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Application of 2-aminopyridine fluorescence labeling in the analysis of in vivo and in vitro metabolism of dextran sulfate sodium by size-exclusion high-performance liquid chromatography

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Abstract

The present study describes a size-exclusion high-performance liquid chromatographic method for the separation and quantification of sulfated polysaccharides, such as dextran sulfate sodium (DSS). Pyridylamination of DSS was achieved without difficulty using 2-aminopyridine as a fluorometric label. In addition, 0.1–0.2 *M* phosphate buffer (pH 3.0) was found to be the mobile phase which produced the best separation. In vitro enzymatic degradation of the pyridylamino-DSS (PA-DSS_{5000}, M_r 5000) using α-amylase and the in vivo metabolism in the rat feces after oral administration of PA-DSS_{5000} were then evaluated. Two small peaks of approximately M_r 380 and 600 appeared after co-incubation with α-amylase, indicating PA-DSS_{5000} may be considerably depolymerized. In vivo, however, PA-DSS_{5000} excreted in the feces was mainly of PA-DSS_{5000} polymer. No peaks of less than M_r 5000 were not clearly detectable in the feces because of background fluorescence attributable to gut lumen contents. This method of fluorometric analysis allows fairly selective detection of sulfated polysaccharides in biological materials.

Keywords: 2-Aminopyridine; Dextran sulfate sodium

1. Introduction

The pathogenesis of inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD), remain unknown. The etiology of these disorders appear to be complex and multifactorial [1].

Animal models of UC can be induced by oral administration of sulfated polysaccharides such as dextran sulfate sodium (DSS). DSS is a heparin-like polysaccharide containing approximately 17% sulfur with up to three sulfate groups per glucose molecule (Fig. 1). It has been reported that DSS induced-colitis exhibits some of the clinical and histological features of UC. For example, several studies have reported that, similar to UC patients, DSS initially induces colonic lesions in the distal colon, which then spread to the whole colon [2,3]. In this model, it has been observed that the development of colitis is dependent on the molecular mass (M_r) and sulfation of the DSS, in addition to the dosage and duration of administration [3]. Therefore, DSS is widely used in

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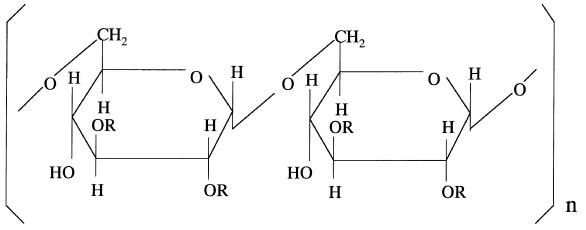

R: SO_3Na or H

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

the investigation of the pathogenesis of IBD and the efficacy of therapeutic agents for IBD.

With respect to pathogenic factors in the development of DSS-induced colitis, previous reports have postulated the importance of various factors such as local immunological disturbances [4], the activation of mucosal macrophages [5], effects related to the strong negative charge of DSS [6], obliteration of the crypt lumina [7], and changes in the intestinal microflora [8]. However, the precise mechanisms responsible for DSS-induced colitis remain unclear. In addition, to our knowledge, there have been few detailed studies of DSS metabolism in the gut lumen. For example, a previous study reported that ingested DSS is very poorly absorbed in the human gut lumen after oral administration, as measured by a competitive binding assay [9]. On the other hand, another report revealed uptake of dextran sulfate by glomerular intracellular vesicles during kidney ultrafiltration. This study was performed using tritium-labeled DSS and gel chromatography columns [10]. However, it is not at all clear whether DSS is degraded by digestive enzymes, such as α-amylase, or in what form DSS exists in the feces.

The separation of DSS from other sulfated proteoglycans in the gut lumen, such as mucin, is potentially very difficult. Therefore, detection of sulfated polysaccharides, including DSS, in biological materials has been rarely reported by high-performance liquid chromatography (HPLC) systems.

The purpose of the present study was to develop methods of detection of DSS in biological materials, and to elucidate the metabolism of DSS in the gut lumen. Tritium labeling at the reducing end of the sugar molecule shows high sensitivity, but this labeling method is too specific to be practical. Therefore, we used a 2-aminopyridine labeling system. The pyridylamination of the reducing termini of sugar chains has been useful for structural analysis and metabolic studies of *N*- or *O*-glycosidically linked sugar chains [9]. Previous studies have revealed good separation of pyridylaminated derivatives of oligosaccharides by HPLC systems such as: (1) a gel permeation using an ion-exchange resin [11–13], (2) a reversed-phase partition [14,15], (3) a normal-phase partition [16] and (4) a cation-exchange column [17]. In the present study, we used a gel filtration column. Up to the present, no studies of the pyridylamination and metabolism of DSS have been reported.

2. Experimental

2.1. Chemicals

DSS with a molecular mass of 5000 (DSS_{5000}), 2-aminopyridine, phthalic acid, tetra-*n*-butylammonium hydroxide and α-amylase (derived from *Bacillus subtilis*, 20 U/mg) were obtained from Wako (Osaka, Japan). DSS with a molecular mass of 8000 (DSS_{8000}), 10 000 ($DSS_{10\,000}$), and D-glucose 3-sulfate were obtained from Sigma (St. Louis, MO, USA). Tetra-*n*-buthylammonium hydroxide and sodium cyanoborohydride were obtained from Nacalai Tesque (Kyoto, Japan).

2.2. Labeling of DSS using 2-aminopyridine

Labeling of DSS_{5000}, DSS_{8000}, $DSS_{10\,000}$ and D-glucose 3-sulfate using 2-aminopyridine was carried out according to the method in a previous report [18]. Briefly, 0.2 mol of DSS_{5000}, DSS_{8000}, $DSS_{10\,000}$ and D-glucose 3-sulfate were dissolved in 25 ml of a 2-aminopyridine solution (prepared by mixing 5 g of 2-aminopyridine, 3.8 ml of conc. HCl, and 11 ml of distilled water, with a final pH of 6.7) and incubated at 100°C for 13 min. After the addition of 1.5 ml of a reducing reagent (prepared by mixing 600 mg of sodium cyanoborohydride and 1.5 ml of distilled water just before use), the reaction mixture was heated at 90°C for 20 h for reductive amination. After 20 h of reaction, four volumes of NaCl-saturated ethanol were then added to the reaction mix-

ture. The resulting precipitates were recovered by centrifugation at 500 *g* for 5 min and washed with ethanol 10 times to remove excess reagents. The precipitates were dried using a vacuum pump, and dry masses were measured. For HPLC analysis, these dried products were dissolved in 0.2 *M* phosphate buffer to a 3% of final concentration (solution$_{5000}$, solution$_{8000}$, solution$_{10\,000}$, solution$_{D\text{-}Glu}$, respectively). In HPLC analysis, the supernatant was filtered through a membrane filter (pore size 0.45 μm), and a 5 μl of individual solution was injected onto the HPLC columns. At each time point (after 10 min, 3 h, 7 h, 15 h and 20 h), the supernatant was filtered through a membrane filter (pore size 0.45 μm) and was injected onto the HPLC columns in order to evaluate the degree of pyridylamination.

2.3. HPLC conditions

Since pyridylamino-DSS (PA-DSS) is a strongly negatively charged polysaccharide in aqueous solution, strong interaction between PA-DSS and the stationary phase is suggested in this HPLC system. Therefore, in order to evaluate the effects of the counter ion, different concentrations of phosphate buffer at different pH (pH 3.0 and 7.0), were tested as mobile phases, in the PA-DSS separation.

In this study, a HPLC LC6A apparatus (Shimadzu, Kyoto, Japan) was used. The retention time of DSS was determined by gel filtration chromatography (GFC) on a Cosmosil 5Diol-120 packed column (300×7.5 mm; Nacalai Tesque) in two series. 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 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.

2.4. Enzymatic metabolites of PA-DSS$_{5000}$

We evaluated whether the enzymes commonly present in the gut lumen could depolymerize PA-DSS$_{5000}$. Briefly, 2000 U of α-amylase was added to 10 ml of solution $_{5000}$. After gentle shaking at 37°C for 48 h, the mixture was centrifuged at 6000 *g* for 5 min. The supernatant was filtered through a membrane filter, and a 5 μl was injected onto the HPLC columns.

2.5. Fecal metabolites of PA-DSS$_{5000}$ in rats

Specific pathogen-free male Sprague–Dawley rats (n=4), 12 weeks old (410~460 g), were purchased from Nippon Clea (Tokyo, Japan). They were housed in a room with controlled temperature (20–22°C), humidity (50~60%) and a preset light–dark cycle (12 h:12 h). The rats were allowed food and solution$_{5000}$ ad libitum for 5 days. On the final day of the experiment, fecal samples were collected and immediately frozen. PA-DSS in the fecal samples was then measured by HPLC. Briefly, 2 ml of distilled water was added to 1 g of fecal sample. After mechanical shaking and centrifugation at 6000 *g* for 10 min, the supernatant was filtered through a membrane filter and 5 μl was injected onto the HPLC column. As the blank, feces from rats (n=2), which were administered DSS$_{5000}$, were also analyzed in the same manner.

On the other hand, it is possible that PA-DSS binds to some insoluble substances present in the feces. In order to rule out this possibility, we evaluated the binding capacity of PA-DSS$_{5000}$ in solution$_{5000}$ to the feces. Briefly, 2 ml of solution$_{5000}$ was added to 1 g of dried fecal sample derived from non-DSS-treated rats. After mechanical shaking for 30 min, PA-DSS$_{5000}$ in the supernatant was measured with the HPLC system as described above.

This experimental protocol was approved by the Animal Care and Use Committee of the Shiga University of Medical Science.

3. Results and discussion

3.1. Effects of reaction time on the degree of pyridylamination and the DSS chain length

Fig. 2 reveals the chromatogram of solution$_{5000}$ produced by reacting DSS$_{5000}$ with 2-aminopyridine. It is obvious that the degree of pyridylamination of DSS$_{5000}$ increased with prolongation of the reaction time and reached a plateau at 7 h. In addition, the elimination of PA was achieved by the washing of

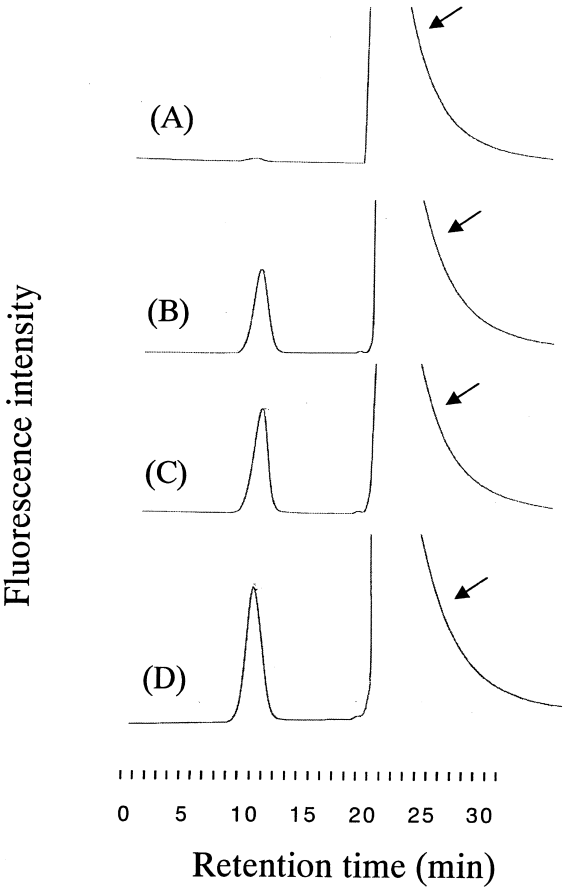

Fig. 2. Chromatogram of PA-DSS_{5000} produced by reacting DSS_{5000} with 2-aminopyridine at 90°C for different reaction times. (A) PA-DSS_{5000} obtained after 10 min of reaction. (B) After 3 h of reaction. (C) After 7 h of reaction. (D) After 15 h of reaction. Arrows indicate the peaks of 2-aminopyridine (PA). HPLC conditions as described in Experimental.

the reaction mixture with ethanol 10 times. This washing did not eliminate the PA-DSS_{5000} (less than 0.2%).

Fig. 2 also shows that no remarkable difference in the elution position of PA-DSS_{5000} was observed with prolongation of the reaction time, indicating no alteration in the length of the PA-DSS_{5000} chain during the reaction.

This pyridylamination is based on equilibrium reaction [19]. Therefore, far exceeding dose of PA compared to that of DSS results in enhanced production of PA-DSS. However, we could not evaluate the molar ratio of PA-DSS to non-labeled DSS in the 20 h reacted mixture. On the other hand, since 2-aminopyridine has a low molecular mass (94.12), it is strongly suggested that there are little structural differences between PA-DSS and non-labeled DSS. Therefore, it is strongly suggested that the molar ratio of PA-DSS_{5000} to non-labeled DSS in vitro and in vivo is similar to the molar ratio in $solution_{5000}$. Therefore, we used this reacted mixture of PA-DSS and non-labeled DSS in in vivo or in vitro experiments.

3.2. Effects of the buffer concentration and pH on the PA-DSS running status

In order to evaluate the effects of the counter ion, different concentrations and pH of phosphate buffer were tested as mobile phases.

PA-DSS_{5000} and PA-$DSS_{10\,000}$ were particularly difficult to separate. Only 0.1–0.2 *M* phosphate buffer (pH 3.0) managed to elute these molecules separately. Less than 0.05 *M* or more than 0.3 *M* phosphate buffer (pH 3.0) resulted in even poorer separation. In addition, pH 3 buffer could better separate the peaks than pH 7.0 buffer (Fig. 3). Therefore, 0.2 *M* buffer (pH 3.0) was used as the mobile phase. At concentrations less than 0.2 *M*, as the concentration of counter ion increased, better separation of the peaks resulted. Since PA-DSS has a strong negative-charge, we speculated that the greater the concentration of counter ions increases, the better the separation of the peaks. However, at concentrations exceeding 0.3 *M*, poor separation occurred. This phenomenon may suggest that the increased viscosity of the buffer at high concentrations potentially interferes with the separation of these peaks. The precise mechanism, by which this occurs however, is unclear.

In the present system, it was impossible to distinguish between PA-DSS_{5000} and PA-DSS_{8000}, or between PA-DSS_{8000} and PA-$DSS_{10\,000}$.

3.3. Analysis of in vitro enzymatic metabolites of PA-DSS_{5000}

Fig. 4A shows the chromatogram of mixture of $solution_{5000}$ and α-amylase at the start of the experiment. No detectable peaks with a molecular mass of less than 5000 were found. Fig. 4B also shows the

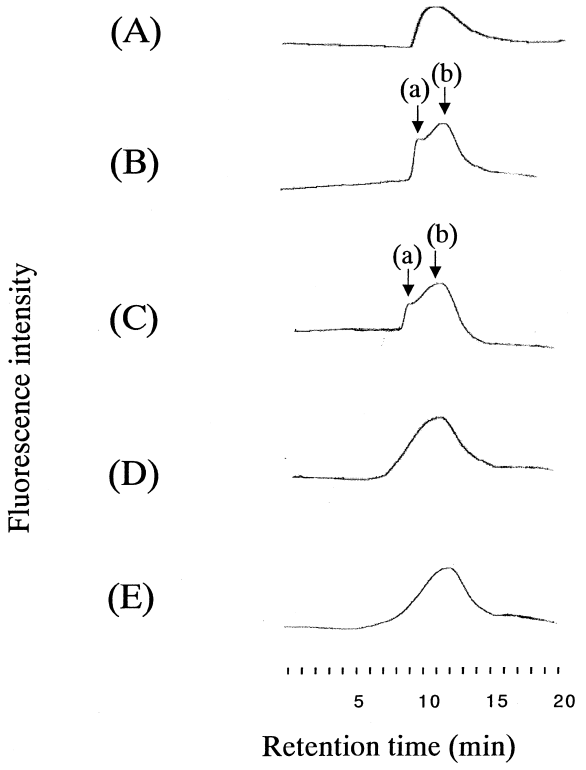

Fig. 3. Chromatogram of PA-DSS of different molecular masses. Mobile phases were (A) 0.05 *M* phosphate buffer (pH 3.0), (B) 0.1 *M* phosphate buffer (pH 3.0), (C) 0.2 *M* phosphate buffer (pH 3.0), (D) 0.3 *M* phosphate buffer (pH 3.0), and (E) 0.2 *M* phosphate buffer (pH 7.0). Peaks (a) and (b) represent PA-$DSS_{10\,000}$ and PA-DSS_{5000}, respectively. HPLC conditions as described in Experimental.

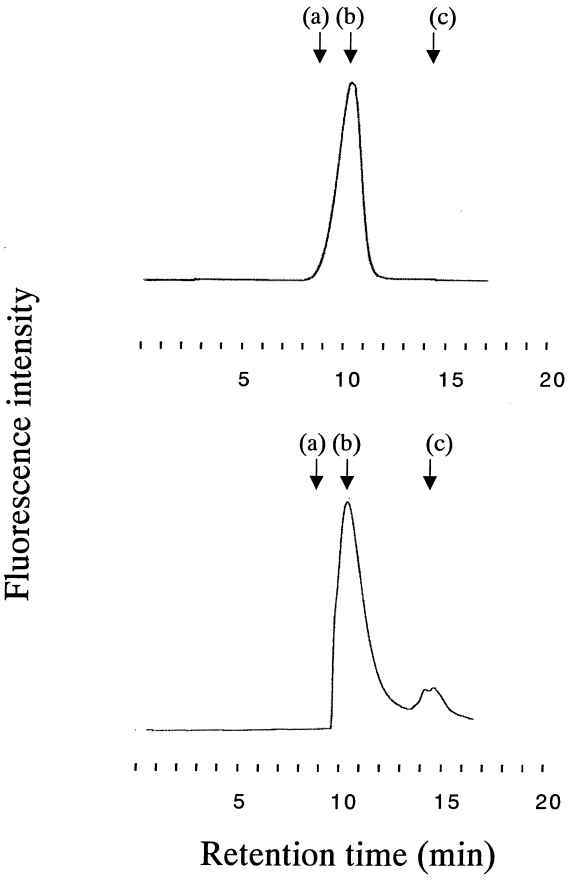

Fig. 4. Chromatogram of PA-DSS in $solution_{5000}$ after co-incubation with α-amylase. (A) At the start of the co-incubation. (B) After 48 h of co-incubation. Arrows indicate the elution times of (a) PA-$DSS_{10\,000}$ (b) PA-DSS_{5000} and (c) PA-D-glucose 3-sulfate, respectively. Two small peaks, peak (d) and (e) were detected. HPLC conditions as described in Experimental.

chromatogram of supernatant after 48 h of enzymatic reaction. After 48 h of reaction, two small peaks were detectable (peak d with an earlier retention time, peak e with a later retention time). We calculated the area of these small peaks, and decided that area of these peaks had approximately 10% of that of PA-DSS_{5000}.

Fig. 5 shows the relationship between the elution volumes and the molecular masses of PA-DSS_{5000}, PA-$DSS_{10\,000}$ and PA-D-glucose 3-sulfate. The molecular masses speculated from the retention times of peak (d) and peak (e) are approximately 600 and 380, respectively. The calculated molecular masses for PA-dimers of D-glucose 3-sulfate and PA-D-glucose 3-sulfate are 640.4 and 360.2, respectively. Therefore, it is possible that these peaks (d) and (e) may correspond to the PA-dimers of D-glucose 3-sulfate and PA-D-glucose 3-sulfate, respectively. These results may indicate that the α-amylase has the potential to depolymerize the PA-DSS_{5000} to some extent. Because PA-DSS has numerous sulfate esters, the possibility of depolymerization of PA-DSS_{5000} by α-amylase, the enzyme commonly presented in the gut lumen, was unexpected.

3.4. Analysis of in vivo PA-DSS_{5000} in the feces

All the rats showed almost the same clinical course. The onset of diarrhea occurred on day 2–3,

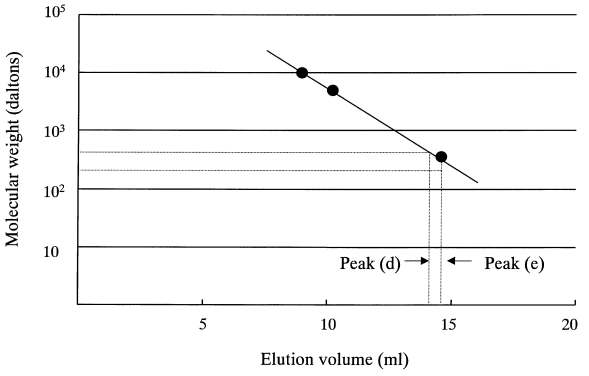

Fig. 5. The relationship between the elution volumes and the molecular masses of PA-DSS_{5000}, PA-$DSS_{10\,000}$ and PA-D-glucose 3-sulfate. Lines (d) and (e) indicate the retention times of peaks (d) and (e), respectively.

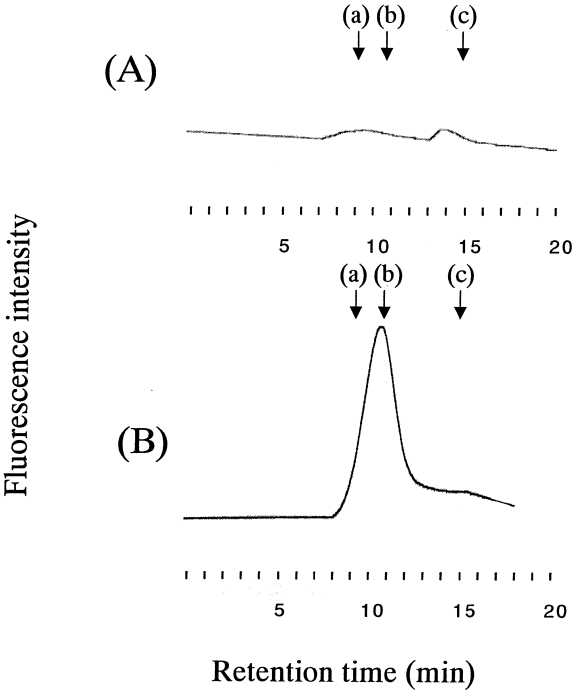

Fig. 6. Chromatogram of PA-DSS in the feces after oral administration of $solution_{5000}$. (A) Blank feces after oral administration of non-labeled DSS_{5000}. (B) Feces after oral administration of $solution_{5000}$. No peaks beyond background fluorescence were detected. Arrows indicate the elution times of (a) PA-$DSS_{10\,000}$, (b) PA-DSS_{5000} and (c) PA-D-glucose 3-sulfate, respectively. HPLC conditions as described in Experimental.

but bloody stools did not appear during the experimental period. None of the rats died during the experimental period. The intake of $solution_{5000}$ per day was 15.3±7.2 ml (mean±SD). On the contrary, the intake of non-labeled DSS_{5000} per day was 24.5±8.8 ml as the blank.

In the analysis of the binding capacity of PA-DSS_{5000} in $solution_{5000}$ on the feces, there were no detectable decreases in the concentration of PA-DSS_{5000} after 30 min of mixing, indicating that PA-DSS_{5000} does not bind to some insoluble substances present in the feces (data not shown).

Fig. 6A shows a chromatogram of feces from a blank rat. Traces of fluorescent content were recognized in the feces. This content may represent some DNA derived from foods, bacteria and so on. Fig. 6B shows a chromatogram of feces from the rat, which was fed $solution_{5000}$. After administration of $solution_{5000}$, a large peak corresponding to PA-DSS_{5000}, was recognized. In this analysis, no clear peaks of lower molecular mass beyond the background fluorescence were recognized. It is not clear whether PA-DSS_{5000} in the feces was not depolymerized or if background fluorescence interfered with the detection of depolymerized PA-DSS_{5000}, which may only be present in minute quantities. The fluorescence signal of PA-DSS_{5000} in the supernatant from the homogenate of 2 ml of distilled water and 1 g of fecal sample corresponded to that in approximately 0.3% $solution_{5000}$. Therefore, 6 mg ($1.2 \cdot 10^{-6}$ mol) of DSS_{5000} (PA- or non-labeled) exists in 1 g of feces.

In conclusion, this paper has demonstrated for the first time a method of separating and analyzing the strongly negative-charged sulfated polysaccharide, DSS, in the biological materials. The pyridylamination of DSS can be performed without difficulty. Furthermore, fluorometric detection with a 2-aminopyridine labeling system allows fairly selective detection of DSS present in the feces.

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