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Journal of Chromatography B, 766 (2002) 351–356

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Short communication

Analysis of α-amylase-derived pyridylamino-dextran sulfate oligomers by the combination of size-exclusion and reversed-phase high-performance liquid chromatography

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Received 19 July 2001; received in revised form 15 October 2001; accepted 16 October 2001

Abstract

In a previous study, we reported a novel method for the separation and quantification of a strong negatively charged material, dextran sulfate sodium (DSS), using fluorometric labeling with 2-aminopyridine and size-exclusion high-performance liquid chromatography. In the present study, we developed a method for the separation of pyridylamino-DSS (PA-DSS) using reversed-phase high-performance liquid chromatography (RPLC). In vitro enzymatic degradation of the PA-DSS was carried out using α-amylase. In RPLC, depolymerized PA-DSS was eluted on the basis of molecular mass (in the order pentamer, trimer, dimer, and monomer of PA-DSS) and separations were more sharply than in size-exclusion chromatography. The combination of RPLC and size-exclusion chromatography also separated depolymerized PA-DSS as effectively as RPLC alone.

Keywords: α-Amylase; Pyridylamino-dextran sulfate oligomers

1. Introduction

The pathogenesis of inflammatory bowel disease (IBD) remains unknown. Its etiology seems to be complex and multifactorial [1]. Animal models of ulcerative colitis (UC) can be induced by the oral administration of sulfated polysaccharides such as dextran sulfate sodium (DSS). DSS is widely used in the investigation of the pathogenesis of IBD and study of the efficacy of therapeutic agents for IBD. DSS is a heparin-like polysaccharide containing approximately 17% sulfur with up to three sulfate groups per glucose molecule (Fig. 1). In addition, many interactions between DSS and the suppression of human immuno-deficiency virus have been reported [2]. However, the precise mechanisms responsible for DSS-induced colitis and the anti-viral effects of DSS as well as the mechanisms by which DSS is metabolized, remain poorly understood.

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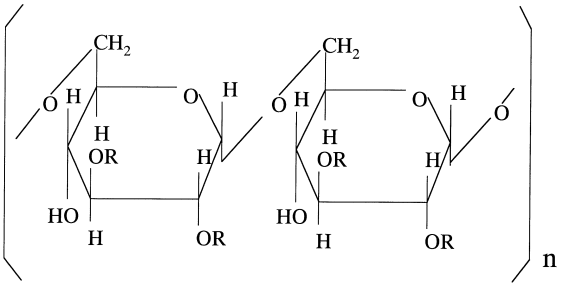
1570-0232/02/$ – see front matter
PII: S0378-4347(01)00511-4



R: SO_3Na or H

Fig. 1. Structure of dextran sulfate sodium (DSS).

In our recent study, we showed for the first time the metabolism of DSS by α-amylase using 2-aminopyridine (PA) fluorescence labeling, and its detection by size-exclusion high-performance liquid chromatography [3]. That study also reported the metabolism of PA-DSS in the gut lumen [3]. However, sufficient separation of PA-DSS oligomers by size-exclusion high-performance liquid chromatography could not be achieved.

Accordingly, the purpose of the present study was to develop methods for the separation of PA-DSS oligomers. Previous studies have achieved good separation of pyridylaminated derivatives of oligosaccharides by high-performance liquid chromatography (HPLC) systems such as (1) a gel permeation using an ion-exchange resin [4–6], (2) a reversed-phase partition [7,8], (3) a normal-phase partition [9] and (4) a cation-exchange column [10]. In the present study, we used reversed-phase high-performance liquid chromatography (RPLC) and a combination of size-exclusion and reversed-phase high-performance liquid chromatography.

2. Experimental

2.1. Chemicals

DSS with a molecular mass (M_r) of 5000, 2-aminopyridine, phthalic acid, and α-amylase (derived from *Bacillus subtilis*, 20 U/mg) were obtained from Wako (Osaka, Japan). D-Glucose 3-sulfate was obtained from Sigma (St. Louis, MO, USA). Sodium cyanoborohydride was obtained from Nacalai Tesque (Kyoto, Japan).

2.2. Labeling of DSS using 2-aminopyridine

Labeling of DSS and D-glucose 3-sulfate using 2-aminopyridine was carried out according to methods described in our previous report [3].

2.3. Purification of PA-DSS using C_{18} solid-phase extraction cartridges

In the course of removing PA from the reaction mixture, washing with ethanol was occasionally insufficient because of coarse particle formation of PA-DSS in ethanol. We paid attention to the structure of PA, particularly NH_3- in the lateral chain and nitrogen in the pyridine structure. Amino functions are protonated in acidic solution. In addition, protonated PA is released from the C_{18} solid phase when the solution is acidic. Therefore, we tried to further purify PA-DSS from PA in the reaction mixture using C_{18} solid-phase extraction cartridge. Solid-phase extraction cartridges (Sep-Pak Plus C_{18} cartridge) were obtained from Waters (MA, USA). We used these cartridges in five series.

2.4. Enzymatic metabolites of PA-DSS

Enzymatically depolymerized PA-DSS was produced according to previously described methods [3]. Briefly, 2000 U of α-amylase was added to 10 ml of 1% DSS solution (2 m*M*). After gentle shaking at 37°C for 72 h, the mixture was analyzed.

2.5. HPLC conditions

We used a Cosmosil 5 Diol-120 packed column (300×7.5 mm; Nacalai Tesque) for size-exclusion chromatography and a Waters 5 C_{18}-AR column (150×4.6 mm; Nacalai Tesque) for RPLC.

In this study, a HPLC LC6A apparatus (Shimadzu, Kyoto, Japan) was used. The mobile phases were delivered isocratically at a flow-rate of 1.0 ml/min. The column temperature was maintained at 60°C.

For the detection of native PA-DSS, depolymerized PA-DSS and pyridylamino-D-glucose 3-sulfate (PA-D-glucose 3-sulfate), a fluorescence detector RF-

535 (Shimadzu) was used at excitation and emission wavelengths of 320 nm and 400 nm, respectively, according to our previous report [3].

2.6. Molecular masses of depolymerized PA-DSS

In order to analyze the molecular masses of depolymerized PA-DSS, we collected the eluted solution representing each peak on the RPLC system. The individual eluted solution was concentrated 10-fold by dehydration, and filtered through a membrane filter. Molecular masses of individual peak were analyzed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Voyager RP, PerSeptive Biosystems, Framingham, MA, USA). 2,5-Dihydroxybenzoic acid (2,5-DHB) and sinapinic acid were used as a matrix in this system. In addition, we analyzed the molecular masses using a gel permeation chromatography (GPC) column. Briefly, the eluted solution representing each peak on the RPLC system was collected and concentrated 10-fold by dehydration, and filtered through a membrane filter. Then, 5 μl of each sample was injected onto the GPC column. We calculated the molecular masses of each peak based on the relationship between the elution volumes and the molecular masses of native PA-DSS (M_r 5000) and PA-D-glucose-3-sulfate (M_r 360.2) as standards.

3. Results and discussion

3.1. Purification of PA-DSS using C_{18} solid-phase extraction cartridges

As expected, PA was retained on the C_{18} solid phase when the solution was neutral or alkaline. In addition, PA was rapidly released from the C_{18} solid phase when the solution was acidic. The procedure was as follows, first, 5 ml of NaOH solution (pH 9) was passed through the cartridges, then 1 ml of PA-DSS (1%) was passed sequentially through the cartridges. Then, 20 ml of NaOH solution (pH 9) was passed through the cartridges. This 20 ml of extracted solution contained PA-DSS without PA. Finally, 20 ml of HCl solution (pH 3) was passed through the cartridges in order to extract residual PA (data not shown).

3.2. Enzymatic metabolites of PA-DSS and effects of the buffer concentration and pH on eluent status in the RPLC analysis

Fig. 2A shows the chromatogram for PA-DSS depolymerized by α-amylase on the GPC column. Peak a represents native PA-DSS (M_r 5000). Two peaks were eluted after native PA-DSS elution, which corresponded to depolymerized PA-DSS [3]. The mobile phase was 0.2 *M* phosphate buffer of pH 3.0.

Fig. 2B shows the chromatogram for PA-DSS depolymerized by α-amylase on the RPLC column. Peak a represents native PA-DSS. Four peaks were eluted after native PA-DSS elution, which corresponded to depolymerized PA-DSS. A 0.2 *M* phosphate buffer of pH 3.0 was also used. Better separation was obtained using the RPLC column than the GPC column.

Fig. 2C shows the chromatogram for PA-DSS depolymerized by α-amylase on the RPLC column using 0.005 *M* phosphate buffer of pH 3.0 as a mobile phase. The peak separation was considerably reduced. In addition, the retention times of each peak were shorter than with 0.2 *M* phosphate buffer of pH 3.0.

Fig. 2D shows the chromatogram for PA-DSS depolymerized by α-amylase on the RPLC column using 0.2 *M* phosphate buffer of pH 7.0 as the mobile phase. Under these conditions, native PA-DSS and depolymerized PA-DSS were eluted en block, indicating very weak interactions between the stationary phase and strongly charged PA-DSS and its derivatives. Therefore, we subsequently used 0.2 *M* phosphate buffer of pH 3.0 as a mobile phase in the RPLC analysis.

3.3. Molecular masses of depolymerized PA-DSS

The relationship between the molecular masses of the PA-DSS polymers derived from the α-amylase reaction and elution volumes on the RPLC system was not unknown. Therefore, we next tried to analyze the molecular masses of the polymers represented by each peak in RPLC (peaks b–e in Fig. 2). The molecular masses of the monomer, dimer, trimer, tetramer, and pentamer of PA-DSS were

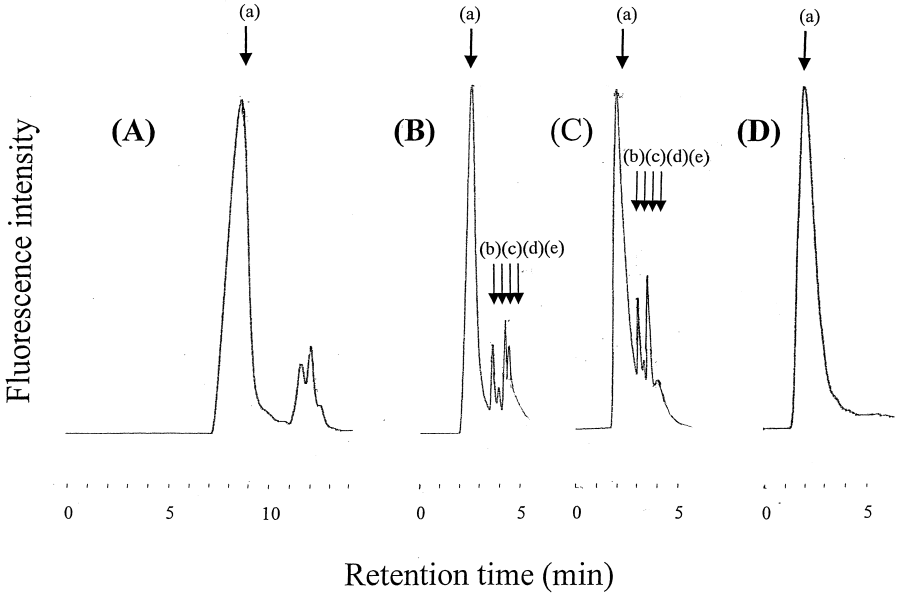


Fig. 2. Chromatograms of depolymerized pyridylamino-DSS (PA-DSS) after α-amylase reaction. (A) Chromatogram by size-exclusion high-performance liquid chromatography. Mobile phase was 0.2 *M* phosphate buffer (pH 3.0). (B) Chromatogram by reversed-phase high-performance liquid chromatography (RPLC). Mobile phase was 0.2 *M* phosphate buffer (pH 3.0). (C) Chromatogram by RPLC. Mobile phase was 0.005 *M* phosphate buffer (pH 3.0). (D) Chromatogram by RPLC. Mobile phase was 0.2 *M* phosphate buffer (pH 7.0). Peak a represents native PA-DSS (M_r 5000). HPLC conditions as described in Experimental.

calculated theoretically to be 398, 719, 1039, 1359, and 1679, respectively.

Fig. 3 reveals the results of mass spectrometric analysis of peak e using 2,5-DHB as a matrix. There is only one small fragmented peak of M_r 394.219, indicating that peak e contained primarily PA-DSS monomer. However, in peaks b–d, there were no other fragmented peaks detectable which corresponded to depolymerized PA-DSS, except for the fragmented peaks derived from 2,5-DHB in MALDI-TOF-MS. The analysis, using sinapinic acid as a matrix, also shows the same results as 2,5-DHB. The high concentration of phosphate buffer may have influenced the detection. The precise reasons for poor detection of fragmented peaks in this mass spectrometric analysis, however, are unknown.

Therefore, we next tried to analyze the molecular masses of depolymerized PA-DSS (peaks b–e by RPLC in Fig. 2) using size-exclusion chromatography. Based on the relationship between the masses of native PA-DSS and PA-D-glucose-3-sulfate, and elution volumes, the molecular masses speculated from the retention times of peaks b–e were approximately 1650, 950, 650, and 390, respectively. Therefore, peaks b–e corresponded to the pentamer, trimer, dimer, and monomer of PA-DSS, respectively.

It is clear that this RPLC technique achieves good separation of depolymerized PA-DSS derived from the α-amylase reaction and polymers are separated in the order of molecular mass. However, the retention times were short. Therefore, we tried to separate depolymerized PA-DSS derived from the α-amylase reaction using a combination of size-exclusion chromatography and RPLC. Fig. 4 shows the chromatogram for depolymerized PA-DSS derived from the α-amylase reaction using a combination of size-exclusion chromatography and RPLC. This method provided separation as effectively as the RPLC technique. This combination provides the added advantage that high-molecular-mass substances present in the biological materials are grossly separated from PA-DSS in the first separation by size-exclusion chromatography in accordance with molecular masses, then more definite separations of native PA-DSS and depolymerized PA-DSS are achieved in the

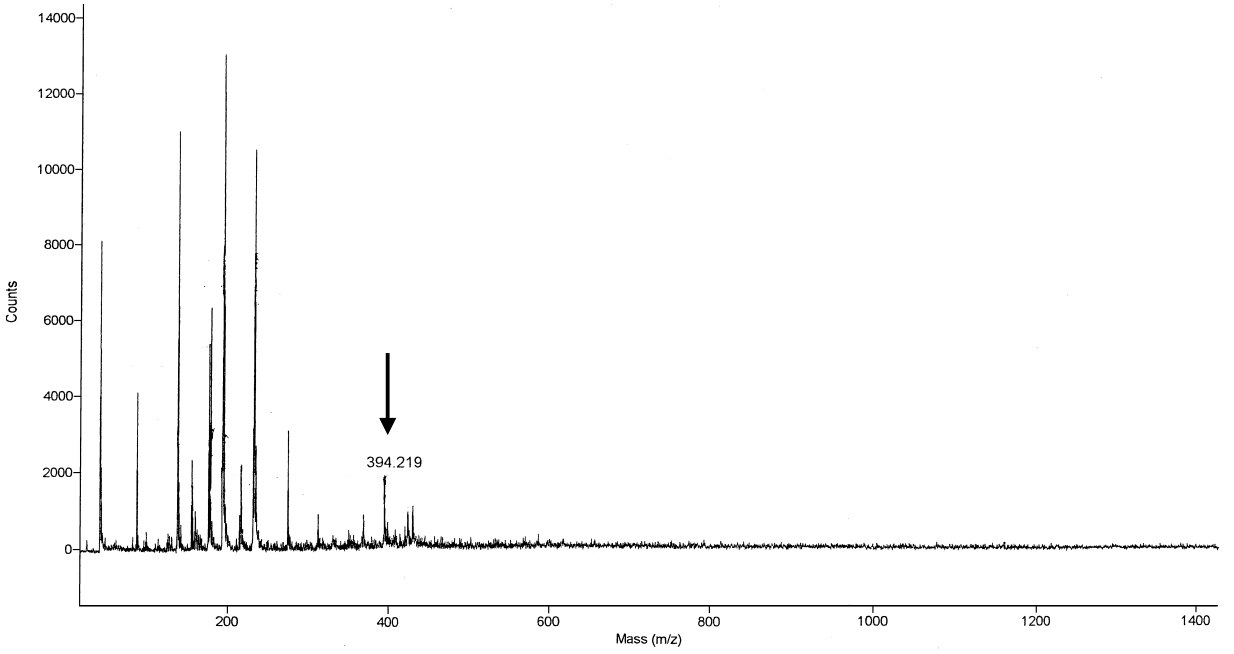


Fig. 3. Mass spectrometric analysis of peak g by reversed-phase high-performance liquid chromatography. Analytical conditions as described in Experimental. Arrow indicates the mass-fragmented peak representing M_r 394.

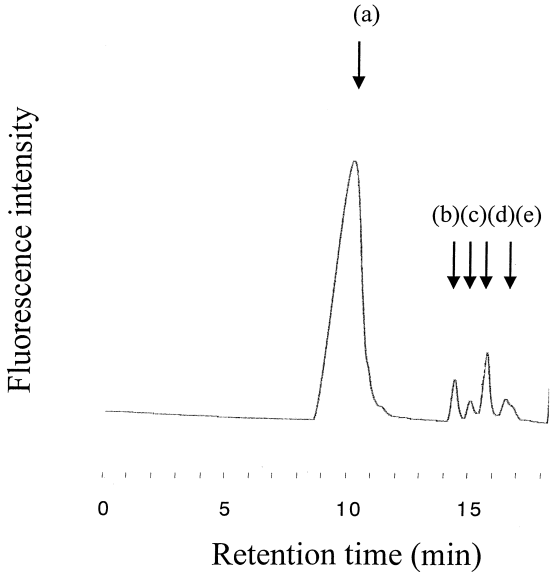


Fig. 4. Chromatogram of depolymerized pyridylamino-DSS (PA-DSS) after α-amylase reaction using a combination of size-exclusion and reversed-phase high-performance liquid chromatography. HPLC conditions as described in Experimental.

second separation by RPLC. We calculated the areas of peaks b–e and 1% PA-DSS standard solution, and decided that the concentrations of peaks b–e corresponded to 0.037% (0.074 m*M*), 0.013% (0.026 m*M*), 0.063% (0.126 m*M*), and 0.030% (0.060 m*M*), respectively. These results indicated that 12.0% of native PA-DSS was totally depolymerized by α-amylase.

In conclusion, this paper has demonstrated a method of separating and analyzing DSS, using PA-labeling and a combination of size-exclusion chromatography and RPLC. RPLC is more suitable for the separation of PA-DSS than size-exclusion chromatography. In addition, the combination of RPLC and GPC is even more advantageous for the separation of PA-DSS.

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