Studies on the Chitinolytic Enzymes of Black-koji Mold

Part VI. Isolation and Some Properties of N-Acetyl-β-glucoaminidase

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Received April 6, 1964

The measurement of N-acetyl-β-glucoaminidase activity upon β-MAGA* was carried out in order to survey oligosaccharidase fraction in the chitinolytic enzymes of Aspergillus niger. N-Acetyl-β-glucosaminidase was purified in parallel with chitobiase activity, being separated from chitinase activity, and some properties of the enzyme in the hydrolysis of β-MAGA and DACB were investigated. The enzyme hydrolysed more rapidly β-glucosaminidic bonds in DACB than that in β-MAGA, but did not decompose α-MAGA.

Evidence of N-acetyl-β-glucosaminidase activity has been demonstrated by several workers in various enzyme sources for chitinase. Zechmeister et al.1,2) found N-acetyl-β-glucosaminidase activity upon β-PAGA in almond emulsin and digestive fluid of the snail Helix pomatia. Lunt & Kent3) observed the enzyme activity in the hypodermis of crab Carcinus maenas, using β-PAGA and MAGA as the substrate. Woollen, Walker and Heyworth4, in their investigation of N-acetyl-β-glucosaminidase action with various enzyme preparations, found its strong activity in molds such as Aspergillus and Lycoperdon sp. which have now been used as chitinase sources. In those studies, however, the interrelation of N-acetyl-β-glucosaminidase activity to the chitinase activity has been obscure.

For the purification of chitinase system of Streptomyces griseus, Berger and Reynolds5) assayed chitobiase activity by measuring the hydrolysis degree of β-PAGA and found that the chitobiase fraction hydrolyses DACB as well as β-PAGA. The present author6) has previously reported that two different enzymes in the chitinase system of Aspergillus niger act successively in the decomposition of glycol chitin to constituent aminosugar and the enzyme responsible for the release of aminosugar does hydrolyse both β-MAGA and DACB. Hence, it is likely that oligosaccharidase fraction can be further fractionated by measuring N-acetyl-β-glucosaminidase activity upon synthetic β-glucosaminide.

The present work was undertaken to purify oligosaccharidase by measuring N-acetyl-β-glucosaminidase activity upon β-MAGA and to clarify its enzymatic roles in the chitinase system of Aspergillus niger. In this paper the author reports that N-acetyl-β-glucosaminidase activity is separated from chitinase activity during enzyme purification and both activities participate in the release of aminosugar from glycol chitin,

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* The following abbreviations are used in this paper: β-MAGA, β-methyl-N-acetylglucosaminide; α-MAGA, α-methyl-N-acetylglucosaminide; β-PAGA, β-phenyl-N-acetylglucosaminide; DACB, N,N-diacetylchitobiose.

6) A. Ōtsukara, This Journal, 27, 454 (1963).
and that oligosaccharidase fraction hydrolyses more rapidly \( \beta \)-glucosaminidic bonds in DACB than that in \( \alpha \)-MAGA.

**MATERIALS AND METHODS**

**Materials.**

Glycol chitin was prepared by glycolation of chitin with ethylene oxide, according to the method of Senju and Oki-masu.\(^7\)

The methyl glycoside of N-acetylglucosamine was synthesized by boiling the mixture of N-acetylglucosamine and dry methanol under reflux for 3 hr in the presence of dried Amberlite IR 120. The separation of \( \alpha \) and \( \beta \)-MAGA was carried out using a column of charcoal-Celite, according to the method of Zilliken et al.\(^8\) \( \alpha \) and \( \beta \)-MAGA were crystallized from dry ethanol (m.p. 187\(^\circ\)C and 196\(^\circ\)C, reported m.p. 187\(\sim\)188\(^\circ\)C and 198\(^\circ\)C, respectively.)

DACB was prepared by saponifying O-acetyl groups of chitobiose octaacetate obtained by acetolysis of chitin according to the procedure of Bergmann et al.\(^9\) The saponification was carried out in methanol saturated with ammonia for 20 hr at room temperature and DACB was separated from the saponified products using charcoal-Celite column, according to the method of Zilliken et al.\(^9\) The compound obtained there was amorphous, decomposing at temperatures above 185\(^\circ\)C. When hydrolysed with \( \text{N HCl} \) in a sealed tube for 5 hr at 100\(^\circ\)C, the compound gave glucosamine, of 97.2\% of theoretical by modified method of the Elson-Morgan reaction.\(^11\)

**Purification of the Enzyme.**

The enzyme was purified from wheat-bran culture of *Aspergillus niger* K14 with a slight modification of the previous procedure\(^6\) that was applied to obtain a chitinase preparation of high saccharifying activity. The procedure is summarized in Table I. The column chromatography applied in the final step was carried out using hydroxylapatite as described in the previous report.\(^6\)

Protein nitrogen was determined by the micro Kjeldahl method and protein concentration on column chromatography was measured by Lowry's method.\(^12\)

**Enzyme Activity Assay.**

N-Acetyl-\( \beta \)-glucosaminidase activity was determined by measuring N-acetylglucosamine formed from \( \beta \)-MAGA under the following condition: To one ml of 0.1 M acetate buffer solution (pH 4.0) containing one mg of \( \beta \)-MAGA, one ml of properly diluted enzyme was added and the mixture was incubated at 40\(^\circ\)C. After 15, 30 and 60 min, one ml of the mixture was withdrawn and N-acetylglucosamine formed was determined using the Tracey's modification\(^11\) of the Morgan-Elson reaction. The time course curves of the formation of N-acetylglucosamine are illustrated in Fig. 1a, showing that the amount of N-acetylglucosamine formed in 30 min is proportional to the amount of enzyme added (Fig. 1b). One unit of enzyme activity was thus defined as the enzyme quantity that formed 10\(\mu\)g of N-acetylglucosamine under the condition.

Chitobiase activity was determined by measuring the hydrolysis of DACB in the same reaction mixture as described above, except that DACB was used instead of \( \beta \)-MAGA. As shown in Fig. 2a, DACB was completely hydrolysed to N-acetylglucosamine by the enzyme. The time required to reach 50\% hydrolysis of the substrate was inversely proportional to the amount of enzyme used. When the amount of enzyme required to hydrolyse 50\% of DACB in 30 min under the condition was defined as one unit of enzyme activity, the activity was found to proportional to the amount of enzyme within six units, as shown in Fig. 2b. In measuring the activity, the enzyme solution was previously diluted so as to reach 50\% hydrolysis within 5 to 20 min and 0.2 ml aliquots of the mixture were taken at 5, 10, 15 and 20 min to determine N-acetylglucosamine formed. Hydrolysis per cent was then plotted against the reaction time and enzyme activity was obtained from the point at which hydrolysis curve intersected the line of 50\% hydrolysis (E in Fig. 2a).

With chitinase, the liquefying and saccharifying activities were determined by measuring the decrease in viscosity\(^13\) and formation of aminosugar,\(^14\) respectively, using glycol chitin as the substrate, as described in detail in the previous papers.

**Paper Chromatography.**

Paper chromatography was run on Tôyô filter paper No. 50 by the ascending method with duplicate developments, using ethyl acetate-pyridine-water (120: 50: 40) as the solvent. Ehrlich reagent was sprayed to detect N-acetylglucosamine by the procedure of partridge.\(^15\) The


\(^9\) M. Bergmann, L. Zervas and E. Silberkweit, *Ber.*, 64, 2486 (1931).


FIG. 1. Time Course Curves of Enzymatic Hydrolysis of β-MAGA (a) and Relationship between the Formation of N-Acetylglucosamine and the Amount of Enzyme (b).

Enzyme used: A, 0.6; B, 0.4; C, 0.3; D, 0.2 and E, 0.1 ml of 400 fold dilution of the enzyme solution at the final step in Table II.

FIG. 2. Time Course Curves of Enzymatic Hydrolysis of DACB (a) and Relationship between the Activity and the Amount of Enzyme (b).

Enzyme used: A, 0.6; B, 0.3; C, 0.2 and D, 0.1 ml of 800 fold dilution of the enzyme solution at the final step in Table II. E indicates the line of 50% hydrolysis.

spots corresponding to DACB, α- and β-MAGA were made visible by the method of Rydon and Smith.16)

RESULTS

Purification of Chitinolytic Enzymes.

The three kinds of activities of N-acetyl-β-glucosaminidase, chitobiase and chitinase were determined at each step of purification presented in Table I, in order to investigate the interrelation of N-acetyl-β-glucosaminidase activity to chitobiase and chitinase activities. The data are
summarized in Table II. The degree of purification of the enzyme was expressed in terms of specific activity, together with the recovery of N-acetyl-β-glucosaminidase activity. At the final step of purification the enzyme activity was concentrated to about elevenfold in 28% yield. During purification, the ratio of liquefying activity to saccharifying activity of chitinase was reduced from 2.22 to 0.86. Whereas, no remarkable changes were observed with regard to the ratios of N-acetyl-β-glucosaminidase activity to saccharifying activity and to chitobiase activity. Therefore, N-acetyl-β-glucosaminidase was partially freed from chitin-liquefying activity, although it was concentrated in parallel with both saccharifying and chitobiase activities.

In order to further separate N-acetyl-β-glucosaminidase from chitinase activity, the final enzyme preparation shown in Table II was chromatographed on a column of hydroxylapatite and chromatographic pattern of enzyme activity was surveyed. As shown in Fig. 3, most of the liquefying activity and N-acetyl-β-glucosaminidase activity appeared at F₁ and F₂, respectively, being distinctly separated each other. Saccharifying activity disappeared during column chromatography and no strong activity was found in any tubes of the effluents, as was observed in the previous experiment⁶) that was carried out to obtain the chitinase preparation of high saccharifying activity. With the three combined fractions, F₁, F₂ and F₃ indicated in Fig. 3, the enzyme activities were again determined as presented in Table III. The ratio of liquefying activity to saccharifying activity with F₁ became greater than that before chromatographic purification, and with F₂ and F₃ the ratio of N-acetyl-β-glucosaminidase activity to saccharifying activity remarkably increased. Thus, N-acetyl-β-glucosaminidase of F₂ was concentrated four times more than before, 72% yield, and was separated

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**Table I. Procedure of Purification**

<table>
<thead>
<tr>
<th>Wheat-bran culture extract</th>
<th>Add solid ammonium sulfate to 0.7 saturation Discarded supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precipitate</td>
<td>Dissolved in distilled water and fractionated with ammonium sulfate between 0.3 and 0.6 saturation</td>
</tr>
<tr>
<td>Precipitate</td>
<td>Discarded in distilled water and dialysed; Added 2.5% its volume of 0.5 N neutral lead acetate solution; Discarded precipitate</td>
</tr>
<tr>
<td>Supernatant</td>
<td>Adjusted the pH of the supernatant to 6.0 with n NaOH</td>
</tr>
<tr>
<td>Precipitate</td>
<td>Fractionated with ammonium sulfate between 0.38 and 0.48 saturation</td>
</tr>
<tr>
<td>Column chromatography</td>
<td>Discared in distilled water and dialysed against running and distilled water</td>
</tr>
</tbody>
</table>

**Table II. Enzyme Activity at Each Step of Purification Presented in Table I.**

<table>
<thead>
<tr>
<th>Purification procedure</th>
<th>Total volume</th>
<th>Chitinase activity</th>
<th>N-Acetyl-β-glucosaminidase activity</th>
<th>Chitobiase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>L. A. (A)</td>
<td>Total</td>
<td>S. A. (B)</td>
</tr>
<tr>
<td>Wheat-bran extract</td>
<td>15,500</td>
<td>310,000</td>
<td>139,500</td>
<td>2.22</td>
</tr>
<tr>
<td>Ammonium sulfate ppt. (0.7 satn.)</td>
<td>1,830</td>
<td>208,600</td>
<td>135,400</td>
<td>1.54</td>
</tr>
<tr>
<td>Ammonium sulfate ppt. (0.3~0.6 satn.)</td>
<td>520</td>
<td>138,800</td>
<td>106,600</td>
<td>1.30</td>
</tr>
<tr>
<td>Lead acetate treatment</td>
<td>465</td>
<td>78,100</td>
<td>70,600</td>
<td>1.11</td>
</tr>
<tr>
<td>Acid treatment</td>
<td>460</td>
<td>50,600</td>
<td>54,200</td>
<td>0.93</td>
</tr>
<tr>
<td>Ammonium sulfate ppt. (0.38~0.48 satn.)</td>
<td>15</td>
<td>36,600</td>
<td>42,300</td>
<td>0.86</td>
</tr>
</tbody>
</table>

FIG. 3. Column Chromatography of the Enzyme Preparation shown in Table II.

Dialysed enzyme solution containing 240 mg of protein was chromatographed on a 28×230 mm column of hydroxyapatite. Elution was carried out stepwise with phosphate buffer solution (pH 6.8) of the following concentrations: (1) 10^{-4} M, (2) 10^{-3} M, (3) 5×10^{-4} M, (4) 0.05 M, (5) 0.1 M and (6) 0.2 M. Flow rate was 20 ml per hour and effluent was collected in 10 ml fractions.

- - Protein, - - A- N-acetyl-β-glucosaminidase activity, - - - - Liquefying activity,  
Saccharifying activity.

from chitinase activity. The fraction F₃ showed low specific activity compared with that before separation. On the other hand, liquefying activity was completely recovered in F₁ and its total recovery exceeded by 6% of the original activity. The yield of saccharifying activity was about 25%, which was somewhat greater than the sum of the activity determined separately with each tube of the effluents.

Properties of the Enzyme and Hydrolysis of β-Glucosaminidic Bonds in β-MAGA and DACB.

For the investigation of enzymatic properties of N-acetyl-β-glucosaminidase F₂ was used.

Optimum pH. The pH-activity curves for hydrolysis of β-MAGA and DACB are shown in Fig. 4. The optimum pH for β-MAGA and DACB was around 3.5–4.2 and 4.0–4.4 respectively.

pH and Heat Stability. The enzyme was kept at various pH's at 30°C for 20 hr and the activities were determined. As shown in Fig. 5, the enzyme was stable over a wide range of pH's from 3 to 9.

The thermal stability of the enzyme is shown in Fig. 6. In the experiment, the enzyme was heated at various temperatures for 10 min at pH 6.8 and the activities were determined. The enzyme was stable at temperatures below 60°C, but was destroyed partially at 63°C and com-
The Mode of Hydrolysis. Since the hydrolysis of DACB by $F_2$ was more rapid than that of $\beta$-MAGA, the rate of hydrolysis of both the substrates was compared using different concentration of enzyme solution. The progressing curve of hydrolysis is illustrated in Fig. 7. DACB was rapidly hydrolysed even by one hundredth of the amount of enzyme which was required for the complete hydrolysis of $\beta$-MAGA. From the initial reaction velocity, the rate of hydrolysis was calculated to be DACB: $\beta$-MAGA = 1:0.012.

Hydrolysis Product. The action of $F_2$ upon DACB, $\alpha$- and $\beta$-MAGA was surveyed by paper chromatography. The results are shown in Fig. 8. N-Acetylglucosamine formed gave purple...
Table III. Enzyme Activity of the Three Fractions Shown in Fig. 3.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Chitinase activity</th>
<th>N-Acetyl-β-glucosaminidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>L. A. (A)</td>
<td>S. A. (B)</td>
</tr>
<tr>
<td>Before chromatography</td>
<td>26</td>
<td>19,500</td>
<td>22,680</td>
</tr>
<tr>
<td>F₁</td>
<td>60</td>
<td>19,800</td>
<td>3,900</td>
</tr>
<tr>
<td>F₂</td>
<td>100</td>
<td>660</td>
<td>1,200</td>
</tr>
<tr>
<td>F₃</td>
<td>100</td>
<td>310</td>
<td>500</td>
</tr>
</tbody>
</table>

F₁, F₂, and F₃ were obtained by combining the tubes of No. 11~16, 40~49, and 55~64, respectively, in Fig. 3.

It is apparent from the present data that the enzyme which hydrolyses β-glucosaminidic bonds in β-MAGA and DACB can be separated from chitinase activity.

In the previous paper, the author demonstrated that the chitin-liquefying activity could be separated from saccharifying activity, although saccharifying activity was measurable only when the chitinase preparation contains liquefying activity. The present paper showed that most of saccharifying activity disappeared during chromatographic purification by which liquefying and N-acetyl-β-glucosaminidase activities were separated each other. However, the total of saccharifying activity determined after combining the effluents was greater than that measured separately with each tube of the effluents (Fig. 3 and Table III). These findings may indicate that the saccharifying activity temporarily appears only in the presence of both liquefying and N-acetyl-β-glucosaminidase activities, supporting the previous paper that glycol chitin is decomposed into constituent aminosugar by successive action of two enzyme fractions in the mold chitinase system.

The ratio of N-acetyl-β-glucosaminidase activity to chitobiase activity was almost unchanged during enzyme purification and no distinct difference was observed in enzymatic properties between the two enzymes. This fact suggests the possibility that the same enzyme catalyses the hydrolysis of β-glucosaminidic bonds in both β-MAGA and DACB. However, the enzyme hydrolysed more rapidly the holoside compound such as DACB, compared with β-MAGA. F₂ is, therefore, oligosaccharidase whose major activity appears to be the cleavage of DACB, though its action upon glycol oligosaccharides is uncertain.

Acknowledgment. The author wishes to express his sincere thanks to Prof. Y. Oshima of Kyūshū University for his encouragement and his interest in this work.

The enzyme attacked DACB and β-MAGA, the substrates gradually disappeared and N-acetylglucosamine became distinguished as the reaction proceeded. This result agreed well with that shown in Fig. 7. On the other hand, no N-acetylglucosamine was detected in the enzyme reaction mixture with α-MAGA.

DISCUSSION

It is apparent from the present data that the enzyme which hydrolyses β-glucosaminidic bonds in β-MAGA and DACB can be separated from chitinase activity.

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