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Article

Purification and characterization of an endo-1,3-β-glucanase from *Arthrobacter* sp.

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An endo-1,3-β-glucanase from *Arthrobacter* sp. was purified by ammonium sulfate precipitation, anion-exchange and gel-filtration chromatographies. SDS/PAGE and gel-filtration chromatography suggested a monomer enzyme with molecular mass of 32,500. N-terminal sequence for 10 residues was APGDLLWSDE. Stable and optimum pHs were 5 to 8 and 6.5, respectively. Optimum temperature was 55℃, however, above 50℃ its activity was lost rapidly. The Michaelis constant K_m for laminaritetraose, laminaripentaose, laminarihexaose, laminariheptaose, and laminarin were 0.12, 0.11, 0.067, 0.066mM, and 0.16mg/ml, respectively. Curdlan and lichenan were also hydrolyzed. The purified enzyme did not give product by digestion with endoglycosidase H (*Streptomyces griseus*), indicating that the carbohydrate moiety was little consisted in the enzyme protein, if any, with N-linked chain or other type linkage. It was suggested that the enzyme belong to the family 16 of glucosyl hydrolase from the properties as above mentioned.

keywords: Endo-1,3-β-glucanase; *Arthrobacter* sp.; Purification; Molecular/Enzymatic properties

Introduction

β-1,3-glucanases are widely distributed among bacteria, fungi and higher plants, and their physiological roles, such as involvement in cell differentiation and defense against fungal pathogens have been suggested in plants.[1-3] In fungi, β-1,3-glucanases seem to have different functions in morphogenetic processes, in β-glucan mobilization and in fungal pathogen-plant interactions. Recently, the first metazoan β-1,3-glucanase, which may be involved in the early embryogenesis, has been described by Gueguen *et al*.[1] In these studies, β-1,3-glucanases have interesting and important physiological roles, and practical uses. For example, the enzymes could be convenient for the degradation of cell wall in fungi, yeasts, and higher plants.

The application of 1,3-β-glucanases is also well established in preparation of protoplasts, [4,5] in analysis of fungal cell wall structures, in clarifying of slimy must, in degradation of barley β-glucan that accumulates during the brewing process, and in treatment of fungal diseases. Furthermore, these glucanases are important tools for use in yeast biotechnological processes such as cell fusion, transformation, and extraction of protein products.

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Some but not all of this class of enzymes are also found to be capable of inducing lyses of viable yeast cells,[4,6] degradation of fungal mycelia (lytic activity), utilization as biomass resources of yeast cell walls wasted by brewing industries, or control of pathogenic fungi infecting plants and rotting foods during storage.[7]

On the other hand, β-1,3(1,6)-glucans are major components in cell wall of yeasts and fungi. In most yeast, especially *Saccharomyces*, the cell wall polysaccharide glucan is consisted with predominantly β -1,3-linked backbone having some branch via β -1,6-linkages.[4,6,8,9] Crude preparations of yeast β-glucans have been known for 40 years to stimulate animal defense mechanisms. It is now generally believed that the active component in these preparations is β-1,3-glucans.[10] β-glucan is known to possess the antimicrobial and antitumorial activities by enhancing the host immune function, and activating macrophages, neutrophils and NK cells.[11]

In recent years, the function of glucan found in baker's yeast cell wall has also been widely noticed for its action of immune stimulation; that is, by utilizing β- glucan of the yeast cell wall to stimulate host immune function for antimicrobe, antitumor and so on. Meanwhile, following these studies, 1,3-β-glucanases are also thought to play more and more important roles not only in food production, but also in more effective utilization of resource what not used and wasted. Thus, it is supposed to be interesting to make use of β-glucanases and β-glucans to produce functional oligomers for bioactive and immune studies.[12] For this purpose, it is necessary to make clear of the molecular and reactionary properties of endo-1,3-β-glucanases, as a first step, here we are concerned with purification and characterization of an endo-1,3- β-glucanases from *Arthrobacter* sp. In fact, several enzymes closely related to food production have been isolated from *Arthrobacter* sp., such as maltooligosyl trehalose synthase, maltooligosyl trehalose trehalohydrolase, glucoseisomerase, and lipase.[13-15] Some kinds of endo-1,3-β-glucanases, such as *Arthrobacter* sp. strain YCWD3(55k),[16] *Oerskovia xanthineolytica* LL G109 (*Cellulomonas cellulans*)(βgll , 27k and βgll A, about 30k),[4,17] and *Oerskovia xanthineolytica* TK-1(40k)[18] have also been obtained from the genus of *Arthrobacter (Oerskovia).* The molecular mass range of these purified glucanases from *Arthrobacter (Oerskovia)* was found to be 12k to 57k.[6,16,19-22]

Based on the action patterns of glucanase-catalyzed hydrolysis reactions, β-1,3-glucanases are classified into the exo- β-1,3-glucanases (β-1,3-glucan glucanohydrolase (EC3.2.1.58)) and the endo-β-1,3-glucanases (β-1,3-glucan glucanohydrolase (EC3.2.1.6 and EC3.2.1.39)).[1,22] By using an enzyme preparation Tunicase® , which is a product from *Arthrobacter* sp., we have purified an endo-1,3-β-glucanase and found that molecular properties of the purified enzyme were different from those of the enzyme samples reported about *Arthrobacter (Oerskovia).*[4,6,16-18,23-30]

In this paper, the experimental results of purification and molecular properties of the enzyme preparation obtained from *Arthrobacter* sp. have been described.

Materials

Crude preparation Tunicase® was purchased from Daiwa Kasei Co., Japan, for purification of the enzyme endo-1,3-β-glucanase. All of the other materials, saccharides, enzymes, and chemicals were purchased as follows that were commercially available and guaranteed products and used without further purification: Soluble laminarin from *Eisenia bicyclis* (Nacalai Tesque, Inc.,Kyoto, Japan), laminari-oligosaccharides from *Poria cocos* (Seikagaku Kogyo Co., Tokyo, Japan), lichenan from *Cetraria islandica* (Sigma Chemical Co., USA), curdlan from *Alcaligenes faecalis*, pustlan from *Umbilicaria papullosa*, carboxymethyl cellulose (CMC), and chitin from crab shells (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The following materials were also the commercial

products: DEAE Toyopearl 650M (Tosoh Co., Tokyo, Japan), Sephacryl S-200 HR (Amersham Pharmacia Biotech Co., USA), the kit of the low-range molecular standard proteins and enzymes for molecular mass estimation (Bio-Rad Lab, USA), endoglycosidase H (abbreviated to endo-H, endo-β-N-acetylgluco-saminidase H, EC3.2.1. 96, mannosyl glyco-protein-1,4- N- acetamidodeoxy-β-Dglycohydrolase) from *Streptomyces griseus* (Seikagaku Kogyo Co., Tokyo, Japan).

Enzyme activity assays

The enzyme-catalyzed reactions were measured with 2.5mg/ml soluble laminarin as a substrate in a 40mM potassium phosphate buffer, pH 7.0, at 30℃, for 15 min to obtain the reaction-time course. The reducing power caused by the enzymatic reaction from the substrate was determined by Somogyi-Nelson methods,[31] of which absorbance at 600nm was measured by a Shimadzu UV-1600 Spectrophotometer. One unit of enzyme was defined as the amount of enzyme, which liberates 1μ mol of reducing sugar equivalent to glucose per 1min under the standard assay conditions.

Enzyme purification

The crude preparation Tunicase® was dissolved in a 10mM phosphate buffer pH7.0 (abbreviated to phosphate buffer, below) at 5% (w/v), mixed with $(NH_4)_2SO_4$ up to 65% saturation with stirring, and stayed for overnight at 4℃. After centrifugation at 20,000×*g* for 30 min at 4℃, the precipitation was recovered, dissolved with phosphate buffer, and dialyzed against the same buffer. The sample dialyzed was loaded on the anion-exchange chromatography, DEAE Toyopearl 650M column (φ 2.5×35cm long) and eluted by the stepwise gradient with 0, 50, 100, 200, 400mM NaCl/phosphate buffer solutions at flow rate about 1.67ml/min. Fractions having laminarinase activity were pooled and concentrated at 2.5-3.0 kgf/cm^2 with the Amicon TAITEC ultra-filter and Tomoegawa Paper-Advantec UP-20 membrane (exclusion limit 20,000, φ 62mm). All operations were carried out at 4℃. The concentrated sample was loaded to the gel filtration chromatography, Sephacryl S-200 HR column (φ 2.5×120cm long) and eluted with a 300mM NaCl/phosphate buffer solution at flow rate about 0.28ml/min. Fractions having laminarinase activity were pooled and concentrated by the same procedures as described above.

Protein concentration was determined by the method of Bradford [32] for all protein samples in the purified steps. When the kinetic examination was carried out with the purified sample, concentration of the enzyme was determined by methods of Lowry [33] and of Bradford simultaneously, for measurement of exact quantity. In the both assays, bovine serum albumin was used as the standard.

For the SDS-Polyacrylamide gel-electrophoresis (SDS/PAGE), protein samples were mixed with equal volume of a 125mM Tris-HCl buffer pH 6.8, containing 4% (w/v) SDS, 10% (v/v) β -mercaptoethanol, 20% (v/v) glycerol, and 0.1% bromophenol blue (BPB). After the mixture was incubated in a Dry Thermo Unit DTU-1C (TAITEC) at 90℃ for 3min, the samples were run on the gel (composing of 10% or 12.5% separating gel pH8.8 and 4% stacking gel) at 20mA as described by Laemmli.[34] Protein bands were stained with Coomassie brilliant blue (CBB). A kit of the low-range molecular standard-proteins/enzymes was adopted for determination of molecular weight.

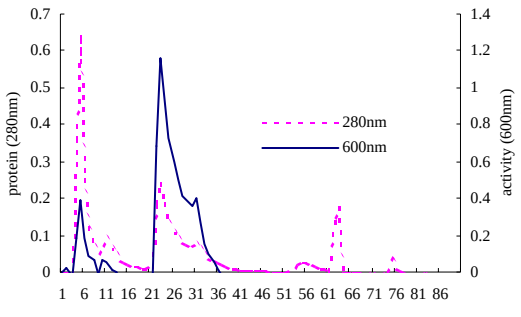

Fig.1. Anion-exchange chromatography on DEAE Toyopearl 650M;each fraction was 20mL, eluted by stepwise gradient of NaCl concentration (0, 50, 100, 200, 400mM).

Table 1
Summary of purification of the endo-1,3- β -glucanase from *Arthrobacter* sp.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor (fold)
5% Tunicase® solution	188	50.0	3.76	1.00
ammonium sulfate precipitation	135	28.2	4.79	1.27
DEAE Toyopearl 650M anion-exchange	111	11.5	9.65	2.57
Sephacryl S-200 HR gel-filtration	26.0	2.06	12.6	3.35

Sequencing of N-terminal amino acids

N-terminal amino-acid sequence of the purified enzyme was analyzed for 10 residues after the SDS/PAGE without staining and with blotting to PVDF membrane for 1h at 100mA. The blotted protein sample was stained by CBB and analyzed by the Shimadzu Model PPSQ-219 amino acid sequencer composed of automated Edman degradation system; Shimadzu LC-10AS Liquid Chromatography and Shimadzu UV-VIS detector Model SPD-10A.

Enzyme kinetic analysis

Initial velocities v of the enzyme-catalyzed hydrolysis reaction were analyzed under the different concentrations $[S]_0$ of laminari-oligosaccharides (G4-G7, concentration range in 0.04-1.0mM) and soluble laminarin (range in 0.3125-5.0mg/ml) as substrates in a 40mM phosphate buffer pH 7.0 at 25℃. The kinetic parameters, Michaelis-Menten constants K_m and molar activity k_0, with the value of standard deviation were obtained from the plots $[S]_0/v$-$[S]_0$ using an NEC PC-9801 personal computer and Bio-graph software (Kyoto Software Co., Japan) for non-linear least-squares method.

Analysis of hydrolyzed products

Products from the enzymatic degradation were analyzed by thin-layer chromatography (TLC) techniques using Silicagel 70 F_{254} Plate (Wako Co., Japan) and ethyl acetate/acetic acid/water (2:2:1, by vol.) as solvent at about 30-40℃. Oligosaccharides on the plate were visualized with the orcinol ferric chloride spray reagent (Sigma Co., USA) at 100℃ for 15 min.

Hydrolysis reaction of saccharides

Laminari-oligosaccharides G2-G7 at a concentration 0.5mM, and chitin, laminarin, lichenan, curdlan, pustlan and CMC at a concentration 1 mg/ml were examined for the enzyme-catalyzed hydrolysis

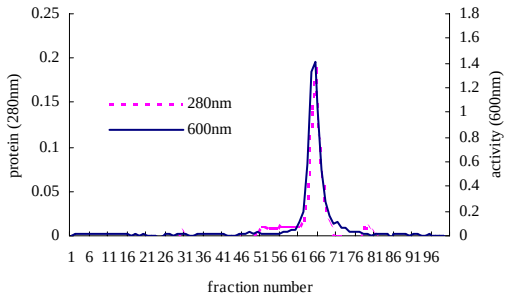

Fig.2. Gel-filtration chromatography on Sephacryl S-200 HR; each fraction was 7.0mL, eluted by 0.3M NaCl/phosphate buffer.

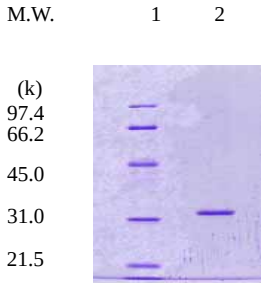

Fig.3. SDS/PAGE of the purified enzyme; lane1: molecular mass marker; lane2: the purified enzyme, MW was 32.5k.

Table 2
Kinetic parameters of the endo-1,3- β -glucanase from *Arthrobacter* sp.[a]

Substrate	K_m (mM or mg/ml)	k_0 (1/sec)	k_0/K_m (1/sec·mM or ml/sec·mg)
laminarin	0.157mg/ml	3.5	22.3 ml/sec·mg
laminariheptaose	0.066	19	288
laminarihexaose	0.067	30	442
laminaripentaose	0.107	43	402
laminaritetarose	0.120	47	392
laminaritriose	n.d.	n.d.	n.d.
laminaribiose	n.d.	n.d.	n.d.

[a] Reaction conditions: enzyme concentration was 1.5×10^{-8}M (by the method of Lowry), at pH 7.0, with 40mM potassium phosphate buffer for 10 min, 25℃. n.d. =not determined.

reaction as a substrate under the experimental conditions described above. Prior to this test for the substrate specificity, lichenan, curdlan, pustlan and chitin were treated with boiling water at about 95℃ for 15min to make a gel state, then cooled to room temperature. On the other hand, for the product analysis the enzyme-catalyzed hydrolysis reactions for laminarin, laminari-oligosaccharides, curdlan, and lichenan were carried out in a 5-8mM phosphate buffer pH 7.0 at 25℃ with the enzyme concentration of 1.5×10^{-7} M. The reaction was stopped in the boiling water at 15, 30, 60, 90, and 120min after the beginning of the reaction.

Effects of pH and temperature on the enzyme-catalyzed reaction and enzyme stability

The optimal pH of the purified enzyme was determined by running the standard enzymatic assay at 30℃ for 10min in a 30mM sodium pyrophosphate-phosphoric acid buffer, of which pH ranges are at 3.0-10.0. Effect of pH on stability of the enzyme was analyzed at pH 4.0 to 10.0 with the pyrophosphate buffer. After incubation at 30℃ for 2h, the residual enzymatic activity was measured by the standard kinetic assay procedures at 30℃.

For analysis of the optimal temperature, the enzyme-catalyzed hydrolysis reactions were carried out by running the standard kinetic assay in a 40mM potassium phosphate buffer (pH 7.0) at 10 to 90℃ for 10 min. Thermo-stability was evaluated by the residual activity after incubation of the enzyme at 10, 20, 30, 40, 45, 50, 55, 60, 70℃ and 80℃ for 2h.

Endoglycosidase H treatment

De-N-glycosylation reaction for the purified enzyme molecule was carried out with a glycochain-scission enzyme endo-H: 5μl of 1U/ml endo-H commercial preparation was dissolved into 250μl of a buffer solution, in which 10μl of 100mM phenylmethylsulfonyl fluoride (PMSF) and 3.3μl of 3mg/ml Pepstatin A were mixed in 500ml of 100mM citrate/phosphate buffer pH5.0. Endo-H solution 50ml was added to equal volume of the purified enzyme solution and was incubated at 37℃ for 22h. Then, the final reaction mixture was analyzed by the SDS/PAGE to detect the molecular mass after endo-H treatment.

Results and discussion

Purification and N-terminal amino acid sequence analysis

5%Tunicase®, was precipitated with 65%-saturated ammonium sulfate solution, followed by centrifugation, dialysis, and finally anion-exchange and gel-filtration chromatographies. In every purification steps, the enzyme activities and protein quantities of all samples are summarized in Table 1.

Fractions eluted from the anion-exchange column are shown in Fig.1, indicating that the fractions from No.21 to 31 were in the high level of the hydrolytic enzyme activity. Protein samples in these fractions were pooled and concentrated to about 5ml using the ultra-filtration apparatus. Subsequently, the sample concentrated was loaded on a column with Sephacryl S-200 HR for the gel-filtration and its chromatogram was obtained as illustrated in Fig.2, clearly indicating that a single peak of protein is consistent with that of hydrolytic enzyme activity. The protein samples in the fractions from No.61 to 67 were collected and concentrated by using the ultra-filtration apparatus. Thus obtained enzyme preparation was analyzed by the SDS/PAGE as shown in Fig.3, clearly indicating a single band of protein, which was, a pure preparation of the enzyme obtained by the purification procedures employed. N-terminal sequence of amino acids was analyzed for the initial 10 residues of the pure enzyme preparation by using a sequence analyzer and found to be APGDLLWSDE, namely Ala-Pro-Gly-Asp-Leu-Leu-Trp-Ser-Asp-Glu-, of which sequence is the same as those of 1) *Oerskovia xanthineolytica* LL G109 (*Cellulomonas cellulans*), a 27k enzyme protein βgIII reported by Parrado et al.,[4] and 2) an *Oerskovia xanthineolytica* TK-1 glucanase, a 40k enzyme protein reported by Saeki et al.[18] This amino acid sequence, however, was different from those of 1) an *Arthrobacter* sp. (strain YCWD3) glucanase, submitted (Dec, 1993) to the GenBank databases (SWISS-PROT, accession number Q59146) by Watanabe et al. and 2) an *Oerskovia xanthineolytica* glucanase, reported by Shen et al.[7] Moreover, the N-terminal amino acid sequence of a glucanase from *Arthrobacter* sp. strain YCWD3, which is coincided with *Arthrobacter citreus*, is almost all the same as that of another glucanase from *Oerskovia xanthineolytica*, MW of these enzymes are 5,5000 and 5,7000, respectively.

Anyway, the purified enzyme obtained here from Tunicase® is called ENG in this study.

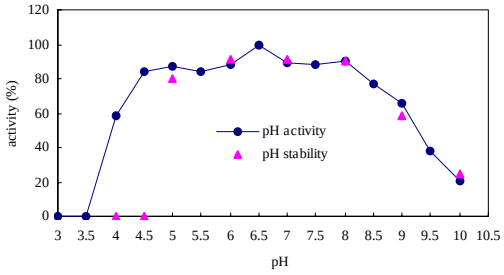

Fig.4. Effect of pH on activity and stablity of the purified enzyme (30℃).

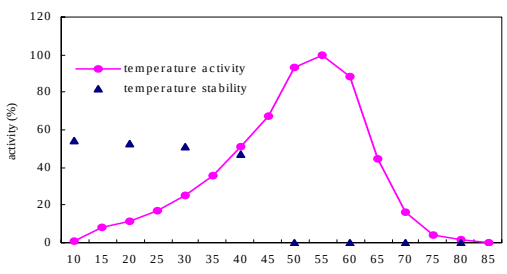

Fig.5. Effect of temperature on activity and stability of the purified enzyme (pH7.0).

Proteochemical and molecular properties of the enzyme ENG

Molecular mass 32,500 was evaluated for the ENG obtained here using the SDS/PAGE procedures as shown in Fig.3. Moreover, another experimental trial was carried out as follows: The ENG obtained was examined using the gel-filtration chromatography with Sepahcryl S-200 HR. Based on the results, its molecular mass of the native state for the ENG was roughly evaluated to be 21,700, suggesting that the ENG is a monomer protein. In this study, it was

Table 3
Substrate specificity of the endo-1,3- β -glucanase from *Arthrobacter* sp.[a]

Substrate	Linkage type	Relative rate (%)
laminarin	β -1,3	100
curdlan	β -1,3	53
lichenan	β -1,3-1,4	64
CMC	β -1,4	0
chitin	β -1,4	0
pustlan	β -1,6	0

[a] Reaction conditions: all substrates concentration was 1mg/ml, curdlan,lichenan, pustlan, chitin were boiled (about 95℃) for 15 min before the assay, enzyme concentration was 1.5×10^{-8}M (by the method of Lowry), at pH 7.0, 25℃, with 40mM potassium phosphate buffer for 10 min.

concluded that the ENG is composed of a monomer having the molecular mass of 32,500.

The ENG was treated with endo-H, at 37℃ in the phosphate buffer pH4.5 for 22h and the reaction mixture was analyzed by the SDS/PAGE procedures. The protein band of the reaction mixture on SDS/PAGE
chromatogram was compared with that of the reference protein, which was the ENG not treated with endo-H. No difference in these protein bands was found between the sample and the reference, suggesting that the ENG could be little digested (the data not shown) by the endo-H-catalyzed reaction. The endo-H enzyme employed has a characteristic property on the substrate specificity, of which catalyzed-reaction rate is very slow for the 3-continuous mannose sequence in the substrate glycochain molecule. In this stage of our investigation, we cannot deny this possibility for the glycochain attached to the ENG obtained. The saccharide attached to ENG was supposed to be not exist, because, in the present studies, the presence of saccharide linkage was not known in endo-1,3-β-glucanase from procaryote(bacteria).

Effects of pH on the ENG-catalyzed reaction and on the molecular stability of ENG were examined by the steady-state kinetic measurement for the substrate

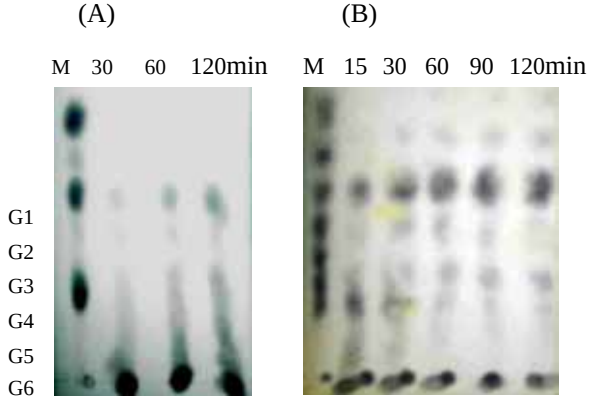

Fig.6. TLC of reaction products obtained from laminarin and lichenan hydrolyzed by ENG.
(A) laminarin(5mg/ml), (B) lichenan(2mg/ml). Reaction conditions:ENG1.5 × 10^{-7}M, phosphate buffer 5-8mM, pH7.0, 25℃. M=maker.

laminarin under various pHs as the results are shown in Fig.4, where the hydrolytic reaction rates are presented by a relative value (%). The ENG-catalyzed reactions were observed to maximum at pH 6.5, thus 6.5 is concluded to the optimum pH of the enzyme ENG. More than 80% activity of the enzyme-catalyzed reaction was observed at pH 5.0-8.0 at 30℃ for 2h as shown in Fig.4. Thus, stable pH of the ENG-catalyzed reaction is concluded to be in the range of 5.0 to 8.0. Effects of temperature on the enzyme-catalyzed reaction and on the stability of the enzyme were observed for the substrate laminarin by the steady-state kinetics under various temperatures in the phosphate buffer pH 7.0, as the results are shown in Fig.5. At 55℃, the ENG-catalyzed reaction exhibited in maximum, and the ENG molecule was stable at less than 40℃ and was very unstable at higher than 50℃.

Kinetic properties of the ENG-catalyzed reactions

The steady-state kinetics of the ENG-catalyzed reaction was carried out for laminarin, lichenan, curdlan, and laminari-oligosaccharides Gn (degree of polymerization n=2-7) as substrates at the enzyme concentration 1.5×10^{-8}M. Under the experimental conditions employed, it was found that all the sacchari- des examined were hydrolyzed by the ENG-catalyzed reaction. The reactions with laminarin and laminarioligosaccharides were confirmed to be obeyed the Michaelis-Menten kinetics over the concentration range 0.04-1.0mM for laminari-oligosaccharides (G4-G7), and 0.31-5.0mg/ml for soluble laminarin. The steady-state kinetic parameters, the Michaelis constant K_m and molar activity k_0 values were evaluated for the substrate laminari-oligosaccharides and laminarin as summarized in Table 2, where the k_0/K_m values were obtained with K_m and k_0. Laminarihexaose G6 has the largest k_0/K_m, thus it should be concluded that G6 is the best oligosaccahride substrate for the ENG-catalysed reaction. G3 and G2 were examined for a substrate and were found not to be hydrolyzed by ENG at any degree of hydrolysis, even though at

higher concentration of enzyme (examined by 1.5×10^{-7}M) for 2h under the experimental conditions employed.

Substrate specificity of ENG

The substrate specificity was analyzed by the steadystate kinetic procedures for the polysaccharides with β-1,3-, -1,4-, and -1,6-glucans as the results are summarized in Table 3, where the relative values are represented instead of the kinetic parameters. These data show that laminarin is the best in the substrate polysaccharide examined, and lichenan and curdlan are good substrates for ENG. The ENG-catalyzed reaction was examined for pustlan, carboxymethyl cellulose (CMC), and chitin as substrates, however, the enzymatic reactions were little observed for these polysaccharides (Table 3). According to the TLC analysis, major saccharides, which are initially produced by the ENG-catalyzed reaction for the substrate laminarin, lichenan, were found to be G3 and G6, and G3, respectively, as shown in Fig.6. On the other hand, G2 was found to produce mainly from curdlan (data was not shown). These findings suggest that β-1,3- and also β -1,3-1,4-glucosidic bonds are susceptible to hydrolysis with the enzyme ENG. The TLC analysis of hydrolyzed products from laminali-oligosaccharides (G2 to G7) was also performed. The major products for every substrates were detected as G3 and G2. The TLC pattern, in case of G7, was shown in Fig.7 as an example.

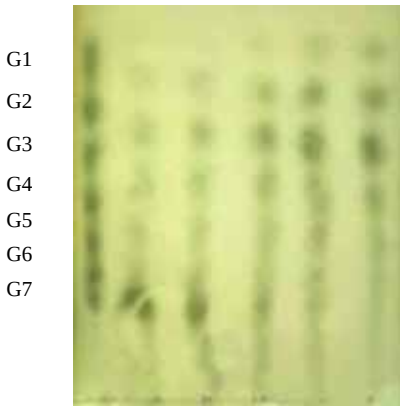

Fig.7 TLC of products obtained from laminariheptaose hydrolyzed by ENG.
Reaction conditions: laminariheptaose 10mM, the others were same as described Fig.6. M=maker.

Meanwhile, when G6 and G7 were employed as substrates for the ENG-catalyzed reaction, the laminari-oligosaccharides products that degree of polymerization larger than 6 and 7 were also confirmed in the TLC chromatograms. This finding suggests that transglucosylation reaction would be occurred in the ENG-catalyzed reaction.

These findings on the steady-state kinetics and the product analysis give the action pattern of the ENG-catalyzed reaction, which is endo-type.

Identification of the enzyme ENG obtained

A yeast-lytic actinomycete *Oerskovia xanthineolytica* is now considered a subjective synonym of C*ellulomonas cellulans* and previously known as *Arthrobacter luteus*.[17] According to the previous descriptions, the N-terminal amino acid sequence of the initial 10 residues of the ENG is just the same as that of *Oerskovia xanthineolytica* LL G109 glucanase (βglⅡ), [4] not only the amino acid sequence but also the other properties are very similar in these two enzymes. For example, lichenan is a kind of suitable substrate for β -1,3-glucanase as well as β-(1,3-1,4)-glucanase-cata- lyzed reactions. However, molecular mass, optimal temperature, and temperature stability of the *Oerskovia* enzyme is very different from those of ENG. The molecular mass of βgl Ⅱ is about 27,000 as determined by SDS/PAGE, the optimal temperature is 65℃, and the enzyme is stable at 55-75℃ at least 80% of its optimal activity but very unstable above 75℃. Thus, the ENG is different from the βgl Ⅱ , that obtained from *Oerskovia xanthineolytica* LL G109. N-terminal sequence for the initial 10 amino acid residues of the ENG obtained here is also the same as that of *Oerskovia xanthineolytica* TK-1 glucanase, but the later MW is 40,000. Thus, those glucanases reported by several researchers have just different proteochemical and enzmymatic properties, MW, optimum and stable pH,

optimum and stable temperature, were different from the ENG obtained here.

The results described above suggest that an intact enzyme molecule could be built as a part with the enzymes from *Oerskovia xanthineolytica* LL G109 (βgl Ⅱ MW 27,000), and from *Arthrobacter* sp. (MW32,500), which is studied here. Glucanase from *Oerskovia xanthineolytica* TK-1 could be regarded as the intact enzyme, thus the other glucanases are derived from *Oerskovia xanthineolytica* TK-1, possibly. In this stage we do not understand how the enzymes are derived from the intact, maybe, by de-amination or proteolysis.

In bacteria, a metabolic function has been reported for endo- β-1,3-glucanase and endo- β-1,3-1,4-glucanase, which are polysaccharide endohydrolases with closely related specificities. β-1,3-glucanases hydrolyze 1,3- β -glucosyl linkages, but they usually require a region of unsubstituted, contiguous 1,3- β-linked glucose residues. In contrast, β-1,3-1,4-glucanases catalyze the hydrolysis of 1,4- β-glucosyl linkages only when the glucose residue itself is linked at the O-3 position.[17] Genes encoding β-1,3-and β-1,3-1,4-glucanases have been cloned and sequenced from different bacterial species, including *Oerskovia xanthineolytica* LL G109 (βglⅢ , NCBI accession number S72212 and AF052745, Japan).

Many bacterial endo- β-1,3-glucanases (laminarinases) have been known to date share sequence similarity with endo- β-1,3-1,4-glucanases (lichenases) and classified in the same family 16 of glycosyl hydrolases.[1,35] It is still unclear for the gene and full amino acid sequence of the ENG now, however, based on the properties of the ENG described previously, suggesting that ENG belong to the family 16 of glycosyl hydrolase.

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