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Isolation and characterization of cyclic α-(1→4)-glucans having degrees of polymerization 9–31 and their quantitative analysis by high-performance anion-exchange chromatography with pulsed amperometric detection

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Abstract

Cyclic α-(1→4)-glucans with degrees of polymerization (DPs) 9–31 were isolated from a mixture of cyclization products formed in the early stage of the action of cyclodextrin glucanotransferase (CGTase) on synthetic amylose, and characterized by matrix-assisted laser desorption ionization time-of-flight MS, ^{13}C-NMR and HPLC of their partial acid hydrolyzates. High-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection for an accurate estimation of cyclic α-(1→4)-glucans was developed using those isolate glucans as quantification standards, and by HPAEC, the time course of the cyclization reaction of CGTase from an alkalophilic *Bacillus* sp. A2-5a on synthetic amylose was determined.

Keywords: *Bacillus* spp.; Glucans; Carbohydrates; Enzymes

1. Introduction

Cyclic α-(1→4)-glucans are produced by the cyclization reaction of cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) on starch. It is well known that the main products by CGTase found in several bacterial species are cyclic α-(1→4)-glucans with degrees of polymerization (DPs) of 6, 7 and 8; they are α-, β-, and γ-cyclodextrin (CD), respectively. In this paper these CDs are abbreviated as CD6, CD7 and CD8, respectively. Although the presence of trace amounts of larger cyclic glucans (δ-, ϵ-, ζ-, η- and θ-CD) in the reaction mixture of CGTase on starch were reported [1], the structures of these larger cyclic glucans were not clarified, because the technique of separation and the method of analysis had not been developed.

Since 1990, larger cyclic glucans of DPs 9–13 have been isolated, and X-ray analysis of δ-CD (DP 9) and ϵ-CD (DP 10), and studies on solubility, hemolytic activity and inclusion reaction of δ-CD were carried out [2–6]. However, the reaction mixture of CGTase on starch contained outer-branched cyclic α-(1→4)-glucans and inner-branched cyclic glucans other than cyclic α-(1→4)-glucans, and the proportion of unbranched cyclic α-(1→4)-glucans

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decreased extremely with increasing DP of the cyclic glucan, and therefore, isolation of larger cyclic α-(1→4)-glucans has been thought to be practically impossible. Endo et al. isolated a new series of large-ring α-(1→4)-glucans (DPs 14–17) from the commercially available CD powder produced by the action of CGTase on starch [7], but their yields were very low and it is impossible to rule out the possibility that such large-ring cyclic glucans might contain small amounts of inner-branched cyclic glucans with α-(1→6) linkages.

On the other hand, Terada et al. investigated the initial action of CGTase from an alkalophilic *Bacillus* sp. A2-5a or *Bacillus macerans* on synthetic amylose, and found production of cyclic α-(1→4)-glucans with DPs ranging from 9 to more than 60 together with the conventional CDs (DPs 6–8) [8].

In this paper, the isolation of cyclic α-(1→4)-glucans with DPs 9–31 from a mixture of cyclization products formed in the early stage of the action of CGTase on synthetic amylose, and characterization and quantitative analysis of those cyclic glucans were described.

2. Experimental

2.1. Materials

Synthetic amyloses with average molecular masses of 10 000 and 320 000 (AS-10 and -320) were obtained from Ajinoki (Aichi, Japan). CGTase from *B. maserance* and glucoamylase were purchased from Amano Pharmaceutical (Nagoya, Japan) and Toyobo (Osaka, Japan), respectively. CGTase from an alkalophilic *Bacillus* sp. A2-5a was purified to a homogeneous state as described previously [9]. All reagents were of analytical-reagent grade. Reagent-grade organic solvents used for preparative chromatography were freshly distilled before use. Water used in solvent preparations was distilled, deionized and redistilled. The eluents for high-performance anion-exchange chromatography (HPAEC) were prepared in the same manner as those in a previous paper [10].

2.2. Preparation of cyclic α-(1→4)-glucan mixtures

Cyclic α-(1→4)-glucan mixtures were produced by the action of CGTase on synthetic amylose as described previously [8] with slight modification. Synthetic amylose AS-320 (5.0 g) was dissolved in 2.5 l of 10 m*M* sodium acetate buffer (pH 5.5) and incubated with 667 μl of *B. maserance* CGTase (2.1 U/μl) for 50 min at 40°C. The reaction mixture was kept at 100°C for 10 min to inactivate the enzyme, then incubated with 400 U of glucoamylase at 40°C for 20 h to degrade linear amylose into glucose. Glucoamylase was heat-inactivated (100°C, 10 min) and removed by centrifugation (8000 *g*, 10 min). Cyclic α-(1→4)-glucans were precipitated by adding nine volumes of acetone to the supernatant and recovered by centrifugation (8000 *g*, 10 min). The glucan pellet was dissolved into the small amount of distilled water and freeze–dried. The glucan pellet (1.7 g) was dissolved in 5 ml of distilled water, loaded onto a Superdex 30 gel-filtration column (600×26 mm I.D., Pharmacia), and eluted by 100 m*M* NaCl with a flow-rate of 2.5 ml/min. Composition of cyclic α-(1→4)-glucans in each fraction (12.5 ml) was analyzed by HPAEC with a CarboPac PA-100 column (250×4 mm I.D., Dionex) and by matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS). The fractions containing cyclic α-(1→4)-glucans with DPs from 9 (CD9) to 31 (CD31) were subjected to further purification. (Hereafter, cyclic α-(1→4)-glucans with DPn are abbreviated as CDn).

2.3. Analysis

High-performance liquid chromatographic analyses of the reaction products were performed with a Jasco 980-PU pump and a Shodex RI-71 monitor. The column used was a Daisopak ODS-BP (150×6 mm I.D., Daiso). For semipreparative high-performance liquid chromatography (HPLC), a Daisopak ODS-BP (5 μm, 250×20 mm I.D., Daiso), a YMC-Pack SH-343-5 (5 μm, 250×20 mm I.D., YMC) and a YMC-Pack A-323-3 (3 μm, 250×10 mm I.D., YMC) was used. HPLC analyses at constant tem-

perature were conducted using a column oven CO-1093C (Uniflows).

HPAEC was performed with a Model 4000i Dionex BioLC system and a Model 2 pulsed amperometric detection (PAD) system (Dionex, Sunnyvale, CA, USA). The pulse potentials and durations used were identical to those described in a previous paper [10]. The columns used were a CarboPac PA-1 (250×4 mm I.D., Dionex) and a CarboPac PA-100 (250×4 mm I.D., Dionex). An 807-IT digital integrator (Jasco, Tokyo, Japan) was used to calculate peak areas.

MALDI-TOF-MS was performed in the positive-ion mode on a Vision 2000 reflector-type TOF instrument (Thermo Bioanalysis, UK). A nitrogen laser (337 nm) with a pulse duration of 5 ns was used for ionization. 2,5-Dihydroxybenzoic acid (10 mg/ml in water) was used as the matrix. The ions generated were accelerated to a potential of 5 kV in the ion source and postaccelerated to a potential of 7 kV for detection with a secondary-ion multiplier. The MALDI-TOF spectra represent the accumulation of six to ten single laser shots. They were calibrated externally by a standard sample (angiotensin I, molecular mass of 1296) that was placed on the same target. The matrix solution (0.5 μl) and the sample solution (1 mg/ml in water, 0.5 μl) were mixed and air-dried on the target.

Nuclear magnetic resonance (NMR) spectra data were recorded for 2–3% solutions in $^{2}H_{2}O$ at 50°C with a JEOL GSX-500 spectrometer. Chemical shifts were expressed in ppm downfield from the signal of $Me_{4}Si$ referenced to external 1,4-dioxane (67.40 ppm). The other conditions for ^{13}C-NMR, ^{1}H–^{1}H correlation spectroscopy (COSY) and ^{1}H–^{13}C COSY measurements were the same as reported in the previous paper [11].

2.4. Preparation of partial acid hydrolyzates of CD17 and CD28

CD17 and CD28 (each 1 mg) were individually hydrolyzed in 1 ml of 0.5 *M* trifluoroacetic acid (TFA) at 100°C for 15 min [12]. After removing TFA by evaporation from 200 μl of the reaction mixture, the hydrolyzate was dissolved in 2 ml of deionized water.

2.5. Time course of cyclization reaction of CGTase from alkalophilic Bacillus sp. A2-5

Synthetic amylose AS-10 was dissolved in 90% (v/v) dimethyl sulfoxide (DMSO) solution, and loaded onto a PD-10 gel-filtration column (Pharmacia) to remove DMSO. The amylose was recovered into distilled water and the concentration adjusted to 0.17% (w/v), then immediately incubated with CGTase (85 U/ml) from an alkalophilic *Bacillus* sp. A2-5a in 0.5 m*M* acetate buffer (pH 6.0) at 40°C. The reaction mixture (200 μl) was taken at indicated time points and boiled for 10 min to terminate the reaction. The sample was treated with glucoamylase (1.8 U/μl) for 3 h at 40°C, boiled for 10 min and centrifuged. The supernatant was filtrated

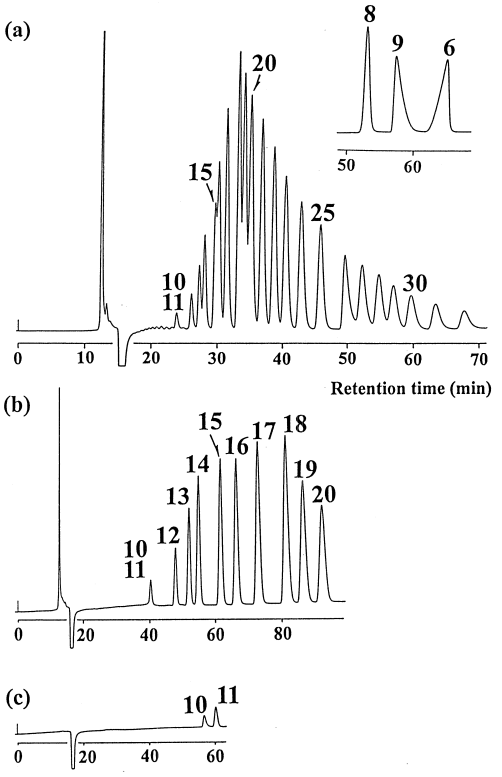

Fig. 1. Chromatograms of a mixture of cyclic α-(1→4)-glucans on an ODS column. Numbers above peaks indicate the DPs confirmed by TOF-MS. Chromatographic conditions: column, YMC-Pack A-323-3 (250×10 mm I.D.); flow-rate, 1 ml/min; temperature, 30°C; detector, refractive index; eluent, methanol–water (a) 6:94, v/v; (b) 4:96, v/v; (c) 3:97, v/v.

through a 0.2-μm membrane filter, then subjected to HPAEC.

3. Results and discussion

3.1. Separation and purification of individual cyclic α-(1→4)-glucans with DPs 9–31

A mixture of cyclic α-(1→4)-glucans which was produced by the action of *B. maserance* CGTase on synthetic amylose and were size-fractionated on a Superdex 30 gel-filtration column (see Experimental), was analyzed on an ODS column with methanol–water systems (Fig. 1).

Fig. 1a is one of the typical elution profiles of the size-fractionated cyclic α-(1→4)-glucans mixture. The elution profile of a mixture of CD6, CD8 and CD9, which were not present in this fraction, was also shown for comparison. Under the same conditions CD7 did not elute even after 140 min. Although a sufficient separation of CD10–CD20 was not achieved with methanol–water (6:94, v/v), a decrease in the methanol content in the eluent resulted in their baseline separation (Fig. 1b and c). As shown in Fig. 1a, cyclic α-(1→4)-glucans larger than CD10 eluted in the order of their DP, while those smaller than CD9 behave differently and eluted slower than expected. The elution order of CD6–CD9 was CD8, CD9, CD6, (and CD7, not shown), and that of CD8 and CD9 was reversed by alterations in methanol concentration of the eluent.

Twenty-three kinds of cyclic α-(1→4)-glucans with DPs 9–31 (CD9–CD31) were individually isolated by repeating chromatography on semipreparative ODS columns. The yield of each cyclic α-(1→4)-glucan was 20 to 40 mg from 1.5 g of a size-fractionated cyclic glucans mixture. It was thought that the isolation of cyclic α-(1→4)-glucans with DPs higher than 32 should be also possible, since they have good solubilities in the eluent unlike linear α-(1→4)-glucans.

During the isolation of cyclic α-(1→4)-glucans, it turned out that CD9, CD10 and CD14 were readily

Table 1
Experimental and theoretical molecular masses of glucans

Compound	Experimental $[M+Na]^+$	DP	Theoretical	
			Cyclic glucan	Noncyclic glucan
CD9	1482	9	1482	1499
CD10	1644	10	1644	1662
CD11	1806	11	1806	1824
CD12	1968	12	1968	1986
CD13	2130	13	2130	2148
CD14	2291	14	2292	2310
CD15	2453	15	2454	2472
CD16	2617	16	2616	2634
CD17	2780	17	2778	2796
CD18	2942	18	2940	2958
CD19	3101	19	3102	3120
CD20	3264	20	3264	3282
CD21	3426	21	3426	3444
CD22	3586	22	3588	3606
CD23	3748	23	3750	3768
CD24	3913	24	3912	3930
CD25	4078	25	4074	4092
CD26	4240	26	4236	4254
CD27	4399	27	4398	4416
CD28	4563	28	4561	4578
CD29	4722	29	4722	4741
CD30	4886	30	4885	4903
CD31	5048	31	5047	5065

crystallized from aqueous solution, and by Jacob et al. crystal structures of CD10 and CD14 were clarified through X-ray analysis [13].

3.2. Characterization of CD9–CD31 by MALDI-TOF-MS, HPLC and NMR

To prove the cyclic structure of each cyclic α-(1→4)-glucan isolated and to confirm its DP, MALDI-TOF-MS and HPLC analysis of partial acid hydrolyzates were carried out.

In Table 1 theoretical and experimental masses of cyclic α-(1→4)-glucans determined by MALDI-TOF-MS are summarized. The experimental value of molecular mass of each cyclic α-(1→4)-glucan was consistent with the theoretical value for the corresponding cyclic glucan but not with that for the noncyclic glucan.

In addition, the DPs of CD17 and CD28 as the representatives were determined by HPLC of their partial hydrolyzates. Fig. 2 illustrates the HPLC elution profiles of partial hydrolyzates of CD17 (left), and partial hydrolyzates of CD28 (right). The last distinguishing peak in each chromatogram of the partial hydrolyzates of CD17 and CD28 were the 17th peak and 28th peak counting from the glucose peak (G), respectively. Consequently, the DPs of CD17 and CD28 were confirmed as 17 and 28, respectively. The cyclic glucan lacks two hydroxy groups which are present in the linear glucan of the same DP, and therefore, the original cyclic glucan remaining was eluted earlier than the corresponding linear glucan on a HPAEC column.

The ^{13}C-NMR spectrum of each cyclic α-(1→4)-glucan showed only one sharp signal for each of the six glucose carbon atoms like those of conventional CDs (CD6–CD8). This result indicated that glucose residues of the individual molecules were identical

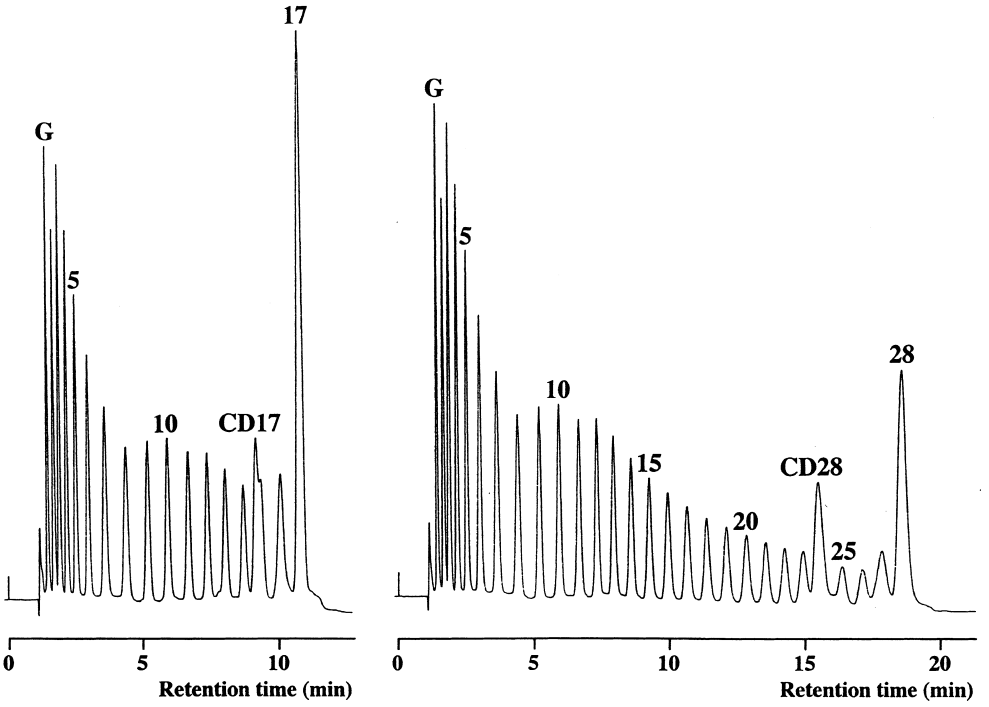

Fig. 2. Elution profiles of partial hydrolyzates of CD17 (left), and partial hydrolyzates of CD28 (right). G is glucose and numbers above peaks indicate the DPs of the hydrolyzates. Chromatographic conditions: column, Dionex CarboPac PA-1 (250×4 mm I.D.); eluent A, 150 m*M* sodium hydroxide solution; eluent B, 150 m*M* sodium hydroxide solution containing 500 m*M* sodium acetate; gradient program, 40% eluent B at 0 min, 50% at 2 min, 60% at 10 min, and 80% at 40 min; flow-rate, 1 ml/min; detector, PAD 2; meter scale, 1 μA; temperature, ambient.

Table 2
^{13}C-NMR chemical shifts (ppm) of cyclic α-(1→4)-glucans in $^{2}H_2O$ at 50°C

DP	C1	C2	C3	C4	C5	C6
6	102.16	72.54	74.15	82.04	72.87	61.30
7	102.57	72.88	73.88	81.93	72.66	61.16
8	102.39	73.11	73.74	81.28	72.63	61.12
9	100.92	73.02	73.69	79.19	72.30	61.23
10	99.71	72.59	73.63	77.97	71.73	61.43
11	99.80	72.50	73.61	78.25	71.69	61.46
12	100.15	72.46	73.61	78.82	71.78	61.47
13	100.36	72.52	73.66	78.99	71.91	61.44
14	100.46	72.59	73.73	78.93	72.00	61.42
15	100.32	72.61	73.77	78.54	72.02	61.40
16	100.09	72.54	73.82	78.12	71.96	61.42
17	100.08	72.50	73.89	78.03	71.97	61.46
18	100.19	72.47	73.93	78.08	72.00	61.44
19	100.32	72.47	73.98	78.17	72.05	61.43
20	100.41	72.49	74.01	78.22	72.08	61.42
21	100.41	72.51	74.01	78.20	72.09	61.42
22	100.33	72.49	73.99	78.10	72.05	61.41
23	100.29	72.46	74.00	78.02	72.04	61.42
24	100.33	72.45	74.03	78.03	72.05	61.42
25	100.39	72.44	74.05	78.09	72.07	61.42
26	100.44	72.45	74.06	78.15	72.09	61.41
27	100.47	72.47	74.06	78.18	72.10	61.42
28	100.45	72.47	74.06	78.16	72.10	61.43
29	100.43	72.46	74.07	78.12	72.10	61.43
30	100.44	72.45	74.07	78.11	72.09	61.43
31	100.45	72.45	74.09	78.11	72.10	61.43

and supported the cyclic structures of these glucans. In Table 2 the ^{13}C-NMR chemical shifts of 23 kinds of cyclic α-(1→4)-glucans (DPs from 9 to 31) together with those of conventional CDs (CD6–CD8) are summarized. These chemical shifts were confirmed by ^{1}H–^{1}H COSY and ^{1}H–^{13}C COSY measurements, if necessary. Fig. 3 shows the variation of chemical shift values at each carbon with DP. Increased DP mainly affects signals of the C1 and C4 atoms, while resonances of the other carbons of cyclic α-(1→4)-glucans are virtually invariant. For CD6, CD7 and CD8, C1 and C4 signals appear at δ~102 and δ~82, respectively, and for CD10 and the higher homologues, they shift upward to δ~100 and δ~78, respectively. The signals for CD9 are intermediate, C1 at δ 100.9 and C4 at δ 79.2. The change in C1 and C4 signals between CD8 and CD10 suggests a clear distinction between their structures. This reasoning was supported by X-ray analysis [13].

3.3. Quantitative analysis of cyclic α-(1→4)-glucans

The successful isolation of CD9 to CD31 described above now enabled us to establish the system to determine these molecules. HPAEC–PAD was selected for this purpose, since the system has been successfully employed for the quantitative analysis of linear α-(1→4)-glucans [14].

First the optimum conditions for an effective separation of individual members of a series of cyclic α-(1→4)-glucans by HPAEC–PAD were investigated. The column used was CarboPac PA-100 which was superior to CarboPac PA-1 or PA-10. As the eluting agent in the eluent sodium acetate [14] and sodium nitrate [15] were compared, and the latter was selected. Several gradient programs were examined and the program summarized in Table 3 gave the best separation. All separations on a HPAEC column were carried out at ambient temperature with a flow-rate of 1 ml/min. The HPAEC elution profile of a mixture of cyclic α-(1→4)-glucans produced by the action of CGTase from *B. macerans* on a synthetic amylose is shown in Fig. 4. The number on each peak, indicating its DP, was confirmed by adding standard cyclic α-(1→4)-glucans obtained prior in the cases of DP≤31. A baseline separation of cyclic α-(1→4)-glucans up to DP>80 was achieved as individual peaks. In general, retention of a homologous series of carbohydrates on this column increases as the DP increases. However, the elution order and pattern of CDs having DPs<12

Table 3
Gradient program for separation of cyclic α-(1→4)-glucans

Time (min)	Eluent[a] (%)		Concentration of sodium nitrate in eluent (m*M*)
	A	B	
0	96	4	8
2	96	4	8
22	92	8	16
38	91	9	18
49	82	18	36
69	72	28	56
89	65	35	70
115	55	45	90
155	37	63	126

[a] Eluents: A=150 m*M* sodium hydroxide solution; B=150 m*M* sodium hydroxide solution containing 200 m*M* sodium nitrate.

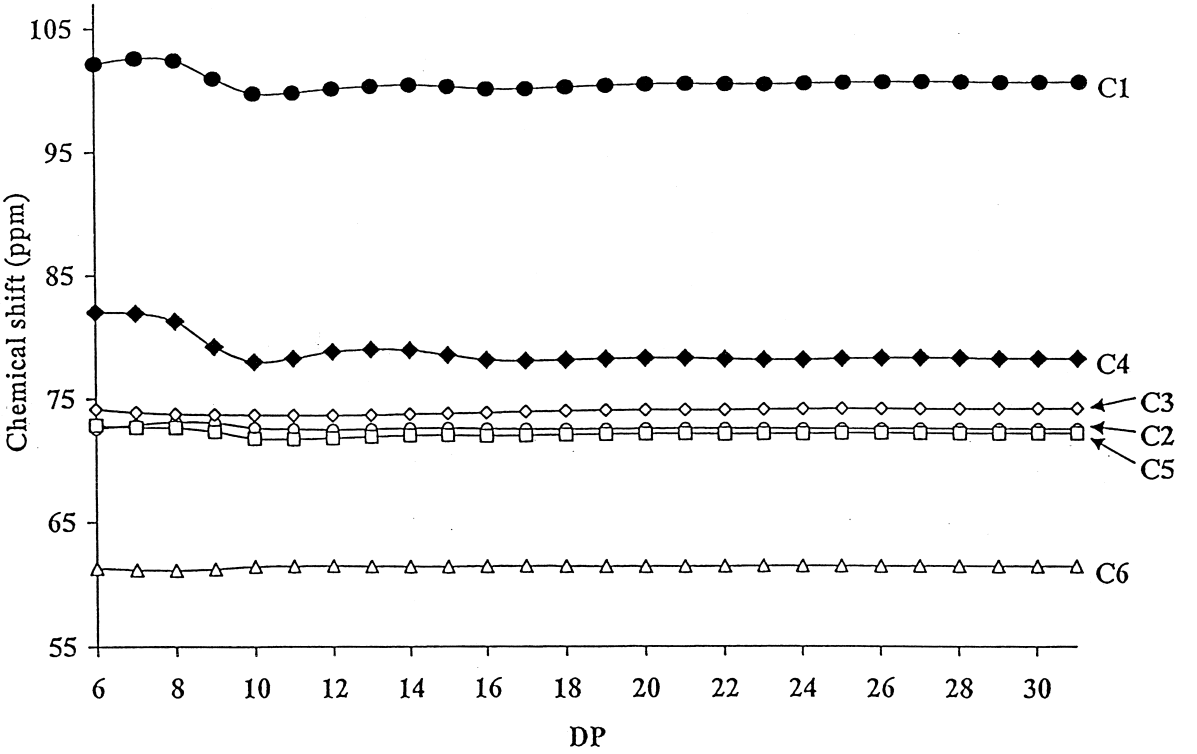

Fig. 3. Variation in ^{13}C chemical shift values of cyclic α-(1→4)-glucans in $^{2}H_2O$ at 50°C.

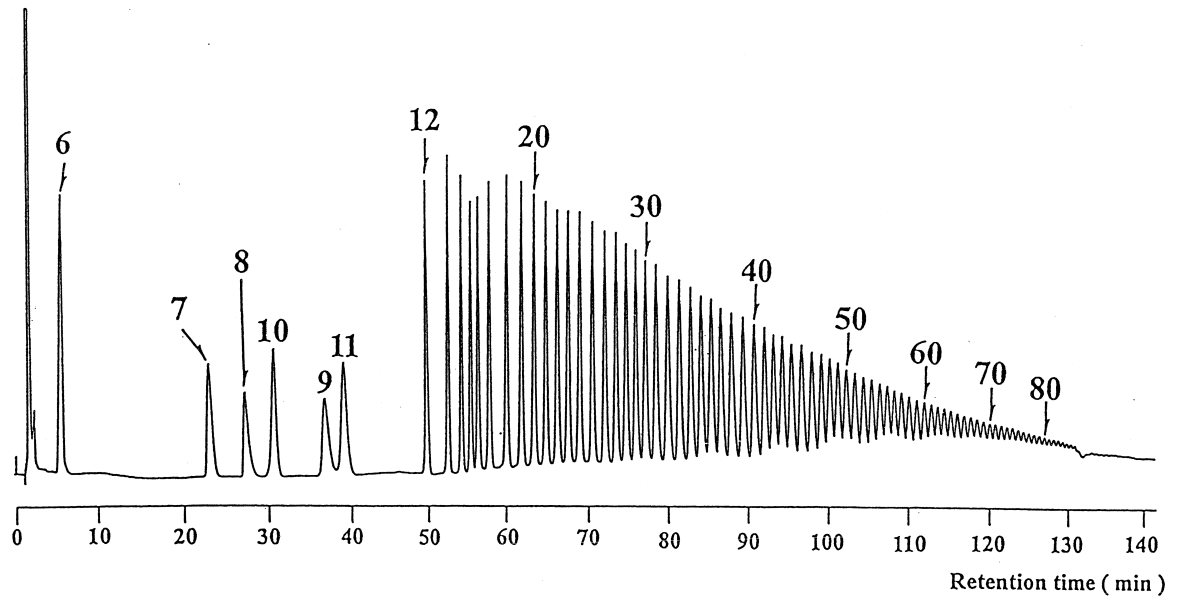

Fig. 4. HPAEC elution profile of cyclic α-(1→4)-glucans produced by the action of CGTase from *Bacillus macerans* on synthetic amylose. Numbers above peaks indicate the DPs. Chromatographic conditions: column, Dionex CarboPac PA-100 (250×4 mm I.D.); eluents and gradient program as in Table 3; other conditions as in Fig. 2.

Table 4
Relative detector responses (RDRs) on molar basis of cyclic α-(1→4)-glucans having DPs 6–31 and maltopentaose (G5)[a]

Saccharide	RDR	Saccharide	RDR	Saccharide	RDR
CD6	0.22	CD15	1.19	CD24	1.73
CD7	0.31	CD16	1.26	CD25	1.80
CD8	0.47	CD17	1.32	CD26	1.84
CD9	0.67	CD18	1.39	CD27	1.90
CD10	0.80	CD19	1.46	CD28	1.94
CD11	0.90	CD20	1.52	CD29	2.01
CD12	1.00	CD21	1.60	CD30	2.05
CD13	1.06	CD22	1.66	CD31	2.12
CD14	1.11	CD23	1.69	G5	0.60

[a] Chromatographic conditions as in Fig. 4. The amounts of saccharides used were 200 pmol each.

were irregular. This result indicates that there are some hydrophobic interactions on this column, but the interaction may be weaker than that on the ODS column.

Previously we have found that PAD responses to a series of glucans increase with increasing DP [10]. Consequently, relative detector responses (RDRs) on molar basis of individual cyclic α-(1→4)-glucans isolated were determined together with that of maltopentaose (G5) used as the internal standard (Table 4). RDRs of CDs (DP>9) increase approximately in proportion to their DP, but the responses per DP of the conventional CDs (DPs 6–8) decrease largely with decreasing DP. The individual peak area obtained from chromatogram was corrected by dividing by the RDR.

3.4. Time course of cyclization reaction of CGTase from an alkalophilic Bacillus sp. A2-5a on synthetic amylose

It has been believed that the major products of CGTase on amylose were CD6 to CD8, and the action of CGTase was often characterized by measuring the amount of CD6, CD7 and CD8. However, recently an investigation clearly demonstrated that CD6 to CD8 were not the major products in the initial stage of CGTase action on amylose, but significant amounts of larger cyclic α-(1→4)-glucans were produced [8].

In order to fully understand the action of CGTase, the quantitative analysis of the cyclic α-(1→4)-glucans in the reaction mixture has been anticipated. We therefore applied the HPAEC system described above, to investigate the action of CGTase on amylose.

As the amylose AS-10 was hard to dissolve directly in 0.5 m*M* acetate buffer, it was first dissolved in sodium hydroxide solution and the solution was neutralized by hydrochloric acid immediately before use. However, splitting of peaks of CD6, CD10 and CD11 occurred due to an influence of the chloride ion, and this phenomenon was found

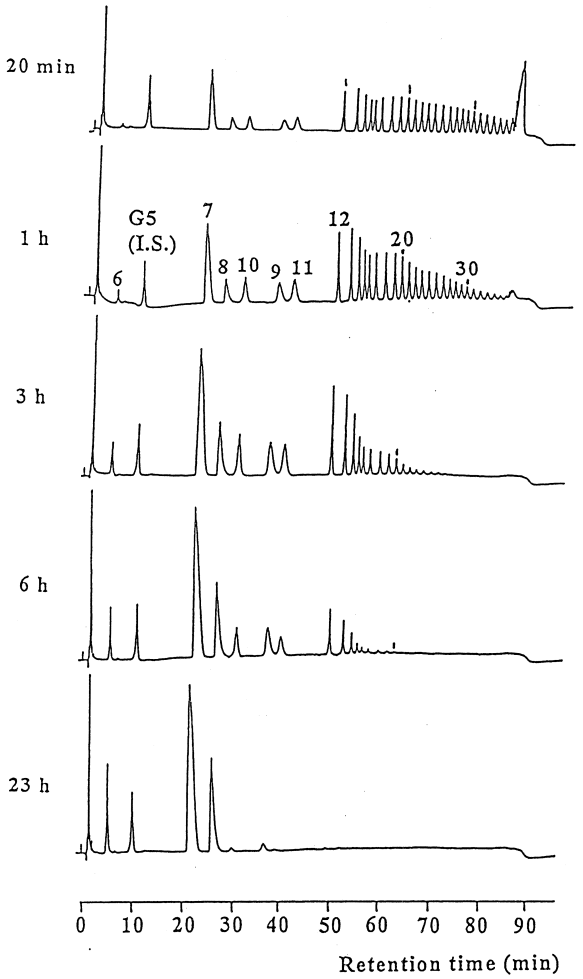

Fig. 5. Time course of cyclization reaction of CGTase from an alkalophilic *Bacillus* sp. A2-5a on amylose AS-10. Maltopentaose (G5) was used as the internal standard. Chromatographic conditions: gradient program, as in Table 3 at 0–69 min, 32% eluent B at 80 min, 100% at 85 min, 4% at 86–100 min to equilibrate at the initial conditions; other conditions as in Fig. 4.

in the case of using acetic acid instead of hydrochloric acid. The amylose was then dissolved in 90% DMSO solution and DMSO was removed by a PD-10 filtration minicolumn immediately before use. To the amylose solution in acetate buffer CGTase from an alkalophilic *Bacillus* sp. A2-5a was added, the reaction mixture was incubated and a portion of it was taken out after 20 min, 1 h, 3 h, 6 h and 23 h. The sample taken out was treated with glucoamylase and then analyzed by HPAEC using maltopentaose as the internal standard. Fig. 5 shows chromatograms at each reaction time. In these chromatograms cyclic α-(1→4)-glucans with DPs higher than 35 are eluted together during 80–85 min for reduction in analytical time. The exact molar concentration of each cyclic α-(1→4)-glucan was estimated by peak area corrected using RDR. The time course of DP distribution are illustrated histographically in Fig. 6.

Thus, it was quantitatively clarified that larger cyclic α-(1→4)-glucans were preferentially produced in the initial stage of the cyclization reaction of CGTase from *Bacillus* sp. A2-5a on synthetic amylose, and were subsequently converted into smaller cyclic α-(1→4)-glucans and finally into the conventional CDs in which CD7 was the major product.

By the way, cyclic α-(1→4)-glucan with DP 5 (CD5), which was chemically synthesized by Nakagawa et al. [16], could not be detected at any

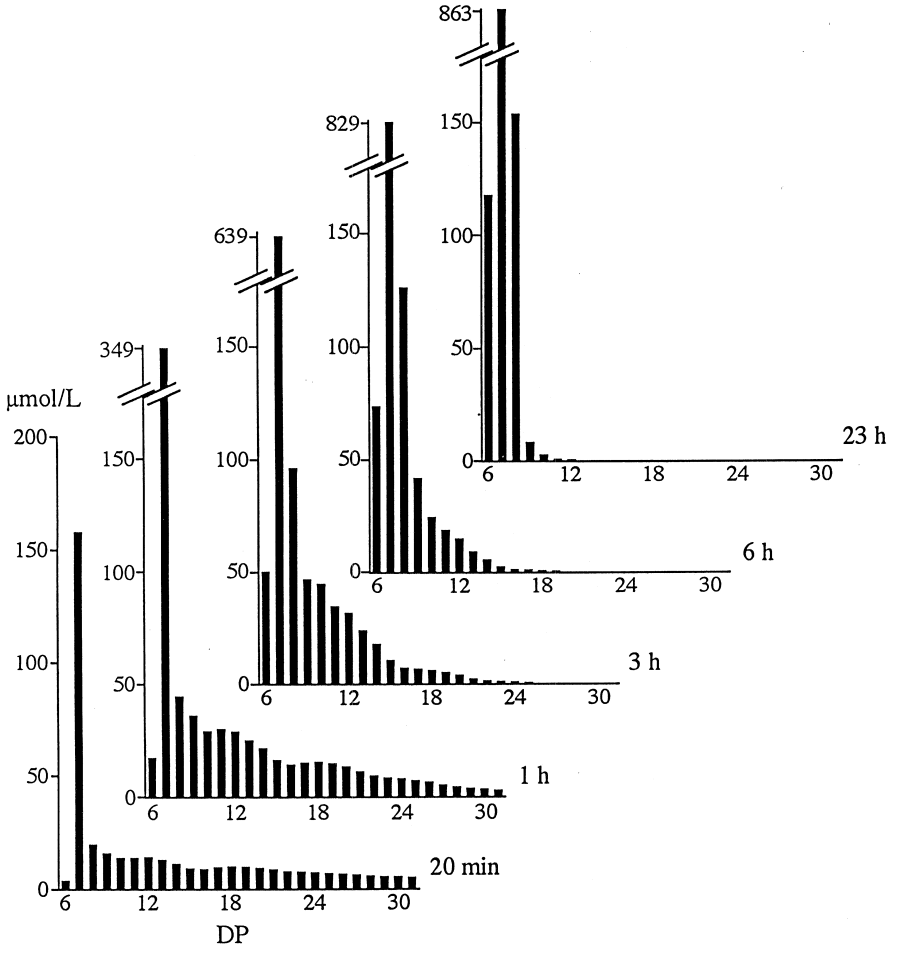

Fig. 6. Time course of molar concentrations of cyclic α-(1→4)-glucans (DPs 6–31) produced by the action of the CGTase on amylose AS-10.

stage of this cyclization reaction, in spite of a detailed search under better chromatographic conditions for CD5, which has a shorter retention time than that of CD6.

4. Conclusion

Twenty-three cyclic α-(1→4)-glucans (CD9 to CD31) were isolated from a cyclic α-(1→4)-glucan mixture produced by the action of CGTase on synthetic amylose, by using gel-filtration chromatography and HPLC on ODS columns. The cyclic structure and DP of each molecule were confirmed by MALDI-TOF-MS and HPLC analysis of partial acid hydrolyzates. Comparison of ^{13}C-NMR chemical shifts suggested a change in their structures between CD8 and CD10. HPAEC–PAD for determination of the accurate molar concentration of each cyclic α-(1→4)-glucan in the cyclization reaction mixture was developed and the time course of reaction of CGTase from an alkalophilic *Bacillus* sp. A2-5a on synthetic amylose was determined. This method will serve to elucidate cyclization reactions of CGTases from different origins.

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