-6}—2×10^{-4}	2.3×10^{-7}
Mannose	$I = 3.12\times10^4 c + 0.03$	0.9969	5×10^{-6}—2×10^{-4}	9.6×10^{-7}
Xylose	$I = 4.52\times10^4 c + 0.02$	0.9972	5×10^{-5}—2×10^{-4}	6.6×10^{-7}

[a] Detection limit was estimated according to three times of signal-noise ratio.

Table 2 Precision of the present method ($n = 6$)[a]

Analyte	Migration time		Peak height	
	Average (min)	RSD (%)	Average (nA)	RSD (%)
Fucose	14.15	0.88	3.64	1.78
Galactose	15.68	0.76	1.44	1.59
Glucose	16.30	0.69	1.32	2.03
Mannose	17.35	1.04	0.80	3.25
Xylose	18.12	1.36	1.16	3.75

[a] Concentrations of fucose, galactose and glucose were 1.0×10^{-5} mol·L^{-1}, and those of mannose and xylose were 2.5×10^{-5} mol·L^{-1}.

Table 3 Contents and molar ratio of laminarin hydrolyzates ($n = 4$)

Component	Measured concentration ($mol \cdot L^{-1}$)	Molar ratio
Fucose	9.63×10^{-6}	10.5
Galactose	2.56×10^{-6}	2.8
Glucose	9.16×10^{-7}	1.0
Mannose	6.70×10^{-6}	7.3
Xylose	3.15×10^{-6}	3.4

Recovery experiments were performed 6 times by adding the five standard monosaccharides into the laminarin sample before hydrolysis and making their analytical concentrations as 5.0×10^{-5} mol $\cdot L^{-1}$. The results are listed in Table 4, showing that the recoveries of all the four monosaccharides were ranged from 94% to 102%, which meant this method was precise and practical for the analysis of laminarin.

Conclusions

The experimental results showed that determining the compositions of laminarin by CZE-AD was of quickness, high sensitivity and high reproducibility when compared to the usual UV-vis method. This method also showed the advantages of low-volume sampling, simple instrument and relatively simple operation when compared to the pre- or post-column derivations in LIF detection. It could be used to study the compositions of other plant polysaccharides and is very potential to be used in the monitoring of polysaccharides medicines during human metabolism.

Table 4 Recoveries of five monosaccharides in sample analysis ($n = 4$)

Component	Added amount ($mol \cdot L^{-1}$)	Found amount ($mol \cdot L^{-1}$)	Recovery (%)	RSD (%)
Fucose	5.0×10^{-5}	5.1×10^{-5}	102.0	1.9
Galactose	5.0×10^{-5}	5.1×10^{-5}	102.0	2.4
Glucose	5.0×10^{-5}	5.0×10^{-5}	100.0	2.1
Mannose	5.0×10^{-5}	4.8×10^{-5}	96.0	3.6
Xylose	5.0×10^{-5}	4.7×10^{-5}	94.0	3.8

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