Precipitate-forming Reaction of \( \beta-(1\rightarrow4)-\text{d}-\text{Glucanase(II)} \)
in Malt

Hiroshi Yamashita,* Tatsuhiko Hiroto*, Fumitaka Hayase
and Hiromichi Kato

Department of Agricultural Chemistry, The University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan

*Central Research Laboratories, Asahi Breweries Ltd.,
13-1 Omori-kitai 2-chome, Ota-ku, Tokyo 143, Japan

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\( \beta-(1\rightarrow4)-\text{d}-\text{Glucanase(II)} \) was isolated and purified to homogeneity on SDS-electrophoresis from brewing barley malt. This enzyme had an endo-hydrolase action pattern on \( \beta \)-glucan prepared from the same malt, while the enzyme formed precipitates in the reaction mixture. These precipitates (P-I) as a whole were composed of \( \beta-(1\rightarrow3) \) and (1\( \rightarrow4 \)) linkages in a molar ratio of 27.6:72.4. The P-I was separated into two parts and analyzed; they had mainly linear \( \beta-(1\rightarrow4) \) linkages. When cellotetraose or 3-O-\( \beta \)-cellotriosyl-D-glucose was used as a substrate for \( \beta-(1\rightarrow4)-\text{d}-\text{glucanase(II)} \), insoluble materials were similarly formed in reaction mixture. These water-insoluble materials were celloctaose and 3-O-\( \beta \)-celloctaosyl-D-glucose, respectively. These results suggest that \( \beta-(1\rightarrow4)-\text{d}-\text{glucanase(II)} \) catalyzed the transfer of \( \beta \)-D-glucosyl residues from each oligosaccharide to C\(_4\)-OH of the non-reducing residue in each of them, and the resulting products from the transglucosylation precipitated in the reaction mixture.

When \( \beta \)-glucanases prepared from malt are incubated with solutions containing malt \( \beta \)-glucan, insoluble materials are formed in the reaction mixture. In a previous paper\(^1\) of this series, we described some properties of three \( \beta \)-glucanases which participated in the formation of precipitates in malt, two of which were different from known enzymes. One of them was \( \beta-(1\rightarrow4)-\text{d}-\text{glucanase(I)} \), with a molecular weight of 37,000 and an optimum pH of 5.0. The other enzyme was \( \beta-(1\rightarrow4)-\text{d}-\text{glucanase(II)} \), with a molecular weight of 49,000 and an optimum pH of 4.5. In addition, it was found that \( \beta-(1\rightarrow4)-\text{d}-\text{glucanase(II)} \) was relatively stable to heat treatment and formed most of the precipitates in the reaction mixture.

MATERIALS AND METHODS

Materials. Two-rowed brewing barley malt was obtained from the Providence Co., France. Standard proteins: bovine serum albumin (mol. wt. 67,000); ovalbumin (mol. wt. 45,000); chymotrypsinogen (mol. wt. 26,000); myoglobin (mol. wt. 17,800) and cytochrome c (mol. wt. 12,500) for the molecular weight estimation were obtained from the Sigma Chemical Co., U.S.A. DEAE-Sephadex A-50, CM-Sephadex C-50, and Sephadex G-75 were obtained from Pharmacia, Sweden. Hydroxyapatite was obtained from Seikagaku Kogyo Co., Ltd., Japan. All other reagents used were guaranteed grade purchased from Seikagaku Kogyo Co., Wako Pure Chemical Co., and Nakarai Chemicals Co., Japan.

Substrates. Carboxymethyl cellulose (CMC-500), lichenin, and barley \( \beta \)-glucan were obtained from Tokyo Kasei Kogyo Co., Ltd., Japan, Sigma Chemical Co., Ltd., U.S.A., and Biocon Biochemicals Ltd., Ireland, respectively. Malt \( \beta \)-glucan and wort \( \beta \)-glucan were prepared by the method described in our previous paper.\(^1\) Cellooligosaccharides were prepared by the method of Millet et al.,\(^2\) and 3-O-celloctiosyl-D-glucose was prepared by \( \beta-(1\rightarrow3), (1\rightarrow4)-\text{d}-\text{glucan 4-glucanohydrolyase which} \) was described in a previous paper.\(^3\)

Measurement of enzyme activity. Glucanase activity was measured from the production of reducing sugars which were assayed by the colorimetric method of Nelson–Somogyi.\(^4,5\)
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**Protein content.** The protein content of the enzyme was measured from the absorbance at 280 nm.

**NMR spectrometry.** The PMR spectrum was measured at 100 MHz and 80°C by the WEFT method in D$_2$O with TSP$_d_4$ as internal standard (JEOL GX-100 NMR Spectrometer). The CMR spectrum was measured at 25 MHz and 80°C in D$_2$O.

**Methylation analysis.** Samples were methylated by the method of Isogai et al.$^6$ Polysaccharide was dispersed in SO$_2$/dimethyl sulfoxide/diethyl amine and to a suspension containing polysaccharide was added freshly powdered NaOH under N$_2$ at room temperature and the mixture stirred for 1 hr. Methyl iodide was then added dropwise at room temperature and the mixture was stirred for 1 hr, and then kept at 40°C for 0.5 hr, at 50°C for 0.5 hr, and at 60°C for 1 hr. The fully methylated glucan was successively hydrolyzed with formic acid and sulfuric acid. The hydrolysate was analyzed as alditol acetate derivatives by GC and GC-MS (EI-MS and CI-MS, JEOL DX-300 Spectrometer).

**Chromatography.** GLC was done using a Hewlett Packard 5790A GC with a column of Methyl silicone (0.2 mm i.d., 25 m) at 170~260°C (10°C/min). TLC was done using silica gel 60 (Merck) and the following mixture: acetonitrile and water (3:1) as the solvent, and sugars were detected by diphenylamine/aniline/phosphate reagent$^7$ followed by heating at 90°C.

**Enzyme purification.** β-(1→4)-D-glucanase(II) was purified from 500 g of barley malt flour by a previously described method$^{11}$ with some modifications (Fig. 1).

**RESULTS**

**Purification and properties of β-(1→4)-D-glucanase(II)**

β-(1→4)-D-glucanase(II) was purified by batchwise adsorption on DEAE-Sephadex A-50, DEAE-Sephadex A-50 column chromatography, hydroxyapatite column chromatography and Sephadex G-75 gel filtration as shown in Fig. 1. On the purification step, the elution pattern of β-(1→4)-D-glucanase(II) with Sephadex G-75 (final step) is shown in Fig. 2. From the symmetrical pattern of protein and enzyme activities, sufficient purity was estimated to be obtained. To check the purity of the enzyme, it was electrophoresed on SDS-polyacrylamide gel. As shown in Fig. 3, the purified β-(1→4)-D-glucanase(II) gave a single protein band. The results of the enzyme purification are summarized in Table I. The enzyme was purified finally about 300-fold. Some properties of the enzyme were described in our previous paper.$^1$ To characterize the catalytic action of the enzyme, the values of $K_m$ and $V$ were measured using the barley, malt, and wort β-glucans as substrates. As shown in Table II, β-(1→4)-D-glucanase(II) has an appreciable affinity for wort and malt β-glucan, and the order of reactivity was barley $>$ wort $>$ malt β-glucan. When CMC was used as substrate, its affinity and reactivity were lower than the other β-glucans. Consequently, β-(1→4)-D-glucanase(II) had a higher affinity for β-glucans which had β-
Table 1. Purification of β-(1 → 4)-d-Glucanase(II)

<table>
<thead>
<tr>
<th>Crude extract (NH₄)₂SO₄ fraction</th>
<th>CM-Sephadex C-50</th>
<th>DEAE-Sephadex A-50</th>
<th>Hydroxylapatite</th>
<th>Sephadex G-75 (II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein (mg)</td>
<td>55,020</td>
<td>7,780</td>
<td>2,610</td>
<td>74</td>
</tr>
<tr>
<td>Total activity (unit)</td>
<td></td>
<td>80,125</td>
<td>44,870</td>
<td>12,169</td>
</tr>
<tr>
<td>Specific activity (unit/mg)</td>
<td></td>
<td>10.30</td>
<td>17.19</td>
<td>150.93</td>
</tr>
<tr>
<td>Yield (%)</td>
<td></td>
<td>100</td>
<td>56</td>
<td>14</td>
</tr>
</tbody>
</table>

Protein concentration of the enzyme was measured spectrophotometrically by measuring absorbancy at 280 nm. An E₁₀₀° value of 10.0 was used.

The enzyme activity was measured by the Nelson-Somogyi method using M-I as substrate. One unit of the enzyme activity was defined as the amount of enzyme catalyzing the production of 1 μg of reducing sugar per min at 37°C.

Fig. 2. Elution Pattern of β-(1 → 4)-D-Glucanase(II) on the Second Sephadex G-75 Column.

(------) absorbance at 280 nm (left scale); (○—○) CMC saccharifying activity (right inner scale); (△—△) malt β-glucan saccharifying activity (right inner scale); (●—●) CMC viscometric activity (right outer scale) Column, 2.5 × 110 cm; flow rate, 10 ml/hr; fraction size, 5 ml.

Fig. 3. SDS-Gel Electrophoresis of β-(1 → 4)-D-Glucanase(II).

A sample of the purified enzyme (5 μl) was put on a gel of 10% polyacrylamide and electrophoresed for 2 hr at 20°C. A, β-(1 → 4)-D-glucanase; B, standard marker protein. Standard marker protein used from right to left were; bovine serum albumin, mol. wt. 67000; ovalbumin, mol. wt. 45000; chymotrypsinogen, mol. wt. 26000; and cytochrome c, mol. wt. 12500.

Malt β-glucan and β-(1 → 4)-D-glucanase (II)

1) Hydrolysis pattern for malt β-glucan. To characterize the mode of action of β-(1 → 4)-D-glucanase(II), the reducing sugar content and viscosity during malt β-glucan hydrolysis by the enzyme were measured. As shown in Fig. 4, viscosity during the initial reaction step rapidly decreased and reducing value increased in an essentially linear manner after approximately 3 hr. Therefore, β-(1 → 4)-D-glucanase (II) had a characteristic endo-hydrolase action-pattern on β-(1 → 3), (1 → 4)-glucan.

(1 → 3) and (1 → 4) linkages than for only β-(1 → 4) linkages. In addition, it was elucidated that wort, malt, and barley β-glucan which had partly cellotetraose units were better substrates than lichenin which had mainly cellotriose units in β-(1 → 3), (1 → 4)-glucan.
Table II. Substrate Specificity of \( \beta(1 \rightarrow 4) \)-D-Glucanase(II)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( K_m ) (%(^a))</th>
<th>( V ) (%(^b))</th>
<th>Linkage ratio ( \beta-(1 \rightarrow 3)/\beta-(1 \rightarrow 4) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley</td>
<td>0.192</td>
<td>3.500</td>
<td>30:70</td>
</tr>
<tr>
<td>Lichenin</td>
<td>0.235</td>
<td>1.674</td>
<td>34.1:65.9</td>
</tr>
<tr>
<td>CMC-500</td>
<td>0.592</td>
<td>1.431</td>
<td>-</td>
</tr>
<tr>
<td>M-I(^c)</td>
<td>0.070</td>
<td>1.960</td>
<td>29.8:70.2</td>
</tr>
<tr>
<td>M-II(^c)</td>
<td>0.382</td>
<td>1.125</td>
<td>-</td>
</tr>
<tr>
<td>W-I(^d)</td>
<td>0.057</td>
<td>2.399</td>
<td>31.8:68.2</td>
</tr>
</tbody>
</table>

The enzyme activity was measured at 37°C and pH 5.0 for 1 hr.

\(^a\) \( K_m \) value was expressed as percent of substrate.

\(^b\) \( V \) value was expressed as the amounts of reducing sugars in the hydrolysis of various substrate.

\(^c\) M-I, M-II were \( \beta \)-glucans prepared from malt by a previously described method, and M-I had a higher molecular weight than M-II.

\(^d\) W-I was \( \beta \)-glucan prepared from wort.

The soluble materials in the reaction mixture were formed after 4 hr of reaction.

(2) Precipitates. When malt \( \beta \)-glucan (M-I, 1 g in acetate buffer, pH 4.5, 50 mm) was incubated with \( \beta-(1 \rightarrow 4) \)-D-glucanase(II) (0.167 units) at 40°C for 2 weeks, precipitates (P-I, 52.4 mg) were produced in the reaction mixture. Furthermore, ethanol was added at 3 volumes to the supernatant of the reaction mixture, and the precipitates (P-II, 462.9 mg) as hydrolyzed products of \( \beta \)-glucan were obtained. P-I was composed of \( \beta-(1 \rightarrow 3) \) and \( \beta-(1 \rightarrow 4) \) linkages and its ratio was 27.6:72.4 by a previously described method with NMR (PMR, WEFT method), and the increase of the ratio of \( \beta-(1 \rightarrow 4) \) linkages was about 8% compared with M-I. P-I was put on a Sepharose CL-6B column and eluted with 0.2 \( \text{n NaOH} \), and it was found that P-I was separated into fractions of mean molecular weight of approximately 2 \( \times 10^4 \) and a lower molecular weight.

Then P-I was partitioned by successive extraction with hot water into two parts. Insoluble materials (P-I-1, 7.7 mg) and soluble materials (P-I-2, 44.7 mg) in P-I were obtained. They were methylated and analyzed as alditol acetate derivatives, respectively. The derivatives obtained from P-I-1 were identified as 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol, 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol, 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-glucitol, as shown in Fig. 5. Accordingly, it was estimated that the molar ratio of nonreducing end glucose: \( \beta-(1 \rightarrow 3) \)-linked glucose: \( \beta-(1 \rightarrow 4) \)-linked glucose was 1:13.0:50.1. P-I-2 was 1:20.2:48.3 in a similar manner. Moreover, the lower molecular weight material which was isolated with...
Table III. Linkage Ratio of Hydrolyzed Products from Malt \(\beta\)-Glucan by \(\beta\)-(1 \(\rightarrow\) 4)-D-Glucanase(II)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nonreducing end glucose</th>
<th>(\beta)-(1 (\rightarrow) 3)</th>
<th>(\beta)-(1 (\rightarrow) 4)</th>
<th>M.W.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malt (\beta)-glucan</td>
<td>29.8</td>
<td>70.2</td>
<td>58 (\times) 10^4</td>
<td></td>
</tr>
<tr>
<td>P-I</td>
<td>27.6</td>
<td>72.4</td>
<td>2 (\times) 10^4</td>
<td></td>
</tr>
<tr>
<td>P-I-1</td>
<td>13.0</td>
<td>50.1</td>
<td>10,386^a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>7.6</td>
<td>1,638^a</td>
<td></td>
</tr>
<tr>
<td>P-I-2</td>
<td>20.2</td>
<td>48.3</td>
<td>11,196^a</td>
<td></td>
</tr>
<tr>
<td>P-II</td>
<td>33.6</td>
<td>66.4</td>
<td>2 (\times) 10^4</td>
<td></td>
</tr>
</tbody>
</table>

* Molecular weight was calculated from number of methylated glucoses.

Sepharose CL-6B was methylated and analyzed by the same methods as described above. From the result, it was 1 : 0.8 : 7.6.

P-II, which was the main hydrolyzed product and an essentially water-soluble component was composed of \(\beta\)-(1 \(\rightarrow\) 3) and \(\beta\)-(1 \(\rightarrow\) 4) linkages and its ratio was 33.6 : 66.4, and the mean molecular weight was estimated to be \(2 \times 10^4\). The higher yield of P-II indicates that malt \(\beta\)-glucan is composed of about 50% of P-II blocks. These results are summarized in Table III.

Transglucosylation

\(\beta\)-(1 \(\rightarrow\) 4)-D-glucanase(II) hydrolyzed \(\beta\)-(1 \(\rightarrow\) 4) linkages of substrates, while insoluble materials, in which the ratio of \(\beta\)-(1 \(\rightarrow\) 4) linkages were increased by the same enzyme, were formed in the reaction mixture during the hydrolysis. Consequently, the reactions of \(\beta\)-(1 \(\rightarrow\) 4)-D-glucanase(II) with the cellooligosaccharides and \(\beta\)-glucan hydrolysates formed by \(\beta\)-(1 \(\rightarrow\) 3), (1 \(\rightarrow\) 4)-D-glucan 4-glucanohydrolase as the substrate were done to investigate the precipitate-forming mechanism. Each reaction mixture containing 4% cellobiose (G_2), cellotriose (G_3), or cellotetraose (G_4) in 5 ml of 50 mM acetate buffer, pH 4.6, was incubated with \(\beta\)-(1 \(\rightarrow\) 4)-D-glucanase(II) (0.105 units) at 37°C. Periodical 500 \(\mu\)l samples were analyzed by TLC, and the precipitates were dried under reduced pressure after washing with distilled water, ethanol, and acetone. Figure 6 shows the course of cellooligosaccharide production by \(\beta\)-(1 \(\rightarrow\) 4)-D-glucanase(II). In the reaction with G_4, G_3 and G_5 were formed as the first products of the reaction. After incubation for...
5 hr, G₆ and a small amount of oligosaccharide (G₃) were formed in the reaction mixture. The β-(1→4)-d-glucanase(II) apparently transferred a glucosyl moiety as a unit to G₄. On the other oligosaccharides, the same patterns were observed (Fig. 6), and the precipitates were formed in the reaction mixture of G₃ and G₄ (Fig. 7).

The susceptibility of precipitates to the cellulase action of “Cellulase Onozuka R-10” (Kinki Yakuruto) was examined. A mixture containing 0.13 units of cellulase in 50 μl acetate buffer (50 mM, pH 6.0) and about 2% of precipitates were incubated at 37°C. Portions (3 μl) were analyzed by TLC. As shown in Fig. 8, the precipitates which remained at the original point of the chromatograms were hydrolyzed by cellulase to give glucose, G₂, and G₃. After incubation for 12 hr, the precipitates were completely solubilized, and the spot at the origin of the chromatogram disappeared. These findings indicate that the precipitates are mainly composed of β-(1→4) linkages. Then 0.02 units of β-glucosidase (from almonds, SIGMA Type I) was added to the mixture of oligosaccharides obtained by incubation for 24 hr with cellulase and hydrolysis products were also analyzed by TLC. As shown in Fig. 8, the oligosaccharides were hydrolyzed to glucose. Therefore, the anomeric configuration of both precipitate-glucosidic linkages was β. Then the precipitate (about 3 mg) from G₄ was methylated and analyzed as alditol acetate derivatives. From the result, the ratio of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-glucitol to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol was calculated to be 6.8. Accordingly, the average degree of polymerization of the precipitate was estimated to be 8.

On the other hand, 3-O-β-celotriosyl-d-glucose, which was the β-glucan hydrolyzate formed by β-(1→3), (1→4)-d-glucan 4-glucanase (II) apparently transferred a glucosyl moiety as a unit to G₄. On the other oligosaccharides, the same patterns were observed (Fig. 6), and the precipitates were formed in the reaction mixture of G₃ and G₄ (Fig. 7).
Canohydrolase was used as the substrate to clarify the transglucosylation reaction. The substrate used was analyzed with NMR spectrometry and is shown in Fig. 9. The two anomeric proton signals appeared at the lowest field on the PMR spectrum. The resonances at δ 4.74 and 4.59 were assigned to glucosidic protons due to the β-(1→3) and β-(1→4) linkages, respectively, and the ratio of the intensity of the former to that of the latter was about 1:2.0. On the CMR spectrum, the signals (δ 88.1 and 85.1) were attributable to C-3 signals of its β- and α-reducing end-units, and this oligosaccharide was preponderantly in the β-anomeric form by their relative intensities.

After incubation for 2 weeks, a precipitate formed in the reaction mixture. The precipitate was dried as mentioned above and analyzed by GC as methylated derivatives. The precipitate from 3-O-β-cellotriosyl-D-glucose was composed of mainly β-(1→4) linkages, and the ratio of nonreducing end glucose: β-(1→3)-linked glucose: β-(1→4)-linked glucose were 1:1.1:7.5. Then the supernatant was put on a Toyopearl HW-40S column, and pentasaccharides were fractionated. The pentasaccharides were dried and analyzed with NMR. The CMR and PMR spectra show that the pentasaccharide is 3-O-β-cellotetraosyl-D-glucose. From these results, it has become apparent that the non-reducing end glucose of the substrate was transferred to the non-reducing end glucose of another substrate to produce a new β-(1→4) glucosidic bond.

**DISCUSSION**

Several kinds of trans-β-glycosylase have been reported to synthesize higher linked oligosaccharides than their substrates. On endo-type β-(1→4)-glucanase, Nishizawa et al. reported that cellulases from Trichoderma viride catalyzed trans-β-(1→4)-glucosylation, but the formation of insoluble materials was not detected. On the other hand, Oi et al. reported that the enzymes from Sclerotinia libertiana and Trichoderma longibrachiatum elongated the β-(1→4) linkages to a higher extent by transfucosylation and formed insoluble materials in the reaction mixture, and the transglucosylases showed only a disproportionation reaction without hydrolytic action on cellooligosaccharides. However, in this paper, β-(1→4)-D-glucanase(II) from malt, which was essentially an endohydrolase, had trans-β-(1→4)-glucosylation activity and formed insoluble materials. Consequently, this β-(1→4)-D-glucanase(II) had not been identified before.

In barley β-glucan longer blocks of adjacent β-(1→4) linkages have been observed at low frequency. However, because β-(1→4)-D-glucanase(II) could hydrolyze CMC these longer blocks of β-(1→4) linkage seemed to be hydrolyzed early in the reaction. Therefore, the formation of precipitates such as P-I-I was assumed to be caused by transglucosylation. Accordingly, we considered the mechanism of the formation of precipitates to be that at the
first step malt β-glucan was endo-hydrolyzed, and at the following step the hydrolyzed lower molecular fractions were used as substrates for transglucosylation to elongate β-(1→4) linkages, and then these products formed precipitates.

Lichenase (β-(1→3), (1→4)-D-glucan 4-glucanohydrolase) was reported to be most important in the brewing processes because it hydrolyzes β-glucan rapidly and because of its abundance in the endosperm of germinating grain.22,23) Because β-(1→4)-D-glucanase(II) catalysed transglucosylation using 3-O-β-cellobiosyl-D-glucose as a product from β-glucan by lichenase, it is estimated that β-(1→4)-D-glucanase(II) synthesizes insoluble materials from that oligosaccharide produced by lichenase without hydrolysis of hydrolyzed β-glucan. Therefore, these insoluble materials which are not removed during the subsequent filtration of wort are brought into beers, and then they become the "nuclei" of frozen beer precipitates24,25)

Further investigation on the frozen precipitates of beer and transglucosylation reaction by the malt β-(1→4)-D-glucanase(I) are now in progress.

REFERENCES