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Synthesis and Properties of Dextran-5-aminosalicylic Acid Ester as a Potential Colon-specific Prodrug of 5-Aminosalicylic Acid

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Dextran-5-aminosalicylic acid ester (dextran-5-ASA) was synthesized as a colon-specific prodrug of 5-aminosalicylic acid (5-ASA) which is active against inflammatory bowel diseases. Chemical stability of dextran-5-ASA in the bath of pH 1.2 or 6.8 was investigated at 37°C for 6 hrs, and 5-ASA was not released on such conditions. Depolymerization (%) of dextran-5-ASA by dextranase with the degree of substitution (DS) of 18, 23, or 30 was 92, 62 or 45 in 8 hrs respectively, but was not affected by the MW of dextran (9,000, 40,600, 80,200 or 580,000). Distribution of 5-ASA in dextran, determined by gel filtration chromatography, appeared to be relatively uniform. Incubation of dextran-5-ASA (DS 18) in cecal contents of rats released 20% (28 g) and 35% (49 g) of 5-ASA in 8 hrs and 24 hrs, respectively, but no 5-ASA was liberated from small intestinal contents.

Key words : Dextran-5-aminosalicylic acid ester, Colon-specific delivery, 5-Aminosalicylic acid, Crohn's disease, Ulcerative colitis, Inflammatory bowel disease

INTRODUCTION

Delivery of orally administered drugs specifically to the colon is desirable for two main reasons; firstly, for the efficient treatment of diseases which develop locally at the colonic site to avoid systemic absorption and reduce side effects, and secondly, for those drugs in which absorption through large intestine is more beneficial (Crotty *et al.*, 1992; Mcleod *et al.*, 1992; Mrsny *et al.*, 1992; Kimura et al., 1994). If we consider the colon as the site of drug absorption, factors such as small surface area, low fluidity of the lipid membrane, low motility, high viscosity of intestinal contents which would hinder the diffusion of drugs from the bulk state to the absorption site, and existence of tight junction are the negative aspects of large intestine compared to small intestine. In contrast, long transit time, low enzymatic activity, responsiveness to the action of absorption enhancers are the positive aspects of large intestine compared to small intestine (Haeberline *et al.*, 1992).

Development of pharmaceutical preparations such as pH-dependant or time-dependant coating is one of the approaches for the design of orally administered-colonic delivery system. The main advantages of pharmaceutical approaches reside in their universal applicability, while inconsistent results are the main disadvantages. Development of a prodrug is another way to deliver drugs specifically to the colon. In prodrug approaches, polymeric or water-soluble carrier is employed to prevent absorption of prodrugs in the upper intestine. After delivered to the colon, the prodrug is presummed to be activated by the enzymes originated from the microbes which are especially abundant in that portion of the alimentary canal (Rubinstein, 1990). Numerous publications have appeared during the last decades (Andrew *et al.*, 1992; Rubinstein *et al.*, 1992; Ryde, 1992; Ashore *et al.*, 1993).

Dextran is a nonstarch polysaccaride of linear α-1, 6-glucopyranose chain with α-1,3-glucopyranose branching. It is nonimmunogenic and biocompatible and has been studied widely as a parenteral polymeric drug carrier (Larsen and Johansen, 1985). It is degraded readily by dextranase which produced by the bacteriods residing only in colon. Release of the drug molecule from dextran-drug conjugate does not take place in the upper intestine, presummably because the steric hindrance of the polymer matrix prevents the enzymic action there. Release of the drug is reported to take place only from the oligomerized dextran-drug conjugate, which is formed by depolymerization of dextran matrix by endodextranase in the colon where the bacterial count is very high (Sery and

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Hehre, 1955; Larsen *et al.*, 1985). Inter- or intra-species variations in the composition and activity of dextranase are very limited. These are very desirable properties for dextran to be adopted as a colon-specific carrier (Andrew *et al.*, 1992).

Corticosteroids, used for the treatment of inflammatory bowel diseases, is well absorbed in the upper intestine and only a limited fraction of the administered dose is delivered to inflammed site in ileum or colon. Long term administration causes serious side effects such as osteoporosis, hypertension, edema, diabetes or decreased immunity (Crotty *et al.*, 1992). For this reason, they are effective only for attack therapy using a large dose, and are not suitable for maintenace therapy to prevent relapse. 5-ASA is known to have antiinflammatory activity, but it is absorbed rapidly and extensively through the upper intestine, and hardly reaches to the colonic site, and systemic absorption of which is reported to be nephrotoxic (Novis *et al.*, 1988). Several colon-specific prodrugs are available (Brown *et al.*, 1983; Kopeckova *et al.*, 1990; Istran *et al.*, 1991; Pelliciari *et al.*, 1993).

In the present study, dextran-5-ASA was synthesized as a potential colon-specific prodrug of 5-ASA, and 1) its chemical stability under upper GI tract pH values, 2) effect of DS and the MW of dextran on the depolymerization by dextranase, and 3) release of 5-ASA after incubation with various intestinal segments of rats was investigated.

MATERIALS AND METHODS

Materials and Instruments

Dextran, dextranase (*Penicillium* sp.), 5-aminosalicylic acid (5-ASA), carbonyldiimidazole (CDI), 2,4-dinitrosalicylic acid (DNS) and anthrone were obtained from Sigma Chemical Co. (St. Louis, USA), Sephadex LH-20 was purchased from Pharmacia Biotech and used as received. All other chemicals and solvents were reagent grade. UV and IR spectra were taken on Shimadzu UV-2101PC spectrophotometer and Bomem MB100 FTIR spectrophotometer, respectively. A Orion 320 pH meter was used for the pH measurements. Thin layer chromatography (TLC) was carried out on Kiesegel 60 F_{254} (Merck) and gel fillteration chromatography was carried out using Sephadex G-75. A Eyela Mazela-Z tissue homogenizer was used for homogenation of tissue and contents of intestinal tracts of rats and a Hanil Supra K-22 centrifuge was used for centrifugation.

Buffer solutions

Buffer solution A, B, or C was prepared as described

Buffer B: 0.2 M citrate buffer(pH 3).

Buffer C: 0.1 M acetate buffer (pH 5.4).

Buffer D: Isotonic phosphate buffer (0.1 M sod. phosphate dibasic and 0.15 M sod. phosphate monobasic were mixed to give pH 6.8)

Buffer E: Phosphate buffer (5.0 mM sod. phosphate dibasic and phosphoric acid were mixed to give pH 6).

HPLC analysis

Concentration of 5-ASA was determined by HPLC (Chungi *et al.*, 1989; Jung *et al.*). The HPLC system consisted of Model 305, 306 pumps, a 117 variable UV detector, a Model 234 autoinjector, a Model 805 manometric module, and a Model 811C dynamic mixer from Gilson. The mobile phase consisted of 10 % methanol in buffer E and 0.5 mM tetrabutylammonium chloride and filtered through 0.45 μm membrane filter before use. A Synchropac ODS (250×4.6, 5 μm) column was eluted with the mobile phase at a flow rate of 1.5 ml/min and at a pressure of about 2000 psi. The column eluent was monitored at 254 nm with a sensitivity of AUFS 0.01 and a Gilson 712 software was employed for data analysis.

DNS method

DNS reagent solution was prepared by dissolving dinitrosalicylic acid (5 g) in 2N NaOH (100 ml) and distilled water (250 ml). To this solution, sodium potassium tartrate tetrahydrate (150 g) was dissolved and the volume was adjusted to 500 ml with distilled water (Bronsted *et al.*, 1995).

Maltose solution in buffer C (0.093 mg/ml~0.75 mg/ml), 200 μl was mixed with 600 μl of DNS reagent solution, boiled for 5 min, cooled for 10 min. and measured the absorbance at 540 nm by UV spectrophotometer. A calibration curve was made from the results.

A portion of the incubated sample was treated with DNS reagent solution according to the same procedure, and the amount of terminal reducing sugar was deduced from the calibration curve.

Preparation of 5-*N*-formylaminosalicylic acid (2, 5-fASA)

5-ASA (**1**) (1 g, 6.5 mmol) in 98% formic acid (10 ml) was refluxed for 30 min and 20 ml of cold distilled water was added. The precipitates were filtered, washed several times with 10% formic acid and cold water, and dried in vacuo (1.19 g 95% yield), mp: 248~251°C; IR (nujol) ν_{max} (C=O): 1682 cm^{-1}.

Preparation of dextran-5-ASA (5)

Preparation of dextan-5-*N*-formylaminosalicylic acid (**4**, dextran-5-fASA) was proceed by method A and

was obtained by the hydrolysis of compound **4** in 0.5 M HCl for 10 min at 80°C.

Method A: To the solution of compound (**2**) (1 g, 5.2 mmol) in DMF (15 ml), carbonyldiimidazole (CDI, 1.5 g, 9.2 mmol) was added slowly, and reacted for 1 hr at room temperature, and dextran (1 g) in DMSO (20 ml) was added dropwise. To the reaction mixture, triethylamine (1.75 ml) was added and stirred for 24 hrs at room temperature, and added excess acetone to produce precipitates. IR (nujol) ν_{max} (C=O): 1682 cm^{-1}, 1689 cm^{-1}.

Method B: Compound (**2**) (1 g, 5.2 mmol) was dissolved in mixed solvent (DMSO 15 ml and benzene 45 ml), added CDI (1.5 g, 9.2 mmol) in small portions, and reacted for 1 hr. To the flask, benzene (55 ml) and Sephadex LH-20 (1300 mg) was added and stirred for 1 hr and filtered Sephadex LH-20 to remove excess CDI (Robert *et al.*, 1988). After benzene was removed by evaporation, triethylamine (1.75 ml) was added and dextran (1 g) in DMSO (20 ml) was added dropwise. It was stirred for 3 hrs at 55°C, cooled, added excess acetone, and separated the resulting precipitates. IR (nujol) ν_{max} (C=O): 1682 cm^{-1}, 1689 cm^{-1}.

Degree of substitution (DS)

DS was defined as mg of 5-ASA bound in 100 mg of dextran-5-ASA. It was determined by measuring the amount of sodium 5-aminosalicylate by UV spectrophotometer at 299 nm, which was released when 100 mg of dextran-5-ASA was placed in 1 N NaOH solution for 1 hr.

Chemical stability

Dextran-5-ASA (1 g) was placed in solution of 50 ml of buffer A (pH 1.2) or buffer D (pH 6.8) and reacted for 6 hrs at 37°C. Reaction mixture (0.1 ml) and methanol (0.9 ml) was vortexed for 2 min, centrifuged at 10,000×g for 5 min, and analyzed the amount of 5-ASA in the supernatant by HPLC.

Degree of depolymerization by dextranase

Effect of MW: Dextran-5-ASA (DS 20), which was prepared from dextran of MW 9,000, 40,600, 80,200 or 580,000, was dissolved in buffer C (6 mg/ml) and incubated with dextranase (15 DU/ml) at 37°C and analyzed by DNS method at appropriate time interval.

Effect of DS: Dextran-5-ASA (MW 70,000) with DS of 30.2, 30, 23, 18.7 or 18 was dissolved in buffer C (equivalent to 3.14 mg of dextran/ml) and incubated with dextranase (15 DU/ml) at 37°C and analyzed by DNS method at appropriate time interval.

Size exclusion chromatography of dextran-5-ASA after incubation with dextranase

Dextran-5-ASA (MW 40609; DS 20) in buffer C (4 mg/ml) and dextranase (10 DU/ml) were placed in a microtube and incubated with shaking at 37°C. At appropriate time interval, it was placed in boiling water to inactivate the enzyme, centrifuged and evaporated. The residue, thus obtained, was dissolved in 100~200 μl of 0.2 M citric acid, loaded on a column packed with Sephadex G-75 (bed volume 30 ml), and eluted with buffer B at a rate of 0.2 ml/min. Each fraction was diluted with buffer C, and the amount of 5-ASA was determined by measuring the absorbance at 307 nm by UV spectrophotometer. Amount of dextran was determined by adding 600 μl of anthrone reagent (35 mg of anthrone reagent in 100 ml of concentric sulfuric acid) to 300 μl of eluent of each fraction, standing for 30 min at 0°C, and measuring the absorbance at 630 nm by UV spectrophotometer.

Determination of dextranase activity of rat cecum contents

Solution of dextran in buffer D (25 mg/ml), 1 ml, was incubated with specified amount of dextranase (*Penicillium* sp.) in a microtube at 37°C for 30 min. The enzyme was inactivated by boiling in water bath for 1 min and the amount of the reducing sugar which produced was determined by DNS method. Calibration curve was obtained in the range of dextranase activity from 0.5 DU/ml to 4.5 DU/ml.

Male Sprague-Dawley rats weighing around 250 g were anesthesized by ether and midline incision was made to remove the cecum after ligating the both ends of it. Cutting and openning of the cecum, and placing 0.1 g of the cecal contents in a microtube were carried out in a glove box which was previously displaced by nitrogen. To each microtube, 1 ml of dextran solution in buffer D (25 mg/ml) was added and incubated at 37°C for 30 min. The reaction was stopped by placing the microtube in boiling water for 1 min and the amount of the reducing sugar which produced was determined by DNS method. Dextranase activity per gram of the cecal contents was determined from the calibration curve.

Release of 5-ASA after incubation with the contents of proximal small intestine (PSI) and distal small intestine (DSI) of rats

A half dilution of PSI or DSI contents of rats in buffer D, 0.2 ml, was placed in a microtube and added 0.8 ml of dextran-5-ASA solution in buffer D (DS 18; 140 μg equivalent of 5-ASA/0.8 ml), and the mixture was incubated for 6 hrs at 37°C. At appropriate time interval, it was centrifuged at 5,000 rpm for 3 min. To the 0.1 ml of the supernatant, 0.9 ml of methanol was added, vortexed for 2 min, centrifuged for 5 min at 10,000×g. The amount of 5-ASA in 20 μl of the su-

pernatant was analyzed by HPLC.

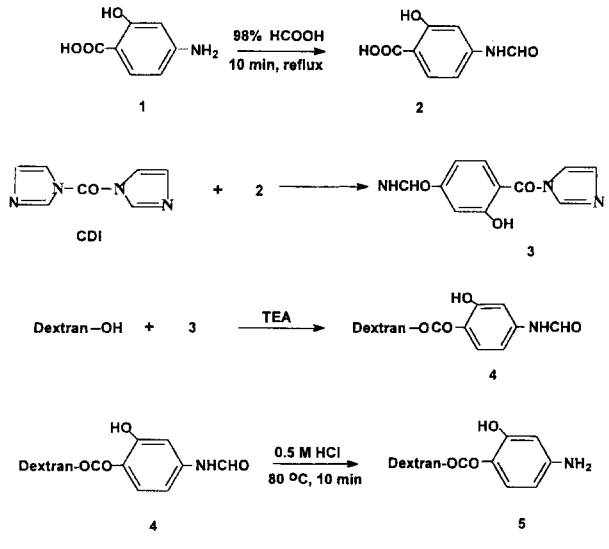
Release of 5-ASA after incubation with the cecal contents of rats

The cecal contents, 0.1 g was placed in a microtube which was previously displaced by nitrogen. To each microtube, 0.9 ml of dextran-5-ASA solution in buffer D (DS 18; 140 μg equivalent of 5-ASA/0.9 ml) was added and incubated at 37°C. At appropriate time interval, the sample was centrifuged at 5,000 rpm for 3 min. To the 0.1 ml of the supernatant, 0.9 ml of methanol was added, vortexed for 2 min, centrifuged for 5 min at 10,000×g. The amount of 5-ASA in 20 μl of the supernatant was analyzed by HPLC.

RESULTS AND DISCUSSION

Preparation

As shown in Scheme 1, preparation of dextran-5-ASA was achieved by the following steps. Amino group of 5-ASA was protected by formylation which proceeded easily in formic acid in good yield. Imidazolide of 5-fASA (**3**) was prepared employing two molar excess of CDI in a nonpolar solvent such as benzene, which reacted with dextran in the presence of triethyamine as catalyst to form dextran-5-fASA (**4**) (method A). Hydrolysis of dextran-5-fASA in 0.5 M HCl for 10 min at 80°C removed only formyl group to give dextran-5-ASA (**5**). In some cases (method B), the reaction mixture was treated with Sephadex LH-20 to remove excess CDI prior to the reaction with dextran to investigate whether the cross-linking of dextran by excess CDI (Robert *et al.*, 1988) might hinder the process of depolymerization by dextranase. Dextran-5-ASA, which prepared either by method A or B, did



Scheme 1. Synthesis of 5-fASA and dextran-5-ASA

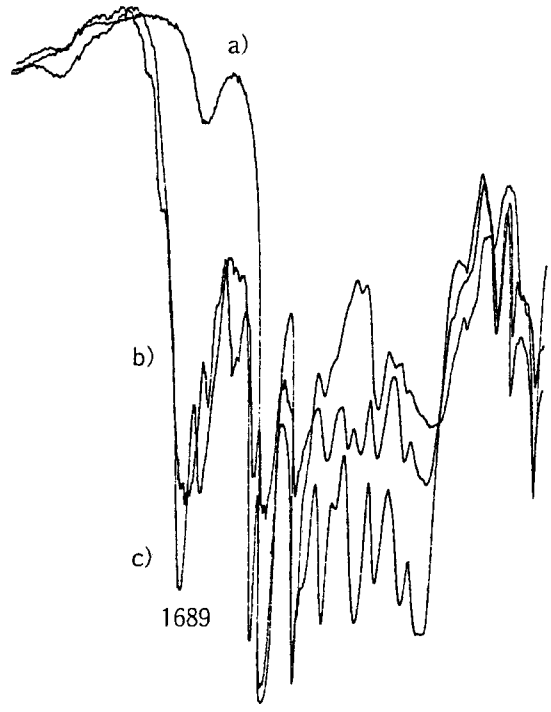


Fig. 1. IR spectra of a) Dextran b) Dextran-5-fASA c) Dextran-5-ASA.

Table I. Weight of 5-formylaminosalicylic acid (5-fASA) per g of dextran used for the coupling of 5-fASA to dextran to prepare dextran-5-fASA and the DS of the resulting product

Dex.: 5-fASA (mole ratio)	mg 5-fASA /g Dex.	DS (5-fASA)[a]		DS (5-ASA)[b]	
		A	B	A	B
1:10	117.7	10.1	8.0	9.90	7.9
1:9	130.7	10.9	8.0	10.0	7.5
1:7	168.2	13.8	11.0	12.1	8.0
1:5	235.6	18.8	12.7	17.8	10.2
1:3	393.3	26.0	20.0	23.4	18.7
1:1	1176.7	45.5	32.1	42.2	30.2

[a]mg of 5-fASA bound per 100 mg of dextran-5-fASA.
[b]mg of 5-ASA bound per 100 mg of dextran-5-ASA which was obtained after removing formyl group of dextran-5-fASA by hydrolysis.
A; Sephadex LH-20 non-treated product.
B; Sephadex LH-20 was used to remove excess CDI in the coupling reaction.

not show any differences in the degree of depolymerization (Table III). Fig. 1 shows the IR spectrum of dextran-5-ASA and ester carbonyl peak is observed at 1690 cm-1 in addition to the peaks originated from 5-ASA and dextran. The degree of substitution was varied by adjusting the ratio of dextran and 5-fASA and the results are listed in Table I.

Chemical stability

To verify the chemical stability of dextran-5-ASA in transit through the upper GI tract, dextran-5-ASA was

placed in buffer solution of pH 1.2 or pH 6.8 representing the pH of the stomach or small intestine, respectively, for 6 hrs at 37°C. No free 5-ASA was detected regardless the value of DS, which suggested that dextran-5-ASA was chemically stable.

Depolymerization by dextranase

Effect of MW of dextran (9,000, 40,600, 80,200 or 580,000) or DS (30.2, 30, 23, 18.7 or 18) on the degree of depolymerization by dextranase was investigated for dextran-5-ASA by DNS method, where the amount of isomaltose produced by dextranses was determined. The results are listed in Table II and III. Depolymerization was not affected by the MW of dextran, but decreased as DS increased. Dextran depolymerized completely in 30 min, but the degree of depolymerization (%) of the sample with DS of 18, 23, or 30 was 92, 62 or 45, respectively, in 8hrs.

As shown in Table IV, depolymerization (%) of dextran-5-ASA was compared with our previously reported data of dextran-5-(4-ethoxycabonylphenylazo) salicylic acid ester (dextran-5-ESA) with same DS value (Jung *et al.*). The degee of depolymerization of dextran-5-ESA was lower than that of dextran-5-ASA, which suggested that the more hydrophobic the molecule was, the lower the degree of depolymerization. This indicates that utilization of dextran as a colon-specific carrier seems to be suitable for hydrophilic drugs or for drugs whose effective doses are not very high if they are hydrophobic.

Table II. Influence of variation in molecular weight (MW) of dextran on the depolymerization (%) of dextran-5-ASA by dextranase[a]

MW \ Time	30 min	1 hr	3 hr	5 hr	24 hr
9000	33.5	39.4	48.5	50.8	67.3
40600	31.5	40.0	46.0	56.5	68.9
80200	33.1	39.2	42.1	52.3	68.3
580000	33.1	37.2	40.4	54.8	65.5

[a]The amount of sample (DS 20) containing 6.0 mg equivalent of dextran was incubated with dextranase (15 DU/ml) at 37°C and the degree of depolymerization was determined by DNS method at specified time interval.

Table III. Influence of DS on the depolymerization (%) of dextran-5-ASA by dextranase determined by DNS method[a]

DS \ Time	0.5 hr	1 hr	3 hr	5 hr	8 hr	24 hr
30.2	-	-	21.6	29.6	32.8	32.9
30.0[b]	28.8	34.8	39.6	42.0	45.6	50.4
23.4[b]	34.7	48.0	50.6	57.8	61.9	69.0
18.7	65.3	69.2	73.2	76.6	80.4	88.8
18.0[b]	75.0	76.4	80.3	86.5	92.2	98.4
Dextran	100					

[a]The amount of sample containing 3.14 mg equivalent of dextran was incubated with dextranase (15 DU/ml) at 37°C and the degree of depolymerization was determined by DNS method at specified time interval.

Table IV. Comparison of dextran-5-ESA and dextran-5-ASA with the same DS on the depolymerization (%) by dextranase determined by DNS method[a]

Time	0.5 min		0.5 hr		1 hr		3 hr	
DS	A	B	A	B	A	B	A	B
10	62.7	38.0	100	58.0	100	94.0	100	100
20	35.5	-	71.8	-	92.2	-	95.0	1.5

[a]The amount of sample containing 2.52 mg equivalent of dextran was incubated with dextranase (15 DU/ml) at 37°C and the degree of depolymerization was determined by DNS method at specified time interval.
[b]Sephadex LH-20 non-treated product.
A: dextran-5-ASA.
B: dextran-5-ESA.

Size exclusion chromatography

Depolymerization pattern of dextran-5-ASA (MW 40,600, DS 20) by dextranase was investigated by determining molecular weight distribution by Sephadex G-75 (bed volume 30 ml). For each fraction, the amount of 5-ASA was determined by measuring the absorbance at 307 nm, and that of carbohydrate was determined by measuring the absorbance at 630 nm which developed after reacting the eluent with anthrone reagent solution. The results are shown in Fig. 2. As the length of incubation time increases, the maxima of the distribution curves moved to the fraction of lower molecular weight, and the pattern of the distribution curves of A (carbohydrate concentration vs fraction volumn) and B (5-ASA concentration vs fraction volumn) was very similar, which suggest that 5-ASA is almost uniformly distributed in dextran backbone.

Release of 5-ASA after incubation with rat intestinal tract contents

It is reported that dextran-carboxylic acid esters are not hydrolyzed by esterases in the upper intestine due to the steric hindrance of the dextran matrix, and hydrolysis by esterases take place only after the dextran backbone is degraded to smaller sizes by the endodextranases of microbial origin in the large intestine (Andrew, 1992).

To determine the dextranase activity in rat cecal contents, dextran was incubated for specific period with varying amount of dextranase (*Penicillium* sp.), and the degree of depolymerization was investigated by DNS method. A calibration curve was constructed from the results. The same amount of dextran was incubated with the specified amount of rat cecal contents at same condition. The degree of depolymeri-

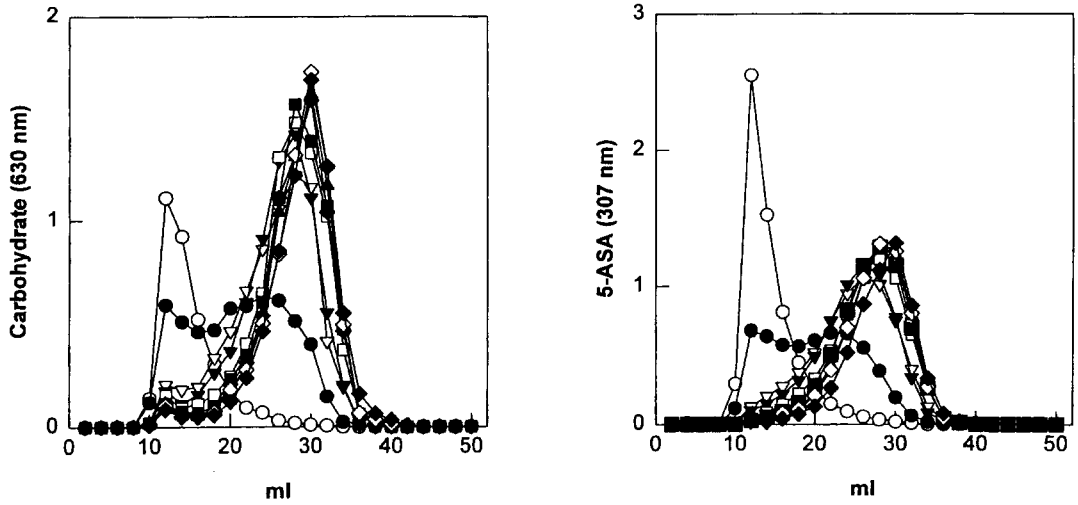


Fig. 2. Size exclusion chromatography of dextran-5-ASA (DS 20, MW 40600) after incubation with dextranase (10 DU/ml) at 37°C. Graph A and B shows content of carbohydrate and 5-ASA, respectively. 0 (○), 0.5 (●), 5 (▽), 15 (▼), 25 (□), 45 (■), 90 (△), 240 (▲), 480 (◇) min and 24 (◆) hr.

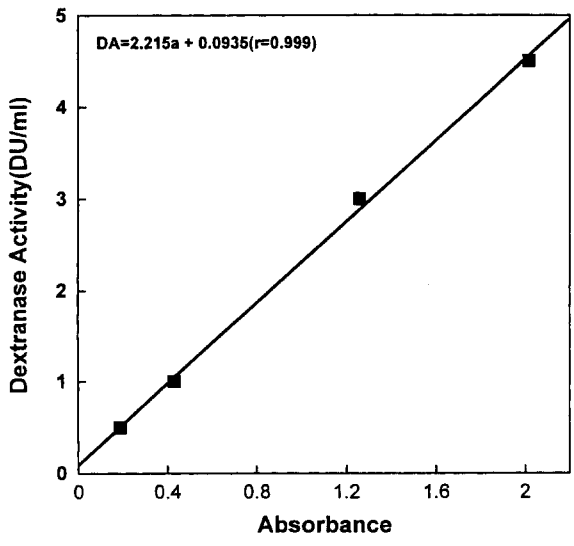


Fig. 3. Calibration of dextranase activity (DA) and determination of DA in rat cecum contents. Dextran (25 mg in 1 ml of phosphate buffer) was incubated with dextranase at 37°C for 30 min and the degree of depolymerization was determined by measuring the absorbance at 540 nm. Calibration curve was constructed at a range of 0.5~4.5 DU/ml of DA. DA of cecum contents was deduced from the calibration after incubation of dextran and cecum contents (0.1 g wet weight) by the same procedure.

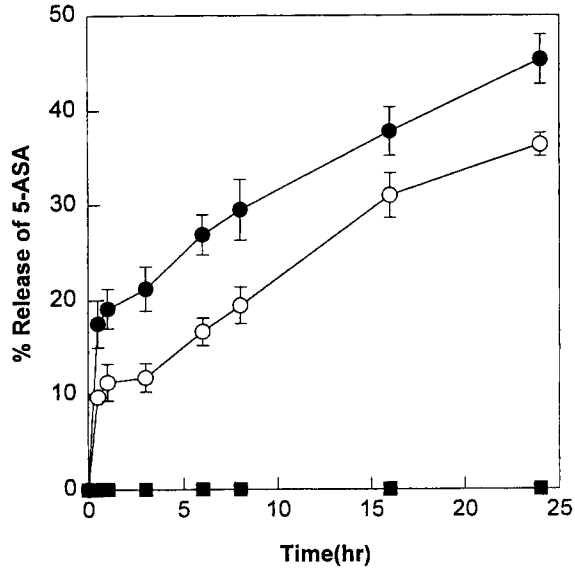


Fig. 4. Release of 5-ASA during incubation of dextran-5-ASA (DS 18, equiv. to 140 μg of 5-ASA) in 1.0 ml of ten-fold dilution in isotonic phosphate buffer (pH 6.8) of GI tract segments. Data are mean±S.E. (n=5). ●: 5-ASA 0.14 mg eqv./100 mg cecal contents, ○: 5-ASA 0.07 mg eqv./100 mg cecal contents, ■: 5-ASA 0.14 mg eqv./100 mg PSI or DSI contents.

zation was determined, and the dextranase activity of rat cecal contents was deduced from the calibration curve. Results are shown in Fig. 3 and the average dextranase activity (DA) of the rat cecal contents was found to be 7.4 DU/g (sd=0.11, r=0.75, n=10). Among the various enzymes existing in rat cecal contents, some are known to degrade reducing sugars which are produced by dextranase (Mortensen *et al.*, 1991). This factor may cause lowering of the experimental value of dextranase activity determined by DNS method than the actual value. If we consider this, actual dextranase activity of rat cecal contents could be higher than the suggested value in this experiment.

Dextran-5-ASA (140 g equivalent of 5-ASA/100 mg cecal contents, which corresponds to 16.6 mg 5-ASA/kg rat) was incubated with 1 ml of cecal contents (10%) at 37°C, and the results are shown in figure 4. The amount of 5-ASA released was 20% (28 g) and

35% (49 g) in 8 hrs and 24 hrs, respectively. The results suggested that most of the active compound might have been liberated if undiluted cecal contents were applied, which amounted to daily dose of 5-ASA. In similar experiments where the contents of PSI or DSI was used instead of cecal contents, no 5-ASA was detected from the incubated sample. Since the release of the active compound had taken place only in the cecum, it was suggested that enzymes of bacterial origin were responsible for the activation of dextran-5-ASA.

In summary, dextran-5-ASA was stable through the upper GI tract, and activated by cecal cotents to liberate 5-ASA, which suggested dextran-5-ASA as a potential colon-specific prodrug of 5-ASA.

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