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PURIFICATION AND PROPERTIES OF ENDO-(1→4)-β-D-GLUCANASE FROM *Ruminococcus albus*

KUNIO OHMIYA, KEIKO MAEDA, AND SHOICHI SHIMIZU

Department of Food Science and Technology, School of Agriculture, Nagoya University, Chikusa, Nagoya 464 (Japan)

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ABSTRACT

An enzyme active against *O*-(carboxymethyl)cellulose (CMC) was purified from a synthetic medium containing ball-milled cellulose wherein *Ruminococcus albus* had been cultivated for 70 h. After 570-fold purification, a homogeneous enzyme was obtained in a yield of 3%. The enzyme degraded CMC (molecular weight, 180,000; degree of substitution, 0.6) to a smaller polymer having a molecular weight of ~20,000, and generated a small proportion of glucose, but negligible proportions of such cello-saccharides as cellobiose, cellotriose, cellotetraose, or cellopentaose. The fact that the enzyme could produce water-insoluble fragments was discovered by dissolving substrate and products in Cadoxen solution. No water-soluble cello-oligomers were detected by thin-layer chromatography after degradation of water-insoluble cellulose by the purified enzyme. Therefore, the enzyme was classified as an endo-(1→4)-β-D-glucanase.

INTRODUCTION

Ruminococcus albus is found to be a potent anaerobic bacterium as it can rapidly solubilize ball-milled cellulose (BMC, 80 g/L) and convert the hydrolyzate within three days into ethanol, acetic acid, cell mass, and such proteins as extracellular cellulases during batch cultivation[1,2], indicating that the organism has an effective enzyme system for rapid utilization of cellulose. This led to our interest in purifying and characterizing the enzymes involved in solubilization of cellulosic materials during cultivation of *R. albus*. The preparation of a large volume of cellulase-containing culture broth by use of a jar-fermentor[2,3] provided a ready source for studies on the putative enzymes. In a previous report[4], we described the purification and characterization of an extracellular cellobiosidase from the culture-supernatant liquor of *R. albus*, and of a β-D-glucosidase[5] from the precipitated fraction containing the cell mass and cellulose. Wood and coworkers[6] had characterized a crude (carboxymethyl)cellulase from *R. albus* SY3 after partial purification[6]. However, details about degradation of cellulose by the highly purified enzyme have not yet been published.

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We now present a method of obtaining a highly purified enzyme having high activity against (carboxymethyl)cellulose (CMC) from a culture-supernatant liquor of *R. albus* F-40, together with a description of the properties of the enzyme (CMCase). Depolymerization of CMC and pure water-insoluble cellulose (KC flock) by the purified cellulase was examined by measuring changes in viscosity.

EXPERIMENTAL

Materials and methods. — Pure cellulose (3% suspension of KC flock W-300, from Sanyo Kokusaku Pulp Co.) was used, after ball-milling for 3 days, as the main source of carbon in the medium. This cellulose suspension was denoted as ball-milled cellulose (BMC). An *O*-(carboxymethyl)cellulose (CMC) having a degree of substitution (d.s.) of 0.6 and a molecular weight of 180,000 (CMC-I) was the main substrate for CMCase. Other samples, namely, CMC-II (d.s. 0.95, mol. wt. 180,000), CMC-III (d.s. 1.40, mol. wt. 180,000), and CMC-IV (d.s. 0.75, mol. wt. 30,000) were also used. All CMC samples were generously provided by Daiichi Kogyo Seiyaku Co.

All other reagents were commercial products of the highest quality available.

Bacterial strain and conditions of growth. — *Ruminococcus albus* F-40, isolated from bovine rumen (kindly supplied by Tohoku University), was cultivated in a BMC (1.5%)-containing synthetic medium at pH 6.5 and 37° for a chosen period. Details of the medium composition and conditions of anaerobic cultivation have been described[7].

Analysis. — *Cellulose determination in culture broth.* — The method of Minato *et al.*[8] was applied for the determination of cellulose in the culture broth. Cellulose was freed of bacterial cells by washing the culture mixture (~2 g) with 0.1% Tween 80 solution (10 mL), and centrifuging at 145*g*. Precipitated cellulose was washed four times for complete separation. The cell-free cellulose (BMC) was quantified by means of the anthrone–sulfuric acid reagent after hydrolysis with 60% sulfuric acid. Cell dry weights were calculated from the nitrogen content, measured with Nessler reagent after decomposition of the cells in a Kjeldahl flask with[9] 98% sulfuric acid.

Enzyme assay. — (Carboxymethyl)cellulase (CMCase) activity was determined by viscosity changes of CMC-containing reaction mixtures at 37°, monitored for 5 min by using a cone-plate type of viscometer (Tokyo Keiki Co.). The reaction mixture contained CMC solution (1%) in 50mM potassium phosphate buffer (5 mL), pH 6.8, with enzyme solution (1 mL). One unit (U) of enzymic activity was defined as the amount of enzyme catalyzing a fluidity increase of CMC solution, in one min, of one centipoise^{-1} (cP^{-1}), as defined by the following equation.

$$\text{Fluidity} = \frac{1}{\text{viscosity (cP)}}$$

To evaluate enzyme activity against pure water-insoluble cellulose (KC flock), the same viscometric method was employed after a special solubilization of the cellulose by a solvent. KC flock (10%) suspended in 5mM potassium phosphate buffer (2.8 mL), pH 6.8, was mixed with enzyme solution (0.2 mL), and incubated at 37° for a given time, after which it was boiled for 5 min to inactivate the enzyme. After centrifugation for 15 min at ~20,000*g*, the amount of soluble reducing sugar (liberated from the cellulose) in the supernatant liquor was determined. The sedimented cellulose was carefully dried by evaporation, and then dissolved completely at ~5° in Cadoxen solution (the supernatant liquor from 5% of cadmium oxide dissolved in 30% aqueous ethylenediamine solution). The viscosity of this mixture was determined by use of a cone-plate viscometer at 37°.

Protein concentrations were determined either by the absorbance at 280 nm, or by the Lowry method[10] as modified by Ross and Shatz[11].

Enzyme purification. — The supernatant liquor from the culture broth was dialyzed, with a cellophane membrane, against 1,000 vol. of 10mM Tris·HCl buffer, pH 7.2, containing 10mM 2-mercaptoethanol. The dialyzate was loaded onto a column (1.5 × 55 cm) of DEAE-Sephadex A-25 (Pharmacia) equilibrated with the same buffer. Linear-gradient elution was conducted with 1 L of the buffer containing KCl (0 to 1.0M; 10-mL fractions). Fractions having high activity against CMC were pooled, and concentrated by ultrafiltration using a Diaflo membrane (Amicon YM 2) under nitrogen gas. The concentrated fraction with activity was passed through a column (2.5 × 100 cm) of Sephacryl S-200 (Pharmacia) with 0.5M KCl in the phosphate buffer (0.05M, pH 6.8). Chromatography on a column (1 × 70 cm) of DEAE-Bio-Gel A (Bio-Rad) was used as the next step. Elution was carried out by linear increase in KCl (0 to 1.0M) in the phosphate buffer. Isoelectric focusing with a column (1.6 × 50 cm) was employed as the final procedure by using Pharmalyte (Pharmacia) covering the pH range of 2.5–5.0 in a sucrose gradient with constant voltage (1450 V) for 72 h at 4°. Sucrose and Pharmalyte in the purified active fractions were replaced with 0.5M KCl in the phosphate buffer by gel filtration through a column (1.7 × 60 cm) of Bio-Gel P-100 (Bio-Rad). When necessary, ultrafiltration was carried out in order to desalt and concentrate the enzyme at appropriate steps.

Polyacrylamidegel electrophoresis (PAGE). — Analytical disc-gel electrophoresis was performed by the method of Davis[12], and gradient slab-gel electrophoresis by the method of Anderson *et al.*[13] at 4°. Protein was stained by dipping the gel in a solution of Coomassie Brilliant Blue (R-250) for 30 min. To detect enzyme activity the gel was sliced into 30 pieces, each 3.0 mm in length, at 4°, immediately after electrophoresis. Protein was extracted from each piece by immersing the sliced gel in phosphate buffer (0.4 mL) overnight, after which the enzymic activity was determined.

Determination of molecular weight. — The molecular weight of the enzyme was estimated by a gradient PAGE method and by gel filtration through a column (1.7 × 60 cm) of Bio-Gel P-100 equilibrated with 20mM phosphate buffer, pH 6.8, containing 0.5M KCl and 10mM 2-mercaptoethanol.

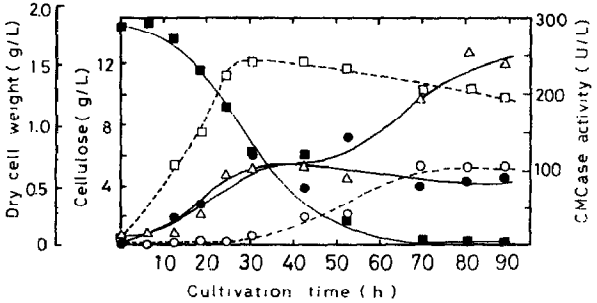

Fig. 1. Time courses of bacterial cell-growth: cell dry weight (□), remaining amount of BMC (■), and CMCase activities in the supernatant liquor (○), in the precipitate (●), and in the whole culture-broth (△), when *R. albus* was cultivated in a BMC-containing synthetic medium.

Molecular weights of CMC hydrolyzates were estimated by high-performance liquid chromatography (h.p.l.c., Hitachi Co.) with use of a refractive-index detector (Japan Analytical Industry Co.) through a gel-permeation column (Gelco W-550, Hitachi Co.; 0.5 × 350 mm; flow rate, 0.7 mL/min), eluted with 0.2M KCl.

Determination of thermal stability, and optimal pH and mercaptoethanol concentration. — The surface-response method described by Mullen and Ennis[14] was employed for determination of the thermal stability, and optimal pH and 2-mercaptoethanol concentration for the purified enzyme, with CMC-1 as the substrate. Enzymic reactions were carried out for 5 min under chosen conditions in the following ranges: pH, 5.6–7.9; temperature, 31.0–54.4°; and 2-mercaptoethanol, 2.5–46.5mM.

RESULTS

Formation of CMCase during cultivation of R. albus. — The time courses for growth (increase in dry-cell weight), remaining amount of BMC, and CMCase activities (in the supernatant liquor, in the precipitated fraction, and in the whole culture-broth) were determined during cultivation of *R. albus* on BMC (see Fig. 1). Almost all of the BMC was solubilized within 70 h, although bacterial cells increased to a maximum at ~30 h and then began to decrease gradually to ~85% of the maximum amount of cells after 70 h of cultivation. CMC-hydrolyzing activity from the precipitated fraction, containing cells and residual cellulose, increased in parallel with the increase in cell mass; the activity was the highest at ~30 h. During this period, the activity in the supernatant liquor was almost negligible, after which it increased gradually. At ~70 h, the activity in the supernatant liquor was higher than that in the precipitate. The activity in whole culture-broth was very close to the sum of activities in the supernatant liquor and in the precipitate. These data suggest that more than half of the CMCase could be released into the culture-supernatant liquor after cultivation beyond 70 h, although the activity from the precipitate decreased slightly. On the basis of these results, the CMCase-containing

TABLE I

SUMMARY OF PURIFICATION OF CMCase FROM *R. albus*

Purification step	*Total volume (mL)*	*Total activity (U)*	*Total protein (mg)*	*Specific activity (U/mg)*	*Recovery of activity (%)*	*Purification (fold)*
Crude extract	3040	345	6030	0.057	100	1.1
Dialyzate	3740	337	5450	0.062	97	1.1
DEAE-Sephadex A-25	204	85.5	357	0.240	25	4.1
Sephacryl S-200	65	42.4	37.4	1.08	12	18.6
DEAE-Bio-Gel A	35	24.2	2.5	9.68	7	170
Isoelectric focusing	1.7	10.6	0.32	33.1	3	572

supernatant from the culture broth of *R. albus* was harvested after 70 h and used as the starting material for purification of the enzyme.

Purification of CMCase. — Chromatography of a CMCase-containing supernatant liquor, prepared by centrifugation and dialysis, on a column of DEAE-Sephadex A-25 provided one peak activity against CMC, eluted at a KCl concentration of 0.18M. The fractions having higher activities were passed through a Sephacryl S-200 column as the second step. A single small peak having activity was eluted with 0.5M KCl in the phosphate buffer in front of the largest (protein) peak eluted. Thereafter, the eluate was loaded onto a DEAE Bio-Gel A column. Enzyme activity was detected in fractions eluted at 0.35M KCl; this led to a dramatic increase in specific activity. Isoelectric focusing of fractions having enzyme activity revealed a sharply symmetrical protein peak coinciding with enzyme activity at a pI of 4.3. This protein was homogeneous, as determined by polyacrylamide-disc gel electrophoresis in the presence and absence of SDS, with enzyme activity corresponding to the protein band. This four-step purification procedure yielded a 570-fold enrichment of the enzyme (see Table I) in a yield of ~0.32 mg. All subsequent studies were carried out with this purified enzyme.

Some properties of the purified enzyme

(i) Molecular weight. A molecular weight of 50,000 was estimated by gradient slab-PAGE with a similar value obtained by gel filtration through Bio-Gel P-100. This value is similar to those for CMCases from *C. thermocellum*[15] and *Cellulomonas fimi*[16] but is larger than that (mol. wt. 30,000) of partially purified CMCase from *R. albus* YS3 (ref. 17).

(ii) Optimal conditions. Maximum enzymic activity was obtained at pH 6.7 in the presence of 23.6mM 2-mercaptoethanol, according to surface-response measurements.

(iii) Thermal stability. Enzyme incubated at pH 6.8 for 10 min lost almost no activity at temperatures below 50°; the activity decreased greatly at temperatures higher than 50°. At 70°, negligible activity was seen. When enzyme activity was determined at various temperatures by the surface response method already mentioned, the use of a 5 min incubation period led to a value of 44° for "optimal temperature".

(iv) Effects of chemical reagents on the activity. Each reagent (1mM) was added just before reaction for 5 min at 37° was initiated by the enzyme. Cu^{2+}, Zn^{2+}, Fe^{2+}, and Hg^{2+} depressed the activity remarkably. Such reducing reagents as 2-mercaptoethanol, 1,4-dithiothreitol, glutathione, and cysteine·HCl led to slight enzyme activation at a concentration of 1mM. Sulfhydryl-reacting reagents (*p*-chloromercuribenzoate, *N*-ethylmaleimide, and iodoacetamide) inhibited the activity extensively. This susceptibility to inactivation indicated that the CMCase may be a thiol enzyme. Similar effects of these reagents on activity were seen also in the cellobiosidase[4] and β-glucosidase of *R. albus*[5], suggesting that both endo- and exo-type cellulases described herein may have some properties in common.

(v) K_m values of CMCase. K_m values of the enzyme against CMCs of the same molecular weight (180,000), but with d.s. values of 0.60, 0.95, and 1.40, were determined to be 0.72, 0.07, and 0.04%, respectively, as determined by Lineweaver–Burk plots, showing a marked decrease with increases in d.s. values of carboxymethyl groups on cellulose, even though the molecular weights of the CMCs were similar.

Degradation of CMC by the enzyme. — CMC-degrading activity of the purified enzyme was determined by viscometric and dinitrosalicylic acid (DNS) methods[19]. The reciprocal values of the viscosity of the reaction mixture (the enzyme and CMC-I) increased linearly during the initial 30 min of reaction (data not shown). Reducing terminals, generated by enzymic cleavage of β-(1→4)-glucoside linkages, were measured by the DNS method (data not shown). The increase in reducing terminals was very small, although the numbers rose linearly with increases in reaction time. Such small values made very difficult the evaluation of CMCase activity during purification. Therefore, the fluidity determination method was employed.

CMCs used as substrates had degrees of substitution of 0.60, 0.95, and 1.40, with molecular weights of ~180,000. The extents of change of fluidity of reaction mixtures were inversely related to the d.s. values (see Fig. 2). This suggests that cleavage of the β-D-(1→4)-glucoside linkage can be suppressed by carboxymethyl groups. CMC-I (d.s. 0.60, mol. wt. 180,000) and CMC-II (d.s. 0.95, mol. wt. 180,000) were extensively hydrolyzed within 30 min by purified enzyme as the fluidity of the reaction mixture increased remarkably (see Fig. 2). In the case of CMC-IV (d.s. 0.75; mol. wt. 30,000), the fluidity change was less than 40% of that of CMC-II (see Fig. 2), although the d.s. value of CMC-IV (0.75) is smaller than that (0.95) of CMC-II. This small change in fluidity may therefore, be due to the lower number of cleavable sites in the smaller substrate, but it may not depend on the numbers of carboxymethyl groups on the substituted cellulose.

Degradation products of CMC-I generated by CMCase after a given reaction time were permeated through a h.p.l.c. column for estimation of the molecular weight. A typical chromatographic pattern of CMC-I without addition of enzyme revealed a broad peak (retention time, 14.1 min) with a shoulder (retention time, 21.6 min), followed by a sharp peak (retention time, 24.8 min) (see Fig. 3-A). The

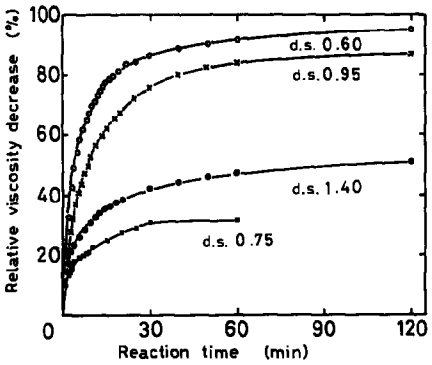

Fig. 2. Time courses of relative viscosity changes of CMC-I (○), CMC-II (×), CMC-III (●), and CMC-IV (■) solutions following hydrolysis by CMCase from *R. albus* at pH 6.8 and 37°. (d.s. = degree of polymerization.)

broad main peak having the shoulder was CMC-I; the later sharp peak was that of salt in the sample. The retention times of the broad main peak increased with longer enzymic reaction times (see Fig. 3-B). After 1 h, one sharply symmetrical peak having retention time at 21.0 min was observed. Further incubation neither prolonged the retention time of this peak further nor changed the chromatographic pattern, indicating that the material with the retention time of 21.0 min was the final product (see Fig. 3-C). The molecular weight of this final product was estimated by use of the retention time. To evaluate the molecular weight of the CMC hydrolyzates, the retention times of CMCs-I and -IV were determined by the same h.p.l.c. method. The molecular weights of CMCs-I and -IV were estimated to be 180,000 and 30,000, respectively, by using a viscosity–molecular weight equation[19]. With the calibration curve drawn with CMCs-I and -IV, the molecular weight of the final product from CMC-I was roughly estimated to be ~20,000.

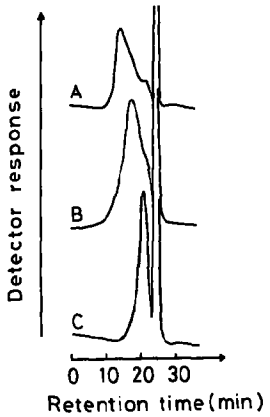

Fig. 3. Gel permeation chromatograms of the products of degradation of CMC-I by purified CMCase. Reactions were carried out for (A) 0, (B) 30, and (C) 120 min at 37° and pH 6.8.

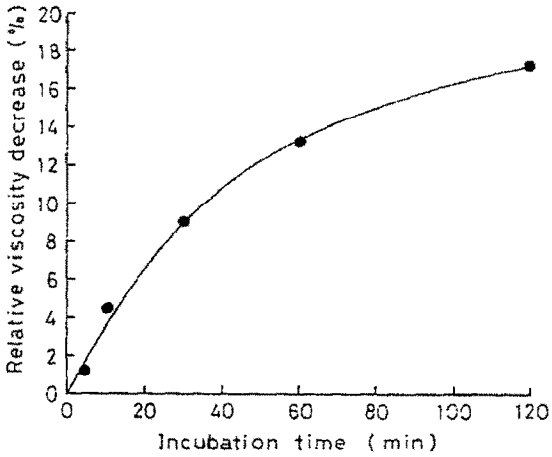

Fig. 4. Time course of the activity of the purified CMCase against pure insoluble cellulose (KC flock) at 37° and pH 6.8. The activity was evaluated by the decrease in viscosity of the mixture of Cadoxen and the cellulose that had been degraded with the enzyme for 2 h.

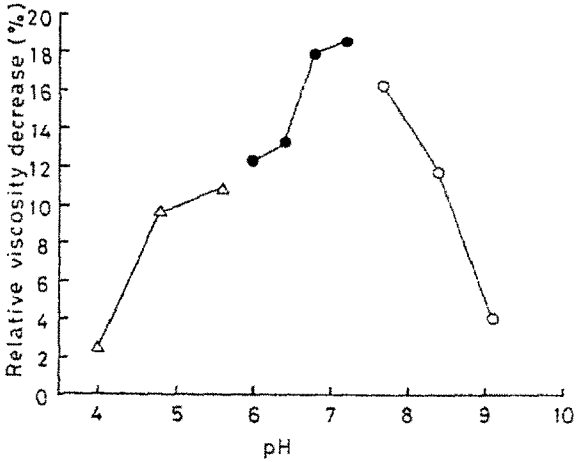

Fig. 5. Optimal pH for the cellulolytic activity of the purified enzyme against pure water-insoluble cellulose (KC flock) at 37°. The activity was evaluated by the decrease in viscosity of the mixture of Cadoxen and the cellulose that had been degraded with the enzyme for 2 h with (△) acetate, (●) phosphate, or (○) Tris·HCl buffer.

Detection of low-molecular-weight hydrolyzates of CMC and pure, water-insoluble cellulose (KC flock). — Purified CMCase was incubated with 1% CMC solution or 10% KC-flock suspension for 1 h at 37°. Thereafter, the whole CMC–enzyme mixture was used to detect reducing sugar by the Somogyi method[20]. Because this method generated a lot of precipitate from the CMC–enzyme mixture, the suspension was filtered. Soluble reducing sugars in the filtrate were found to be negligible by the colorimetric method. Reducing sugars in the supernatant liquor of

the KC flock–enzyme mixture were undetectable by the Somogyi method after 1 h of reaction. Thin-layer chromatography[5] was employed to detect glucose and such saccharides as cellobiose, cellotriose, cellotetraose, and cellopentaose, but none was found in the reaction mixture. However, by using a glucose-detecting enzymic system ("Glucostat", Fujisawa Pharmaceutical Co.), a small proportion (83 μM) of glucose was detected after 2 h of reaction in the reaction mixture containing CMC-I (46μM).

Hydrolysis of pure water-insoluble cellulose (KC flock) by the enzyme. — KC flock was incubated with the purified enzyme at 37° for a given period and thereafter isolated from the mixture and solubilized with Cadoxen solution. The viscosity of the enzyme-treated KC flock–Cadoxen mixtures showed an inverse correlation with the reaction time (see Fig. 4). The optimal pH (see Fig. 5) for enzyme activity against KC flock, by the method already described, was very similar to the value obtained when CMC was used as the substrate.

DISCUSSION

In a cellulose-containing culture-broth, CMCase activity from *R. albus* appeared in the precipitated fraction (cell mass and cellulose), and changed in parallel with changes in cell mass, indicating that the enzyme is either cell- or cellulose-bound. If the enzyme is cellulose-bound, the activity in the precipitated fraction should decrease with decreases in residual cellulose. However, the decrease in CMCase activity in the precipitated fraction was not significant, even after an extensive decrease in residual cellulose (see Fig. 1), suggesting that this enzyme is essentially cell-bound. The existence of cell-bound CMCases from *R. albus* and other rumen anaerobes[21] was reported in the presence of phenylpropanoic acid[22,23] and grass[24], respectively. The latter enzymes became cell-free in the absence of the compounds described. According to Wood and Wilson[17], cell-free CMCase from *R. albus* may be a monomeric form released from the cell-wall-bound enzyme, suggesting that cell-free CMCase harvested from the culture-supernatant liquor is the same as that located in the cell-bound form.

The purified enzyme degraded CMC-I to a smaller polymer with a molecular weight of 20,000 as the main component and also digested pure water-insoluble cellulose (KC flock) to compounds insoluble in water in the absence of Cadoxen. The molecular weights of the water-insoluble products could not be determined, as we know of no way in which to evaluate the sizes thereof after fractionation in the presence of Cadoxen. However, the hydrolyzates must be β-D-(1→4)-glucoside-linked cello-oligosaccharides having a chain longer than that of cellopentaose, which is water-soluble. Moreover, no water-soluble product such as glucose, cellobiose, cellotriose, cellotetraose, or cellopentaose or any such carboxymethylated product was detected in thin-layer chromatograms of the hydrolyzates from either substrate (KC flock or CMC-I). From these results, the present enzyme is identified as an endo-1,4-β-glucanase.

This enzyme is very different from the CMCase or endo-(1→4)-β-D-glucanases purified from a *Clostridium* sp. and other fungi, because the latter can produce, from CMC and pure water-insoluble cellulose, large proportions of compounds that are soluble in water. The production of saccharides (G_3 and G_4) was also found with the endo-(1→4)-β-D-glucanase formed by *E. coli* after cloning the *Clostridium* gene[15]. These cloning experiments revealed that the *Clostridium* strain can produce several kinds of endo-(1→4)-β-D-glucanase, making difficult the identification and definition of endo-type enzymes. This difficulty has been well known in cases of the enzymes from *T. viride*[25,26]. Fortunately, only one endo-(1→4)-β-D-glucanase was isolated from the culture broth of *R. albus* throughout the purification procedure employed in the present report suggesting that only one type of endocellulase may be produced by the organisms. The simplicity of the cellulolytic enzyme system of *R. albus*, compared with that of *Clostridium* sp. and *Tricoderma* sp., may be due to the circumstance that *R. albus* is cultivated only under strictly anaerobic conditions, such as in animal rumen, rather than the conditions in a compost, where clostridia or fungi can grow well under changeable circumstances. Therefore, *R. albus* may not need different types of CMCases for adaptation to its specific milieu.

The decreases in viscosity of pure cellulose dissolved in Cadoxen after enzymic reaction with the purified endo-(1→4)-β-D-glucanase suggested the cleavage of water-insoluble cellulose to afford water-insoluble fragments without any synergistic reaction of a C_1 component as a swelling factor, as suggested by Reese *et al.*[27] and Leatherwood[28].

As already discussed the present enzyme is a typical endo-type of cellulase having a thiol functional group. This may be the first report describing the highly purified endo type of enzyme from *R. albus*, which mainly degrades water-insoluble cellulose to water-insoluble fragments. The enzyme seems to be somewhat different from the endo-(1→4)-β-D-glucanase reported by Wood and Wilson[17], which releases large proportions of cellotriose and percentages of cellotetraose from H_3PO_4-swollen cellulose.

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REFERENCES

1 J. M. LEATHERWOOD, *Appl. Microbiol.*, 13 (1965) 771–775.
2 K. OHMIYA, K. NOKURA, AND S. SHIMIZU, *J. Ferment. Technol.*, 61 (1983) 25–30.
3 M. TAYA, K. OHMIYA, T. KOBAYASHI, AND S. SHIMIZU, *J. Ferment. Technol.*, 58 (1980) 463–469.
4 K. OHMIYA, M. SHIMIZU, M. TAYA, AND S. SHIMIZU, *J. Bacteriol.*, 150 (1982) 407–409.
5 K. OHMIYA, M. SHIRAI, Y. KURACHI, AND S. SHIMIZU, *J. Bacteriol.*, 161 (1985) 432–434.

6 T. M. WOOD, C. A. WILSON, AND C. S. STEWART, *Biochem. J.*, 205 (1982) 129–137.
7 M. TAYA, T. KOBAYASHI, AND S. SHIMIZU, *Agric. Biol. Chem.*, 44 (1980) 2225–2227.
8 H. MINATO, S. ENDO, T. KORIYAMA, AND T. UEMURA, *Nippon Nogei Kagaku Kaishi*, 36 (1962) 101–106.
9 W. W. UMBREIT, R. H. BURRIS, AND J. F. STAUFFER, *Method for nitrogen*, in *Manometric Techniques – A Manual Describing Methods Applicable to the Study of Tissue Metabolism*, Burgess Publishing Co., Minneapolis, 1957, p. 238.
10 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
11 E. ROSS AND G. SHATZ, *Anal. Biochem.*, 54 (1973) 304–306.
12 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404–427.
13 L. O. ANDERSON, H. BORG, AND M. MILCAELESON, *FEBS Lett.*, 20 (1972) 199–202.
14 K. MULLEN AND D. M. ENNIS, *Food Technol.*, 33 (1979) 74–80.
15 J. MILLET, D. PÉTRÉ, P. BÉGUIN, O. RAYNAUD, AND J. P. AUBERT, *FEMS Microbiol. Lett.*, 29 (1985) 145–149.
16 N. R. GILKES, M. L. LANGSFORD, D. G. KILBURN, R. C. MILLER, JR., AND R. A. J. WARREN, *J. Biol. Chem.*, 259 (1984) 10,455–10,459.
17 T. M. WOOD AND C. A. WILSON, *Can. J. Microbiol.*, 30 (1984) 316–321.
18 S. FUKUI, *Quantitative Method for Reducing Sugar*, University of Tokyo Press, Tokyo, 1973, pp. 19–20.
19 G. SITARAMAIAH AND D. A. I. GORING, *J. Polym. Sci.*, 58 (1962) 1107–1131.
20 M. SOMOGYI, *J. Biol. Chem.*, 195 (1952) 19–23.
21 W. R. SMITH, I. YU, AND R. E. HUNGATE, *J. Bacteriol.*, 114 (1973) 729–737.
22 R. E. HUNGATE AND R. J. STACK, *Appl. Environ. Microbiol.*, 44 (1982) 79–83.
23 R. J. STACK AND R. E. HUNGATE, *Appl. Environ. Microbiol.*, 48 (1984) 218–223.
24 D. E. AKIN AND H. E. AMOS, *Appl. Microbiol.*, 29 (1975) 692–701.
25 G. OKADA, K. NISHIZAWA, AND H. SUZUKI, *J. Biochem. (Tokyo)*, 63 (1968) 591–606.
26 G. S. RAUTELA AND K. W. KING, *Arch. Biochem. Biophys.*, 132 (1968) 589–601.
27 E. T. REESE, R. G. H. SIU, AND H. S. LEVINSON, *J. Bacteriol.*, 59 (1950) 485–497.
28 J. M. LEATHERWOOD, *Adv. Chem. Ser.*, 95 (1969) 53–59.