ISOLATION AND ACTION PATTERN OF MALTOHEXAOSE PRODUCING AMYLASE FROM *AEROBACTER AEROGENES*

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1. Introduction

During studies on the fine structure of amylopectin and Nägeli amylodextrin [1], we discovered that an unusual hydrolase activity was contained in a pullulanese preparation obtained from *Aerobacter aerogenes* by the method of Wallenfels et al. [2]. This hydrolase produced large amounts of maltohexaose (G_6) from amylose, amylopectin and whole starch. Using crude enzyme extract, we could easily get more than 35% of G_6 from amylopectin.

After examining more than 30 strains of *Aerobacter aerogenes* and *cloacae*, both of which organisms had the G_6-producing activity, we obtained one strain of *A. aerogenes* which had strong and stable enzyme activity. The enzyme was separated completely from other starch-hydrolyzing activity by ammonium sulfate precipitation and DEAE-cellulose column chromatography. We report here the experimental data and evidence which support an exo action pattern of the new enzyme, analogous to that of β-amylase and *Pseudomonas stutzeri* amylase, the latter as reported by Robyt et al. [3].

Symbols and abbreviations:
G_1, G_2, G_3 - - - - etc. are glucose, maltose, maltotriose - - - - etc. [^{14}C]G_2, [^{14}C]G_3 - - - - etc. are reducing-end-labeled maltose, maltotriose - - - - etc. O: D-glucose unit; –: 1,4-α-glucoside linkage; Ø: reducing-end glucose unit.

2. Experimental

2.1. *Partial purification of the enzyme*

A. aerogenes was grown essentially by the method of Wallenfels et al. [2] at 30° for 12 hr around pH 7.0. The culture medium was slightly modified to use maltodextrin (dextrose equivalent 20) and ammonium acetate instead of the glucose–maltose mixture and sodium nitrate respectively.

Harvested *A. aerogenes* cells were shaken with 0.1% sodium lauryl sulfate solution to liberate the enzyme from the cell wall. Ammonium sulfate-precipitated fractions between 0.3–0.7 saturation were collected and dialyzed overnight against 10 mM Tris-maleate buffer (pH 6.6) in a cold room, then put on the top of DEAE-cellulose column (1 × 25 cm). The column was eluted by stepwise increasing concentrations of NaCl and 7-ml fractions were collected. The elution profile of the G_6-producing activity and A_{280} are shown in fig. 1. In order to detect the G_6-producing activity, 0.5 ml of each fraction was incubated at 37° with 0.5 ml of 1% soluble starch solution, adjusted to pH 7.0 by Tris-maleate buffer, for 30 minutes. Ten μl was spotted on thin filter paper (Toyo Filter Paper No. 51), then irrigated twice in 65% aqueous propanol. The dried paper chromatogram was treated with glucoamylase solution to convert maltosaccharides into glucose [4], then revealed by the silver-nitrate dip method [5]. Intensities of the spot were determined by a densitometer (Ozumor-8, Atago, Japan) and are indicated as the integrator reading in fig. 1 [6].

Three active peaks of the G_6-producing amylase

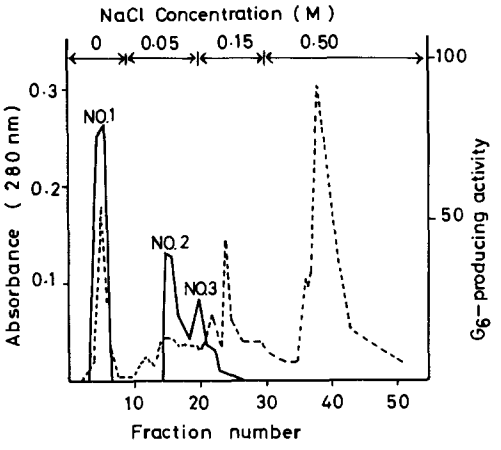

Fig. 1. Elution profile of the G_6-producing amylase from DEAE-cellulose. (——): G_6-Producing amylase activity in terms of integrated densitogram reading as described in the text; (- - - -): absorbance at 280 nm. See experimental section for details.

were obtained by DEAE-cellulose column separation. Although these peaks may well be isozymes of the G_6-producing amylase, we need further purification of each peak to make this clear.

Peak no. 1 had the highest total activity, however it was eluted right after the void volume and had a lower specific activity which suggested less homogeneity than the peaks no. 2 and 3. The peak no. 2 fraction was used for the determination of enzyme properties. Pullulanase and α-amylase-like activity were eluted from the column in the large protein peak of 0.5 M NaCl concentration.

2.2. *Preparation of a series of maltosaccharides and reducing-end-labeled maltosaccharides*

A series of maltosaccharides ranging upwards from maltose was obtained by the partial hydrolysis of potato amylose (Nagase Co., NK-110) by three times recrystallized *Aspergillus oryzae* α-amylase purchased from Sankyo Co.

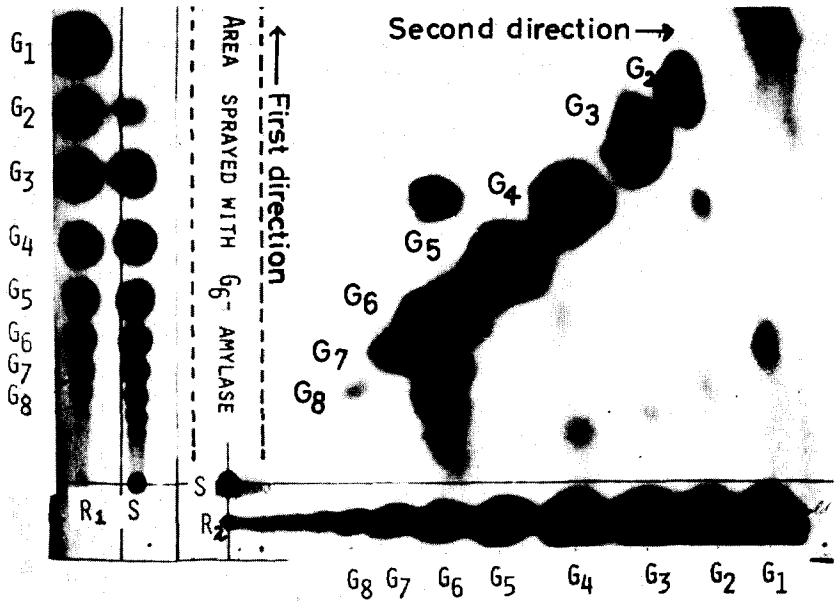

Fig. 2. Two-dimensional chromatogram, showing action of the G_6-producing amylase on maltosaccharides. R_1 and R_2 are reference series for the first and second direction of chromatogram. S is the point of application of the sample. After irrigation in the first direction, the left side of the chromaogram containing R_1 and one of the S channels, was cut off for reference. The remaining S channel was sprayed with the enzyme solution. After allowing enzyme action on the paper, the chromatogram was dried, reference R_2 was applied and the chromatogram was redeveloped in the vertical direction.

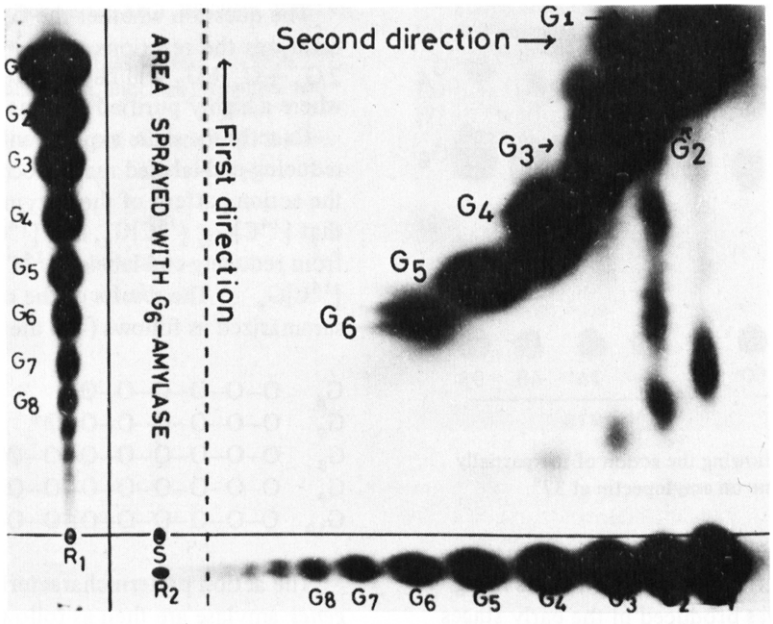

Fig. 3. Radiograph showing action of the G_6-producing amylase on reducing-end-labeled maltosaccharides. Symbols are as in fig. 2.

Reducing-end-labeled maltosaccharides were prepared by the *Bacillus macerans* amylase coupling reaction [7]. Ten μCi of [U-^{14}C]glucose (Daiichi Pure Chemical Co.) and 10 mg of recrystallized cyclohexaamylose were incubated with four Tilden-Hudson Units [8] of *B. macerans* amylase in a total volume of 1.2 ml at 37° for 3 days. A small grain of thymol was added to inhibit microbial growth.

2.3. *Two-dimensional paper chromatography*

To survey the action of the enzyme on maltosaccharides, the two-dimensional paper chromatographic method was used [9]. The chromatographic arrangement is illustrated in figs. 2 and 3.

The maltosaccharide mixture and reference series were subjected to two ascents in 65% aqueous propanol. After irrigating in the first direction and removing the reference channels, the area containing oligosaccharides was sprayed with the partially purified enzyme solution. The enzyme-sprayed chromatogram was stored in a moisture-saturated chamber for 24 hr at 37° to allow the enzyme react on the paper. After drying and applying a new reference spot, the chromatogram was irrigated in a direction perpendicular to the first direction by the same solvent system.

The sugar spots were revealed as described in the Experimental section. In the case of reducing-end-labeled maltosaccharides, the dried chromatogram was contacted with X-ray film (Fuji X-ray Film, Kx., Medical use, Fuji Film Co.) in a dark room for 3 weeks. The results of the two-dimensional chromatograms are shown in figs. 2 and 3.

3. Results and discussion

3.1. *Criteria of freedom of the enzyme from other starch-hydrolyzing activity*

A. aerogenes is known to produce pullulanase (EC 3.2.1.9) and an α-amylase-like activity which acts on starch to produce G_1, G_2, G_3 and other maltosaccharides. Besides the G_6-producing amylase, we could observe these two activities in the crude enzyme preparation. These contaminating activities could be separated on DEAE-cellulose at pH 6.6, when the G_6-producing activity was bound weakly and the pullulanase and α-amylase bound strongly (fig. 1). Other protein was removed at low ammonium sulfate concentration during the initial purification.

The second of the three peaks of G_6-producing

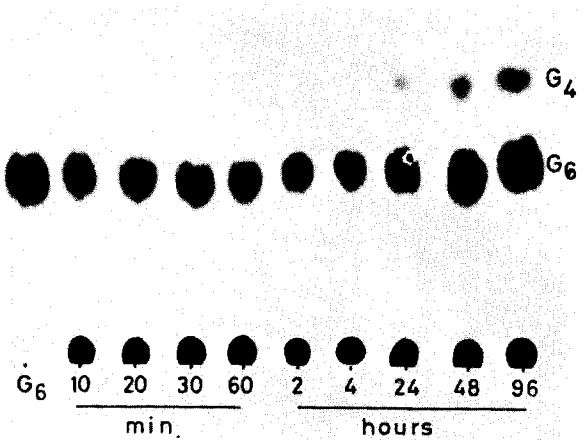

Fig. 4. Chromatogram showing the action of the partially purified enzyme on amylopectin at 37°.

amylase was incubated with defatted waxy-maize starch solution for various times. The results in fig. 4 show that only G_6 was produced in the early stages of the reaction, until 4 hr; then G_6 was hydrolyzed very slowly to G_4 and G_2.

No reaction was observed on pullulan by this enzyme fraction. Though the specific activity was as low as 0.4 IU/mg at this stage, which was only 5–7-fold higher than the crude enzyme, we did not find any other starch-splitting activity except the G_6-producing amylase.

The optimum activity of the enzyme was at pH 7.0 and 45°. The enzyme was stable between 20–45° for 1 hr. Fifty percent of the activity was lost at 50°.

3.2. *Action pattern of the enzyme*

The results of the enzyme action on maltosaccharides are illustrated in figs. 2 and 3. As seen in fig. 2, all maltosaccharides larger than G_6 formed G_6. It was easily observed that:

$$G_7 \rightarrow G_6 + G_1$$
$$G_8 \rightarrow G_6 + G_2$$
$$G_9 \rightarrow G_6 + G_3$$

until $G_{11} \rightarrow G_6 + G_5$. Faint spots of G_4 and G_2 were observed from G_6. This indicated the slow reaction $G_6 \rightarrow G_4 + G_2$. Also, we observed G_6 and G_2 formation from G_4. This could be considered as due to $2\,G_4 \rightarrow G_6 + G_2$.

The question whether the G_6-producing amylase catalyzes the reactions of $G_6 \rightarrow G_4 + G_2$ and $2\,G_4 \rightarrow G_6 + G_2$ will be discussed in a later paper where a highly purified enzyme will be described.

Exactly the same experiment was carried out with reducing-end-labeled maltosaccharides to determine the action pattern of the enzyme. It is seen from fig. 3 that $[^{14}C]G_1$, $[^{14}C]G_2$, and $[^{14}C]G_3$, ... were formed from reducing-end-labeled $[^{14}C]G_7$, $[^{14}C]G_8$, $[^{14}C]G_9$ .... The results of the radiograph may be summarized as follows (↓ is the point of cleavage):

G_6 O–O–O–O–O–O–Ø*
G_7 O–O–O–O–O–O↓–Ø* → Ø*
G_8 O–O–O–O–O–O↓–O–Ø* → O–Ø*
G_9 O–O–O–O–O–O↓–O–O–Ø* → O–O–Ø*
G_{10} O–O–O–O–O–O↓–O–O–O–Ø* → O–O–O–O*

The action pattern characteristics of the *A. aerogenes* amylase are then as follows:

1) The formation of a high-molecular-weight limit dextrin from amylopectin as shown in fig. 4. No oligosaccharide was observed between G_6 and high-molecular-weight limit dextrin.

2) A slow decrease in the iodine reaction of the amylopectin substrate compared with a rapid increase in the amount of G_6.

3) The specific hydrolysis of the sixth bond from the non-reducing ends of G_7, G_8, G_9, ...

These observations strongly support the idea that the reaction mechanism is exo. This action mechanism is similar to that of β-amylase and *Ps. stutzeri* amylase [3].

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