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Examination of the Active Center of a (1→3)-β-D-Glucanase Preparation, Zymolyase

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Zymolyase is a commercially available *endo*-(1→3)-β-D-glucanase preparation, and is widely used to lyse fungal cell walls. In this paper, the active center of this enzyme preparation was examined by using chemical modification of functional groups of amino acid residues, ultraviolet (UV) spectroscopy, optical rotation measurement and analysis of the enzymic products. First of all, chemical modifications of the amino acid residues of Zymolyase, by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide–HCl or 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide metho-*p*-toluenesulfonate for carboxyl, and *N*-bromosuccinimide (NBS) for tryptophan residues, caused the loss of (1→3)-β-D-glucanase activity. These inactivations were prevented by the addition of substrate to the reaction mixture. In the presence of the substrate analogues, *e.g.* laminarabiose or laminaratriitol, a UV difference spectrum attributed to tryptophan residues was observed. This difference spectrum disappeared after NBS oxidation. Secondly, when the specific rotation of carboxymethylated curdlan, a linear (1→3)-β-D-glucan obtained from *Alcaligenes faecalis*, was measured during the enzymic reaction, the value was more negative than that after mutarotation. These results suggest that carboxyl and tryptophan residues are essential to the enzyme activity and the anomeric specificity of the reducing end of the product is β.

Keywords——(1→3)-β-D-glucanase; Zymolyase; carboxyl residue; tryptophan residue; carboxymethylated curdlan

(1→3)-β-D-Glucans are now used clinically in Japan as immunomodulating anticancer agents, such as lentinan and schizophyllan.[1] Many studies are being carried out to develop new immunomodulating anticancer agents of this type; for example we are studying the antitumor activity of grifolan from *Grifola frondosa* and SSG from *Sclerotinia sclerotiorum*.[2] (1→3)-β-D-Glucanase is used as a tool to prepare protoplasts (to obtain new kinds of plant) and to entrap external deoxyribonucleic acid (DNA) segments,[3] and also as an additive to degrade the insoluble glucans in some foods.[4] There are many applications of (1→3)-β-D-glucanase, and several enzymes are now commercially available in Japan, such as Zymolyase,[5] Kitalase,[6] Uskizyme.[7] However, little information is available concerning the active center of these enzymes.[8]

Recognition of carbohydrate structures by proteins, such as enzymes, lectins, and receptors, is an important area of research.[9] Wheat germ agglutinin (WGA),[10] which is a lectin, reacts with *N*-acetyl-D-glucosamine residues. WGA contained a tryptophan residue in the carbohydrate binding site. Lysozyme,[11] and *endo*-chitinase, binds to 6 carbohydrate units on the active centher (cleft), and His and Asp are important for its enzymic activity. In the case of amylase,[12] α-amylase releases the α-anomer (α-oligosaccharides) from starch, and β-amylase releases the β-anomer.

In this paper, to elucidate the interaction between Zymolyase and (1→3)-β-D-glucans, essential amino acid residues for enzymic activity and the anomeric specificity of the reducing end of the reaction products were examined by means of chemical modifications, ultraviolet

(UV) spectroscopy, characterization of the products and optical rotation measurements.

Materials and Methods

Materials——Zymolyase 20T and 100T (laminaraoligosaccharides) were purchased from Seikagaku Kogyo Co., Ltd. Curdlan, laminarin, Bio-Gel P-2 and TSK-HW 55F were purchased from Wako Chemical Co., Ltd., Nakarai Chemical Co., Ltd., Bio Rad Co., Ltd., and Toyo Soda Kogyo Co., Ltd., respectively. Carboxymethylated curdlan (CM-curdlan) was prepared by reacting curdlan with sodium monochloroacetate in 0.5 N sodium hydroxide at 65 °C for 3 h.[13] The degree of substitution was about 0.2, as determined by the method of Eyler *et al.*[14] Alditols of laminaraoligosaccharides were prepared by reaction with sodium borohydride. Other materials were purchased from Wako Chemical Co., Ltd.

Enzyme Assay——The reaction mixture, containing 0.1% laminarin or 0.5% CM-curdlan, 0.05 M acetate buffer (pH 6.0), and enzyme solution, was kept at 37 or 40 °C for 30 min. The reducing power produced was determined by literature methods.[15]

Chemical Modifications——(a) By 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide–HCl (EDC)[16]: The enzyme (2.5 mg) was dissolved in distilled water (1 ml) and adjusted to pH 5.0 with hydrochloric acid. EDC (final concentration of 0.1 M) and glycine ethyl ester–HCl (final concentration of 1 M) were added to the enzyme solution and the mixture was kept at room temperature for 1 h. Then the reaction was stopped by the addition of 1 M acetate buffer (pH 5.0).

(b) By 1-Cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide Methotoluene-*p*-sulfonate (CMC)[17]: a procedure similar to that of Irie *et al.*[17] was used. A solution of the enzyme (2.5 mg) in 1 ml of 0.1 M 3-(*N*-morpholino)-propanesulfonic acid (pH 5.0) was treated with 40 mM CMC at room temperature for 30 min. Aliquots, withdrawn at intervals, were added to 1 ml of 1 M acetate buffer (pH 5.0) in order to stop the reaction.

(c) By *N*-Bromosuccinimide (NBS)[18]: NBS (stock solution) was added to a solution of enzyme (1.8 mg) in 1 ml of 0.1 M acetate buffer (pH 5.0) to give a final concentration of 10 mM, and the mixture was kept for 30 min at room temperature.

Other chemical modifications, *e. g.* with formaldehyde,[19] acetic anhydride[20] and succinic anhydride[21] for amide, with 1,2-cyclohexanedione[22] for arginine, with sodium tetrathionate,[23] 5,5-dithiobis(2-nitrobenzoic acid) (DTNB),[24] and *p*-chloromercuribenzoic acid (PCMB)[25] for sulfhydryl, with diethyl pyrocarbonate[26] for histidine, and with *p*-toluenesulfonchloramide[27] for methionine, were performed by the literature methods.

Analysis of Reaction Products——The enzymic digests were analyzed by gel filtration using Toyopearl HW 55F and Bio-gel P-2 or by high performance liquid chromatography (HPLC) using a Silica-NH_2 column (Merck). The elution profiles from gel filtration were monitored by the phenol sulfuric acid method,[28] and those from HPLC by fluorometric detection (Shimadzu FID-1) after reaction with borate–ethanolamine complex.[29]

Other Methods——UV spectra were measured with a Hitachi 557 spectrophotometer. Optical rotations were measured with a DIP-4 (JASCO). Carbon-13 nuclear magnetic resonance (NMR) spectra were measured with a JEOL-FX-200 spectrometer.[2]

Results

Effect of Various Chemical Modifications on the (1→3)-β-D-Glucanase Activity of Zymolyase

To investigate the essential amino acid residues for the (1→3)-β-D-glucanase activities of Zymolyase, several chemical modifications were performed on Zymolyase 20T and the remaining activity was examined (Table I). As shown in Table I, treatment with EDC, CMC and NBS suppressed the glucanase activity. Other modifications did not affect the glucanase activity. To examine whether the tryptophan and the carboxyl residues were present in the active center of Zymolyase, the modification reactions were performed in the presence of substrate, laminarin. As shown in Fig. 1, inactivation of Zymolyase 20T by NBS was suppressed by the addition of laminarin, and the remaining activity increased dose-dependently. Inactivation was not suppressed by the addition of soluble starch (data not shown). The results suggest a specific interaction between the enzyme and substrate anomer.

After the NBS oxidation of Zymolyase, the molecular weight of the enzyme was not decreased from the native value measured by 12.5% SDS-PAGE, suggesting that the conditions for oxidizing tryptophan residues were appropriate (data not shown). Further, the modifying reaction by CMC was also suppressed by the addition of laminarin, time- and dose-dependently (Fig. 2). These results suggest that the tryptophan and carboxyl residues are

TABLE I. Effects of Chemical Modifications of Amino Acid Residues on (1→3)-β-D-Glucanase Activity of Zymolyase[a)]

Inactivators	Residues	Remaining activity[b)] (%)
Formaldehyde, sodium borohydride	$-NH_2$ (Lys)	98.7
Acetic anhydride	$-NH_2$ (Lys)	106.1
Succinic anhydride	$-NH_2$ (Lys)	117.3
EDC	–COOH (Asp, Glu)	0.1
CMC	–COOH (Asp, Glu)	12.9
1,2-Cyclohexanedione	Arg	133.3
Sodium tetrathionate[c)]	Cys	96.1
Diethyl pyrocarbonate	His	102.6
Chloramine T	Met	92.0
NBS	Trp	2.6
None	—	100.0

a) Zymolyase 20T was used. The reaction conditions were described in Materials and Methods. *b*) The modified enzyme dissolved in acetate buffer was mixed with 100 μg of laminarin and incubated at 40 °C for 30 min. *c*) Ellman's reagent (DTNB) or PCMB shows a similar value.

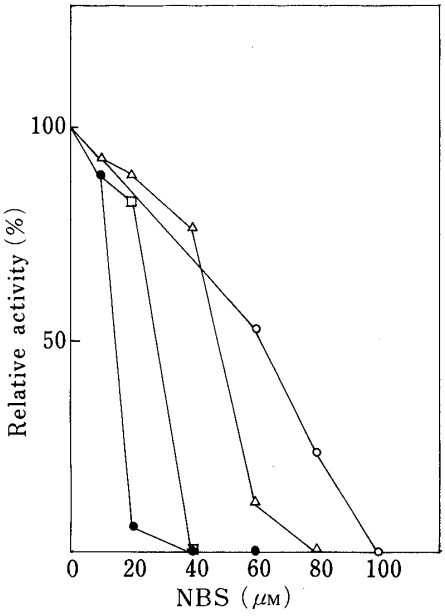

Fig. 1. Inactivation of Zymolyase 20T with NBS and Protection by Laminarin

Laminarin concentration: —●—, 0%; —□—, 0.1%; —△—, 0.5%; —○—, 1.0%.

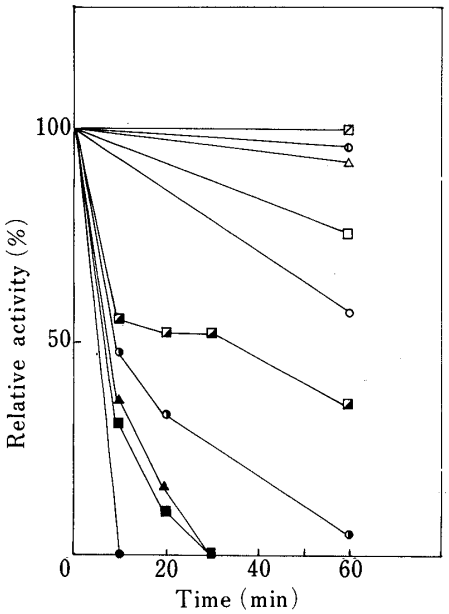

Fig. 2. Inactivation of Zymolyase 20T with CMC and Protection by 0.1% Laminarin

CMC concentration in the absence of laminarin: —●—, 50 mM; —■—, 40 mM; —▲—, 30 mM; —◑—, 20 mM; —◪—, 10 mM.
That in the presence of laminarin: —○—, 50 mM; —□—, 40 mM; —△—, 30 mM; —①—, 20 mM; —◪—, 10 mM.

present in or near the active center of Zymolyase.

UV Spectroscopy of Zymolyase Coupled with Laminaraoligosaccharides

Involvement of tryptophan residues in the active center of Zymolyase was further examined by measuring the difference spectrum with or without laminaraoligosaccharide. The end product formed by Zymolyase from (1→3)-β-D-glucan, pachyman, was thought to be laminarapentaose,[30)] but the reactivity of the enzyme preparation toward laminaraoligosaccharides, laminarabiose to hexaose were examined with Zymolyase 100T. As shown in Fig. 3,

laminaratriose to hexaose were good substrates for Zymolyase 100T. Subsequently, alditols of these oligosaccharides were used as substrates. Laminaratetraitol, pentaitol, and hexaitol were readily hydrolyzable (Fig. 4). Similar results were also obtained by HPLC analysis as follows. Each oligosaccharide was reacted with Zymolyase 100T for 30 min and the reaction mixture was applied to a column of Silica-NH_2. As shown in Fig. 5, (A) laminarahexaose, (B) pentaose and (C) tetraose were hydrolyzed mainly to biose and triose. (D) aminaratriose and (E) biose were poorly hydrolyzable under these conditions. These results indicated that the main end products of this enzyme preparation were laminaratriose and laminarabiose (rather than laminarapentaose). Thus, we examined the ability of laminarabiose and laminaratriitol to inhibit the Zymolyase activity. As shown in Table II, both laminarabiose and laminaratriitol inhibited the (1→3)-β-D-glucanase activity of Zymolyase.

TABLE II. Effect of Laminarabiose or Laminaratriitol on the Activity of Zymolyase

Inhibitor	Ratio[a)]	Remaining activity[b)] (%)
Laminarabiose (DP-2)	50/100	83.6
	70/100	54.5
	90/100	27.2
Laminaratriitol (DP-3-OH)	50/100	73.8
	70/100	58.9
	90/100	32.7
None	—	100.0

a) The values indicate the quantity of inhibitor (μg) *versus* that of substrate (μg). Carboxymethylated curdlan was used as the substrate. *b*) The enzyme (15 μg) dissolved in acetate buffer was mixed with the inhibitor and/or substrate and incubated at 37 °C for 30 min.

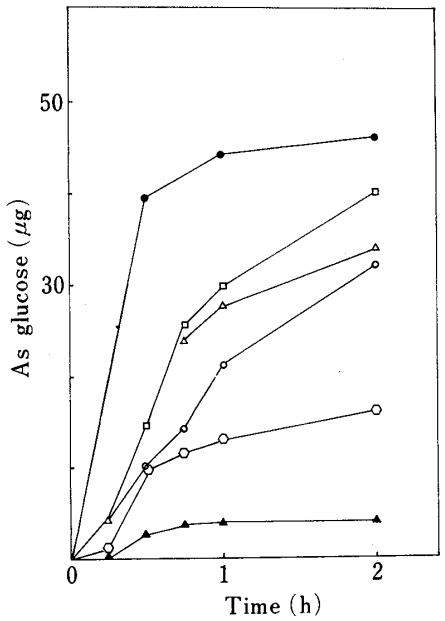

Fig. 3. Time Course of Laminaraoligosaccharides Digestion by Zymolyase 100T

Laminaraoligosaccharide (100 μg) was mixed with 10 μg of Zymolyase 100T in 200 μl of 50 mM acetate buffer (pH 6.0) and the mixture was incubated at 37 °C. The degree of hydrolysis was measured by Somogyi and Nelson's method.
—●—, CM-curdlan; —▲—, G_2, —○—, G_3; —○—, G_4; —△—, G_5; —□—, G_6.

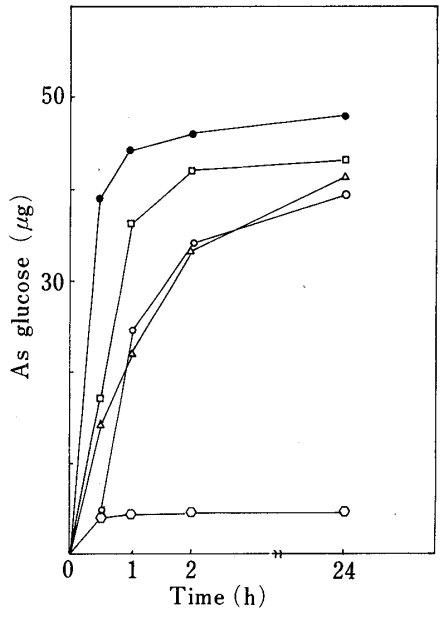

Fig. 4. Time Course of Alditols Digestion by Zymolyase 100T

Alditol (100 μg) was mixed with 10 μg of Zymolyase 100T in 200 μl of 50 mM acetate buffer (pH 6.0) and the mixture was incubated at 37 °C. The degree of hydrolysis was measured by Somogyi and Nelson's method.
—●—, CM-curdlan; —○—, G_3-OH; —○—, G_4-OH; —△—, G_5-OH; —□—, G_6-OH.

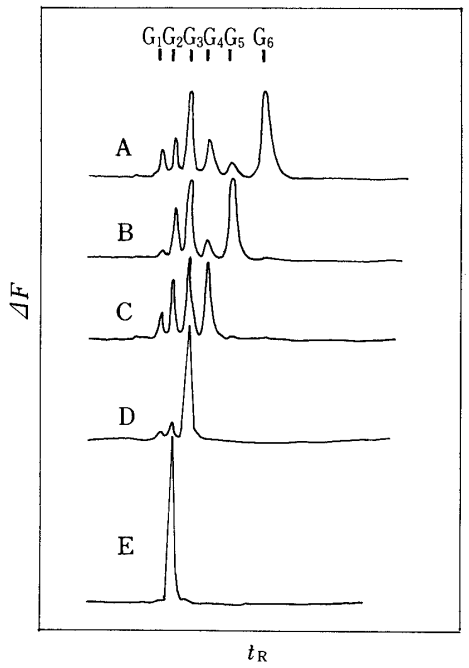

Fig. 5. Elution Profiles of Zymolyase 100T Digests of Laminaraoligosaccharides from a Column of Silica-NH_2

Laminaraoligosaccharide (100 μg) was mixed with 10 μg of Zymolyase 100T in 200 μl of 50 mM acetate buffer (pH 6.0) and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by heating at 100 °C for 3 min, and then a 25 μl aliquot was injected into the column.

A to E: laminarahexaose to laminarabiose.

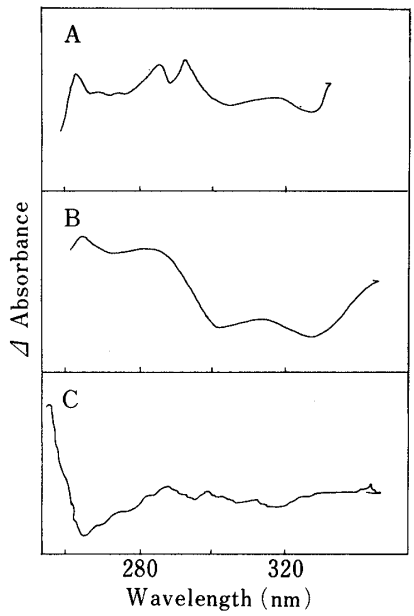

Fig. 6. UV Absorption Difference Spectra of Zymolyase Together with Competitive Inhibitors

(A) The difference spectrum of Zymolyase 100T (2 mg/ml) with or without laminaratriitol (10 mg/ml) at room temperature.

(B) The difference spectrum of NBS-oxidized Zymolyase 100T (2 mg/ml) with or without laminaratriitol (10 mg/ml) at room temperature. NBS-oxidized Zymolyse was prepared by adding NBS (10 mM) to Zymolyase (2 mg/ml) at room temperature.

(C) The difference spectrum of Zymolyase 100T (2 mg/ml) with or without laminarabiose (10 mg/ml) at room temperature.

Figure 6 shows the difference spectrum with and without laminarabiose or laminaratriitol. In the case of laminaratriitol (Fig. 6A), two representative peaks were observed between 280 to 300 nm, suggesting that some tryptophan residues of Zymolyase play a part in substrate (analogue) binding. The difference spectrum of Zymolyase 100T with or without laminarabiose is shown in Fig. 6C. This was not the same as that in the case of laminaratriitol, suggesting different binding characteristics of triitol and biose; tryptophan residues are less important in the binding of biose than that of triitol. It is assumed that the β-conformer of the laminaribiose is required as the minimum structure to stabilize the substrate on the reactive center of the enzyme, because laminaribiose shows mutarotation in aqueous solution. It is also assumed that the binding constant of laminaribiose would be lower than that of laminaritriitol. The difference spectrum of NBS-oxidized Zymolyase 100T with or without laminaratriitol is shwon in Fig. 6B; no representative peaks can be observed. These results suggest that the tryptophan residues are involved in the reactive center of Zymolyase.

Anomeric Specificity of the Reducing End of the Zymolyase Digest

α-Amylase releases α-oligosaccharides (such as α-maltose) and β-amylase releases β-oligosaccharides. It is known that the phenomenon reflects the characteristics of enzyme-substrate binding. Thus, to clarify the anomeric specificity of the reducing end of Zymolyase digest, the optical rotation value was measured immediately and compared with the values after mutarotation. Carboxymethylated curdlan was used as the substrate because of its

solubility in water. Figure 7A shows the time course of the appearance of reducing power in the reaction mixture, and Fig. 8A shows the elution profiles of the reaction products for 30 min from Bio-gel P-2. From the results of reducing power measurements, more than a half was found to be degraded during 1 h. The elution profiles of the digests from Toyopearl HW 55F show the disappearance of macromolecules within a short time, and also suggest that the hydrolysis of the enzyme may be endogenous (data not shown). The elution profiles from Bio-gel P-2 shows that the major products were laminarabiose and triose. Further, Fig. 5 also suggests that the main Zymolyase products from oligosaccharides are laminarabiose and -triose within a short time.

Optical rotation values during the reaction described above were measured and are shown in Fig. 7B. The values quickly decreased (within 1 h) and then increased gradually. This phenomenon can be explained in terms of two factors: one is the optical rotation of the reducing end unit, and the other is the conformational change from helix to random coil of the glucan segments. Generally, (1→3)-β-D-glucan having helical conformation possesses positively shifted optical rotation values,[31] which decrease to a constant value after conformational change to the random coil which is usually attained by ① the addition of 0 to 0.2 N sodium hydroxide or ② degradation to small molecular weight fragments. The 1st step of decreasing optical rotation would result from the helix to random coil transition of the glucan chain because of the endogenous digestion of polymer chains. The lowest value (at 60 min)

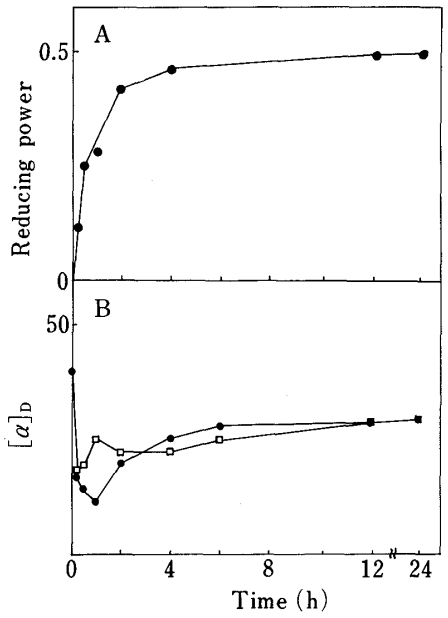

Fig. 7. Time Course of the Differences of the Reducing Power (as Glc) and the Optical Rotation ($[\alpha]_D$) during Digestion of CM-Curdlan with Zymolyase 100T

CM-curdlan (4 mg/ml) was digested with Zymolyase 100T (3 mg/ml) at 37 °C. At appropriate time intervals, the reducing power of the reaction mixture, and the optical rotation before (immediately) and after (100 °C for 3 min) mutarotation were measured.

(A) Enzymic hydrolysis as the appearance of the reducing power.

(B) Specific rotation: —●—, at once; —□—, after mutarotation.

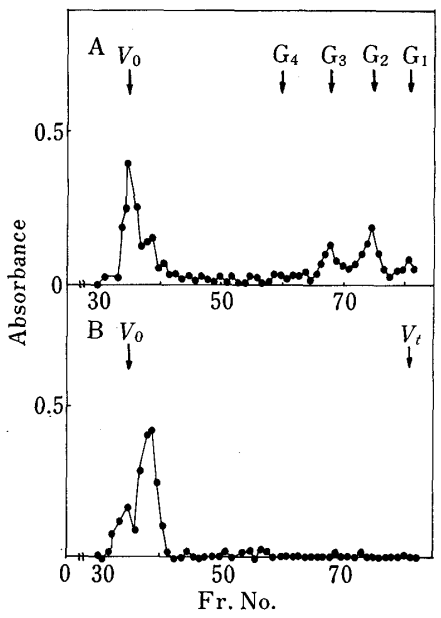

Fig. 8. Elution Profiles of the Zymolyase Digest of CM-Curdlan from a Column of Bio-Gel P-2 (1.7 × 137.5 cm) Eluted with Distilled Water

(A) CM-curdlan (4 mg/ml) in 50 mM acetate buffer (pH 6.0) was digested with Zymolyase 100T for 30 min. The reaction was stopped by boiling at 100 °C for 3 min, and the mixture was applied on the top of the column. The elution profile was monitored by the phenol–H_2SO_4 method. Arrows indicate void volume and the elution volumes of laminaraoligosaccharides (G_4, G_3, G_2, G_1) respectively.

(B) Elution profile of DEAE-Sephadex adsorbed fraction. The procedures were similar to those in the case of Fig. 8A. Fractions 33 to 36 (void) and fractions 38 to 42 (main) were used for further experiments.

was quite similar to the value of random-coiled CM-curdlan, which was obtained by measurement in 0.25 N sodium hydroxide ($[\alpha]_D$: +500 in water; +240 in 0.1 N NaOH; +76 in 0.2 N NaOH; +22 in 0.25 N NaOH). The second step of increasing optical rotation (60 min to 6 h) would be attributable to the appearance of reducing end groups. Comparing the values after mutarotation, each value obtained immediately after the reaction tends to be negatively shifted. These facts suggest that the anomeric specificity of the reducing end group is β.

Characterization of the Carboxymethylated Oligosaccharide Produced from CM-Curdlan by Zymolyase Digestion

The structure of the enzymic products provides information about the mode of action. Thus, structural elucidation of the products was performed. Figure 8A shows the elution profiles of the products from a column of Bio-gel P-2. Even after a 24 h reaction, peaks eluted near the void volume were still observed (data not shown). Figure 9 shows the elution profile of the enzymic digest from a column of DEAE-Sepadex A-25. The acidic fraction, which contained the carboxymethylated oligosaccharides, was applied to a column of Bio-gel P-2. As shown in Fig. 8B, acidic oligosaccharides were eluted near the void volume. The main oligosaccharide showed a single spot on paper chromatography (data not shown). After reduction with sodium borohydride, the carbohydrate content of the acidic oligosaccharide (main) was decreased to 53%, suggesting the acidic oligosaccharide to be a dimer. It is not surprising that the acidic laminarabiose (carboxymethylated) was eluted faster (fr. 40) than natural laminaribiose (fr. 75), because the carboxyl group would repel the gel matrix. After hydrolysis of the reduced oligosaccharide with 1 N trifluoroacetic acid at 100 °C for 5 h, the

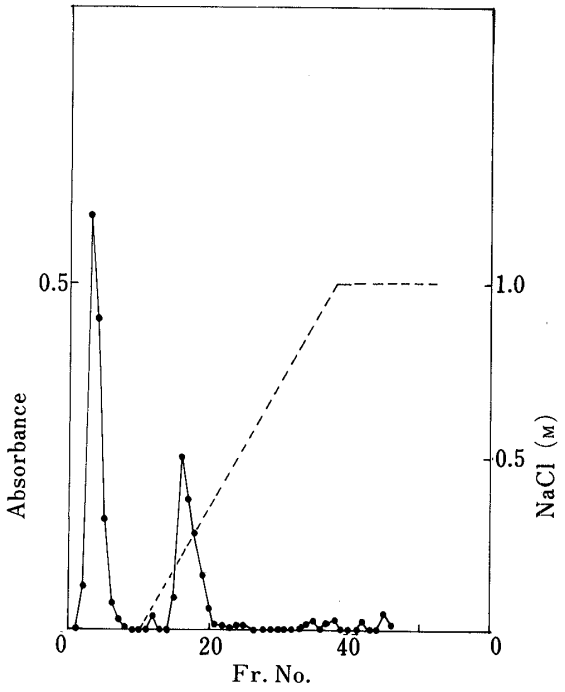

Fig. 9. Elution Profiles of the Zymolyase Digest of CM-Curdlan from a Column of DEAE-Sephadex A-25 (Cl^-) (7 ml)

The digest (10 mg) dissolved in distilled water was applied to a column of DEAE-Sephadex A-25 (Cl^-) and eluted with (1) distilled water and (2) 0 to 1 M sodium chloride. Fractions of 2 ml each were collected and fractions 15 to 19 were concentrated. The residue was applied to a column of Bio-gel P-2 (Fig. 12). The elution profile was monitored by the phenol–H_2SO_4 method.

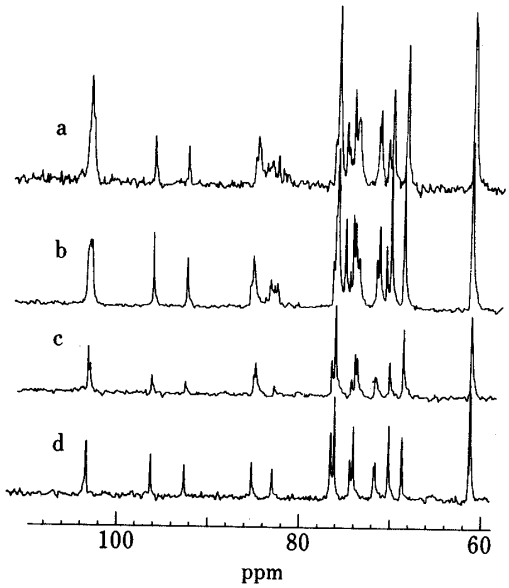

Fig. 10. ^{13}C-NMR Spectra in H_2O of the Acidic Fraction of Zymolyase-Digested CM-Curdlan

(a) void; (b) main.

carbohydrate portion was eluted to obtain passed through (62%) and adsorbed (38%) fractions. These results suggest that the carboxymethylation site of the disaccharide is not uniform. Figure 10 shows the ^{13}C-NMR spectrum of the acidic disaccharide, and is quite similar to that of the laminarabiose. Therefore, this is the carboxymethylated disaccharide and th carboxymethyl group was bound to either reducing or non-reducing end glucosyl units. The heterogeneity would result from the heterogeneity of the carboxymethylation sites in the parent glucan. These findings suggest that the carboxymethyl group interfered with the enzymic activity only a little.

Discussion

The data presented in this paper show that (1) the active center of Zymolyase contains essential Trp and carboxyl residues, (2) the anomeric specificity of the reducing end of Zymolyase digest is β, and (3) CM-curdlan and laminaraoligosaccharides digested with Zymolyase preparation produced firstly laminaratriose and -biose and small oligosaccharides and these were gradually degraded into laminarabiose and glucose.

There are many glucanohydrolases, in which tryptophan and carboxyl group are important for enzymic activity.[32] Zymolyase presumably has a similar mechanism of action to those of other glucanohydrolases except for the substrate binding structure. Amylases can be separated into several groups, such as *endo*- and *exo*- types, and α- and β-types. Zymolyase is an *endo*-type glucanase. The action pattern of Zymolyase is similar to that of α-amylase, in which the anomeric conformation of the reducing end is maintained.

(1→3)-β-Glucans are widely distributed in microorganisms and some of them are important natural polymers, which may be useful immunomodulating anticancer agents. The glucan must interact with other biological polymers, such as proteins, lipids, and cell membrane components, when it shows its biological activities, and it is important to elucidate these interactions. (1→3)-β-Glucanase may be regarded as a representative model protein which interacts with (1→3)-β-D-glucans. The precise characterization of the hydrolytic mechanism of (1→3)-β-D-glucanase is also important in the field of fermentation. Unfortunately, little information is yet available concerning the sequence and the action mechanism of the enzymes, although other kinds of hydrolytic enzymes such as amylase, lysozyme and cellulase have been quite well characterized. Recently, Doi and Doi cloned the glucanase gene from *Arthrobacter* sp. [33] It should be possible to establish the action mechanism of (1→3)-β-D-glucanase precisely in near the future.

Futher work on the active center, subsite structure, and conformational changes of Zymolyase and the substrate are in progress.

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