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SHORT COMMUNICATION

Chiral separation of phenyllactic acid by helical structure from spring dextrin

Jin Xu · Xueming Xu · Qiang Wang · Xuerong Fan

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Abstract We performed the enzymatic synthesis of spring dextrin (SD) with a helical conformation and investigated its chiral-recognition properties in relation to the stereoselective phenyllactic acid (PLA), using reversed-phase high-performance liquid chromatography. The effects of column temperature, buffer pH and methanol content on enantioselective separation were investigated. Baseline chromatographic separation was achieved on an Inertsil ODS-SP column using 1 % SD as the chiral mobile-phase additive. Helical structure was necessary for chiral separation of PLA, according to visible spectroscopy. Molecular dynamic simulations to predict the interactions between SD and PLA showed that van der Waals attractions played an important role in enantiomer separation.

Keywords Spring dextrin · Helical structure · Chiral discrimination

Introduction

The chromatographic resolution of enantiomers is one of the most difficult challenges in analytical chemistry [1]. As living organisms often show different biological activities towards different enantiomers [2], the detection and assignment of chirality at the molecular level continues to be an important issue. Phenyllactic acid (PLA) is frequently used as the component of pharmaceuticals and natural antibiotic agents [3]. L-PLA is used for the synthesis of antiviral and antitumour compounds (pederin, onnamides and theopederins) [4]. However, D-PLA is an integral part of a cyclic depsipeptide (a novel Gq/11 protein inhibitor) [5]. Therefore, it's important for chiral separation of the PLA enantiomers to synthesize the pharmaceuticals.

Dextrins, such as cyclodextrin (CD) and maltodextrin (MD) (Fig. 1a), are optically-active polymers that can be readily exploited for the chiral resolution of racemic compounds. Their torch-like structure with a hydrophobic internal surface and hydrophilic external surface gives CDs the ability to form complexes with a variety of chiral compounds [6, 7]. CDs are therefore widely used for the chiral separation of enantiomers in the pharmaceutical industry, as mobile-phase additives or as chiral stationary phases [8–11]. MDs, which are mixtures of malto-oligo and polysaccharides, can also be used as chiral selectors [12, 13]. The mechanism of chiral recognition has been shown to be attributable to the fact that the helical structure of the MD mimics the cavity responsible for chiral recognition in CDs [14, 15]. Amylose forms a helical structure with small molecules, such as iodine, which can be included in the hydrophobic interior of MDs (iodine color reaction), as in the cavity of CDs. The helix structure of amylose is therefore important in resolving racemic mixtures [16–18].

Spring dextrin (SD) [19] is a spiral polymer composed of repeating (1-4)-α-D-glucose units that can be produced by the enzymatic hydrolysis of amylose (by α-amylase) [20] or by enzymatic synthesis (with amylosucrase) [21]. Recent studies demonstrated effective single-walled carbon

J. Xu · Q. Wang · X. Fan (✉)
Key Laboratory of Eco-textiles of Ministry of Education, College of Textiles and Clothing, Jiangnan University, 1800 Lihu AVE, Wuxi 214122, People's Republic of China
e-mail: wxfxr@163.com

X. Xu
State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, People's Republic of China

X. Xu
School of Food Science and Technology, Jiangnan University, 1800 Lihu AVE, Wuxi 214122, People's Republic of China

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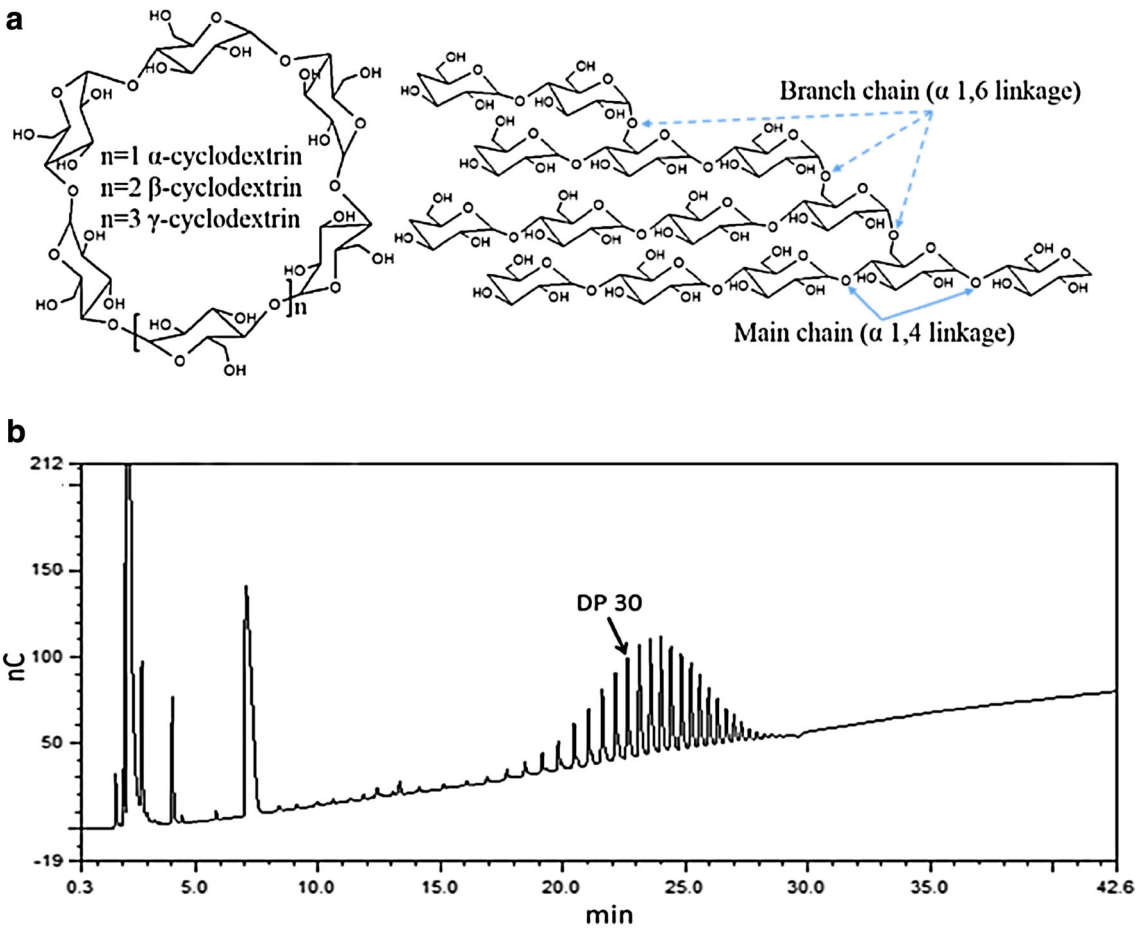

Fig. 1 **a** Chemical structure of the cyclodextrin (*left*) and maltodextrin (*right*); **b** HPAEC-PAD profiles of the SD (DP values are indicated above the *peaks*.)

nanotube (SWNT) dispersion with SD, and reported that SD wrapped around SWNTs in a helical manner [22]. Further, SD has been used to improve the stability of polyunsaturated fatty acids by encapsulating them within with helical SD [23]. Importantly, analogs of SD (MD or amylose) have been used for the chiral separation of dozens of enantiomers [24–26]. Therefore, the current study aimed to clarify the molecular mechanisms responsible for SD, with a view to the future development of chiral mobile-phase additives capable of resolving enantiomeric compounds such as the PLA enantiomers.

Experimental

Chemicals and reagents

D-(+)-3-Phenyllactic acid (D-PLA), L-PLA, DL-PLA, trifluoroacetic acid (TFA), triethylamine (TEA), methanol, phosphorylase and glucose 1-phosphate were purchased from Sigma-Aldrich (Shanghai, China). Maltotetraose was purchased from Hui Cheng Biological Technology Co., Ltd. (Shanghai, China). All other chemicals were analytical reagents purchased from commercial sources.

Enzymatic synthesis of SD by phosphorylase and chain-length distribution

SD was synthesized from glucose 1-phosphate (G-1-P) using glucan phosphorylase. Briefly, an aqueous solution of G-1-P (10 mL, 4 mg/mL) was adjusted to pH 6.5 with 2 mol/L acetic acid and mixed with 0.1 M citrate buffer pH 6.5 (4 mL). Twenty milliliters of the phosphorylase solution was mixed with an aqueous solution of maltotetraose and made up to 100 mL with water, and incubated at 40 °C for 24 h. After the reaction, the mixtures were centrifuged at 5000*g* for 10 min, and the precipitate was washed five times with distilled water and then freeze-dried. The chain-length distribution of the SD was characterized by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [20].

High-performance liquid chromatography analysis and optimization of mobile-phase parameters

Stock solutions of L-PLA, D-PLA and DL-PLA were prepared (1 mg/mL) by dissolving in distilled water, and then stored at 4 °C. The stock solutions were diluted with distilled water to the required concentrations before use.

Separation and detection of L-PLA and D-PLA were performed on a Shimadzu liquid chromatography system consisting of an LC-20AT pump, an SIL-10A injector, Inertsil ODS-SP column (150 × 4.6 mm, particle size 5 μm) and an SPD-10A UV–Vis detector (254 nm) (Shimadzu Corp., Tokyo, Japan). Data were analyzed using LC solution software (Shimadzu Corp.). The injection volume was 20 μL. The mobile phase was a mixture of 0.05 % TFA buffer (adjusted with TEA, pH 2.5) containing 1 % SD and methanol (90:10, v/v) at 30 °C with a flow rate of 1.0 mL/min (unless otherwise specified). The effects of column temperature, pH and methanol content on the enantiomeric separation ability [resolution (Rs)] were studied. The Rs was based on the average of at least three independent determinations of each solute.

Molecular dynamics simulations

The interactions between the two enantiomers of PLA and SD were simulated using the molecular dynamics module in HYPERCHEM 8.0 software (Hypercube Inc., Waterloo, Canada). Simulations were performed in a periodic box (30 × 30 × 50 Å), according to the size and shape of PLA and SD. All established models were energetically pre-optimized and obtained using the AMBER force field. The pre-optimized configurations were heated for 1 ps to 303.15 K (30 °C) and then simulated at 303.15 K for 2 ps at a step size of 0.001 ps containing water molecules. Information for the equilibrated conformations was then computed and parameters such as non-bonded interactions, including van der Waals attractions (VdWs), hydrogen bonds (HBs), and electrostatic forces (EFs) were subsequently analyzed.

Results and discussion

SD chain-length distribution

Synthetic SD was analyzed by HPAEC-PAD (Fig. 1b). The individual SD homologs were baseline resolved on a CarboPAC PA200 column (Thermo Fisher Scientific, Inc., United States). Individual and sharp peaks were well-resolved, strongly suggesting that the SDs were linear [27].

Method development

We investigated the effects of column temperature, buffer pH and methanol content on separation of the PLA enantiomers. The effect of column temperature on enantioselectivity was investigated using 0.05 % TFA buffer (adjusted with TEA, pH 2.5) with 1 % SD containing 10 % methanol as the mobile phase. Rs decreased with increasing temperature (20–60 °C), and baseline separation was observed at 30 °C (Fig. 2a). The temperature effects on enantioselectivities usually depend on the thermodynamic interactions between the analytes and chiral selectors, an enthalpy-driven process or an entropy-driven process [28]. The enantioselectivity for this assay in the relative lower temperature ranges can probably be attributed to the slower rotation of the guest and host molecules, hence a steric fit is possible [29]. We therefore fixed the temperature at 30 °C for further optimization. Since PLA has one carboxylic acid functionality (pKa 3.46) [30], the molecule dissociates in aqueous solution by releasing a proton. The effect of buffer pH on the Rs of the PLA enantiomers was investigated using 0.05 % TFA buffer (adjusted with TEA) containing 1 % SD and 10 % methanol as the mobile phase. The Rs decreased with increasing pH from 2.0 to 4.0 (Fig. 2b), indicating better separation at lower pH values. This phenomenon can be possibly attributed to the decrease of the formation of inclusions caused by the increasing dissociation of the solute [31]. A pH value of 2.5 was used for further studies, given that higher pH reduces life-cycle costs. The effect of methanol concentration on the Rs of the enantiomers was also investigated using 0.05 % TFA buffer (adjusted with TEA, pH 2.5) with 1 % SD containing 10–30 % methanol as the mobile phase. The Rs of PLA decreased with increasing methanol concentrations in the mobile phase (Fig. 2c). This finding was consistent with previous research [31] showing that the Rs of analytes decreased with increasing methanol concentrations, which could be probably attributed to the increase of SD-analytes inclusion complexes caused by the organic modifier [32]. Mobile-phase buffer containing 10 % methanol was therefore used in subsequent experiments. Based on the above results, baseline separation of PLA (Fig. 3a) was performed on an Inertsil ODS-SP column (150 × 4.6 mm, particle size 5 μm) under the specified conditions (0.05 % TFA buffer (1 % SD, 10 % methanol, pH 2.5, 30 °C).

Calibration, precision and accuracy

Calibration curves for D-PLA and L-PLA were linear over concentrations ranging from 0.5 to 15 μg/mL, according to linear regression analysis of the peak area (y) of each enantiomer versus the concentration (x). The regression equations of the calibration curves were

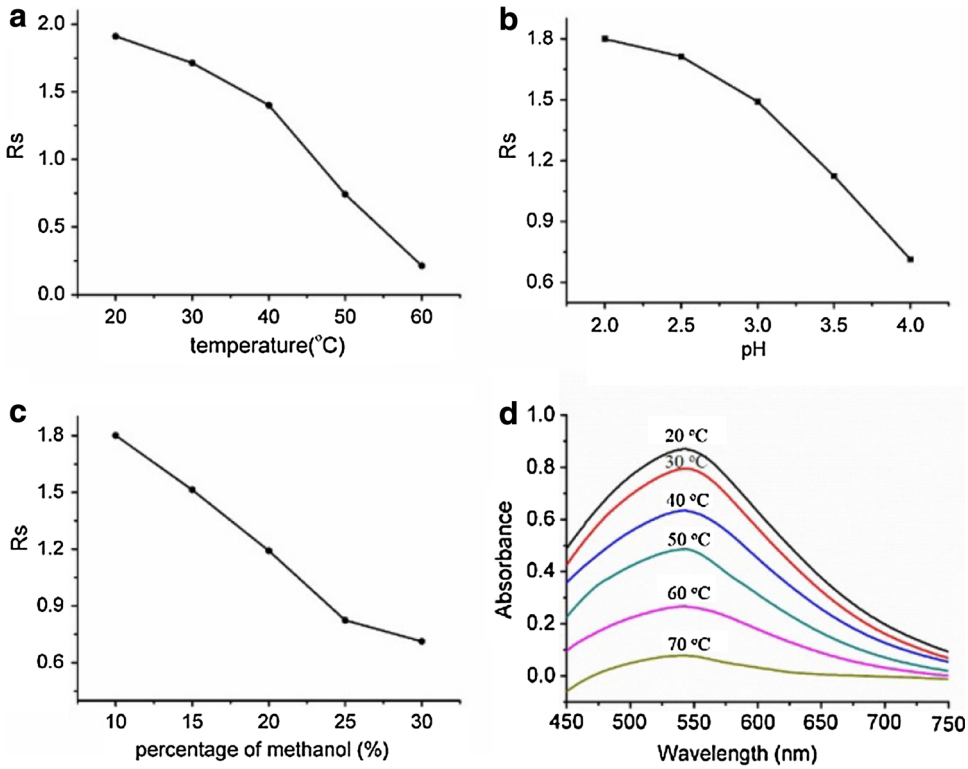

Fig. 2 The influences of column temperature (**a**), pH (**b**) and methanol content (**c**) on the HPLC separation. Vis spectra of the aqueous solutions of SD-iodine complexes at different temperatures (**d**)

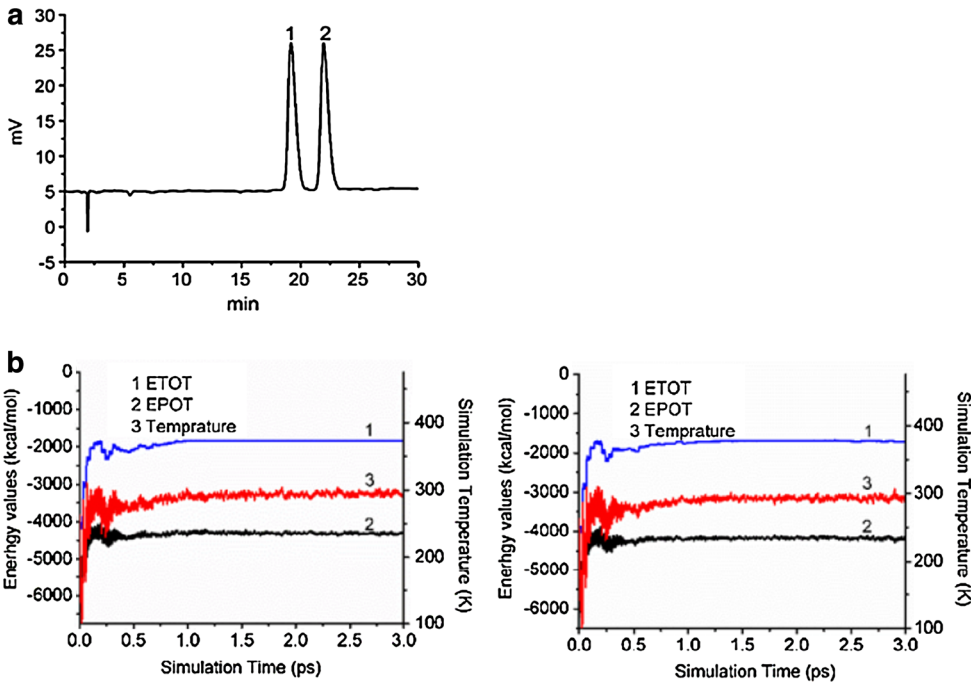

Fig. 3 **a** Chromatographic separation of PLA optical isomers; **b** Curves of total energy, potential energy, and simulation temperature during the simulation performed at 293.15 K in 3 ps (*left* L-PLA; *right* D-PLA)

$y = 29{,}375x + 512{,}473$, with a regression coefficient (r^2) of 0.9984 for D-PLA, and $y = 31{,}529x + 513{,}016$ ($r^2 = 0.9951$) for L-PLA. The limits of detection (LOD) were 0.4 and 0.8 μg/mL in receptor medium for D-PLA and L-PLA, respectively, measured based on a signal-to-noise ratio (S/N) ≥3. The limits of quantitation (LOQ) (S/N ≥ 10) were 0.9 and 1.3 μg/mL for D-PLA and L-PLA, respectively, with a relative standard deviation (RSD) <9.7 %. The intra- and inter-day precisions and accuracy of the method are shown in Table 1. The RSD values for

intra- and inter-day assays were <5 %, indicating the reproducibilities of the chromatographic conditions and the method (Table 1). The accuracy of the method was demonstrated by a percentage recovery of 93.7–102.6 %, determined by spiking D-PLA to 1.0 mg/mL L-PLA at 0.07, 0.2, and 0.5 % levels.

Conformational transition of SD with temperature

The transition of SD conformation with temperature was investigated using the visible spectrum, with iodine as a probe for helical structure. The visible spectra profiles of the SD-iodine complexes at 20, 30, 40, 50, 60 and 70 °C, respectively, are shown in Fig. 2d. The absorption at 548 nm decreased with increasing temperature, indicating that the helical structures were gradually destroyed at higher temperatures.

Evaluation of inclusion complex formation

The organic molecules could undergo non-covalent interactions with dextrin leading to the inclusion complex formation. The chiral HPLC method with chiral mobile phase additive is an efficient way to determine the binding constant of a solute to a dextrin molecule [33]. For a 1:1 complex the following equation could be used for determination of formation constant and the stoichiometry for the inclusion complex.

Table 1 Precision and recovery data of this assay

Spiking level of the D-PLA (%)	Parameter	RSD (%)	
		D-PLA	L-PLA
Precision data			
Repeatability (n = 5)			
0.3	Retention time	0.12	0.15
	Peak area	3.6	2.9
0.07(LOD)	Retention time	0.16	0.19
	Peak area	4.7	3.2
Inter-day precision (n = 3)			
0.3	Retention time	0.14	0.21
	Peak area	4.4	3.5
0.07 (LOD)	Retention time	0.23	0.19
	Peak area	4.1	3.4

Added (μg/mL)	Recovered (μg/mL)	% Recovery	% RSD
Recovery data			
0.696	0.652	93.7	4.6
2.114	2.038	96.4	2.9
5.070	5.202	102.6	4.1

Table 2 Changes of single point energies of SD and PLA complex

SD complex	Single point energies (kcal/mol)[a]		
	ΔE_{VdW}[b]	ΔE_{HB}[b]	ΔE_{Ef}[b]
L-PLA	224.3 ± 0.2	−5.8 ± 0.1	1368.9 ± 0.2
D-PLA	154.4 ± 0.1	−3.3 ± 0.2	1378.0 ± 0.2

[a] Values are means ± standard deviations from three experiments of simulation

[b] Van der Waals attractions, Hydrogen bonds, Electrostatic forces

$$\frac{1}{k'} = \frac{1}{\phi k[A]} + \frac{k1[SD]}{\phi k[A]}$$

where k′ is chromatographic definition of capacity factor, ϕ is the phase ratio, A is a stationary phase adsorption site, SD stands for spring dextrin, and k, $k_1$and k_2 are the respective equilibrium constants. Plots of 1/k versus SD concentration for the enantiomer gave the straight lines (regression equation $1/k_{1'} = 5.3218[SD] + 0.1012$; $1/k_{2'} = 3.7956[SD] + 0.1061$), and the correlation coefficients of plots were greater than 0.998 indicating that the stoichiometry was 1:1 for the inclusion complex. The binding constants were 54.428 and 37.674 L/g, respectively.

Molecular dynamic simulations

Stable conformations were obtained at 303.15 K (30 °C) after heating for 1 ps to 303.15 K followed by simulation at 303.15 K for 2 ps, according to the stable levels of total energy, potential energy, and simulation temperature finally achieved. The curves of these parameters during simulation at 303.15 K for 3 ps are shown in Fig. 3b. The optimized conformation was in equilibrium after about 1.1 ps simulation at 303.15 K. Table 2 summarizes the single-point energies of SD with the L- and D- enantiomers (the symbol '-' in Table 2 represents the force orientation). Electrostatic energy showed a maximum value, indicating that electrostatic energy was the main driving force responsible for complex formation. However, the maximum change between the L-PLA complex and D-PLA complex was ΔE_{VdW}, suggesting that VdW interactions played an important role during enantiomer separation.

Conclusions

SD as a chiral mobile-phase additive provides a flexible alternative for the separation of enantiomers by allowing separations to be performed on conventional columns. We developed a simple, specific, precise and rugged reversed-

phase high-performance liquid chromatography method involving SD for separating DL-PLA. This represents the first use of SD as a chiral mobile-phase additive for the separation of chiral compounds in high-performance liquid chromatography. The results of this study indicating that helical structures are necessary for recognizing enantiomers.

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