Novel Maltose-producing Amylase from *Bacillus megaterium* G-2[†]

Yoshiyuki TAKASAKI

Fermentation Research Institute, Agency of Industrial Science and Technology,
Ministry of International Trade and Industry, 1-1-3, Azuma,
Tukuba City, Ibaraki 305, Japan

Received July 18, 1988

A novel maltose-producing amylase, which strictly hydrolyzes the α -1,4-glucosidic linkages in starch with maltose units and releases α -maltose, was found in the culture filtrate of a strain of *Bacillus megaterium* newly isolated from soil. The enzyme was purified to almost homogeneity by means of ammonium sulfate fractionation, DEAE-Sepharose column chromatography and Bio-gel A 0.5 m column chromatography. Its optimum pH and temperature were around 7.0 and around 60°C, respectively. The enzyme was inhibited by sulfhydryl reagents such as mercuric chloride and *p*-chloromercuribenzoate, and the inhibition was recovered by cysteine. Its molecular weight was estimated to be about 60,000 by the gel filtration method. Its enzymatic properties, including its action pattern, were very similar to those of β -amylase, except for the configuration of the maltose produced.

There were two hitherto known kinds of maltose-producing amylase. One is β -amylase, which is present in soybeans, wheat, barley, etc. This enzyme strictly hydrolyzes the α -1,4-glucosidic linkages in amylose and amylopectin with maltose units, from their non-reducing ends, and releases β -maltose. The same kind of enzyme is also produced by some *Bacillus* strains.^{1~5)} The other maltose-producing amylase is a kind of α -amylase which yields α -maltose by splitting the α -1,4-glucosidic linkages in starch through an endo-mechanism. Some amylases of this type, such as those from *Streptomyces hydroscopicus*⁶⁾ and *Bacillus stearothermophilus*,⁷⁾ can produce maltose in higher yields from starch than β -amylase does. However, these amylases also produce glucose, maltotriose and other oligosaccharides at the same time. The author found a novel maltose-producing amylase in the culture filtrate of a strain of *Bacillus megaterium* isolated from soil. This paper describes the purification and characterization of this new type of maltose-producing amylase.

Materials and Methods

Microorganism used. The microorganism used in this study was isolated from soil in Chiba prefecture in Japan and identified as *Bacillus megaterium* G-2.

Cultivation method. The strain was cultivated in a medium (30 ml, pH 7.0) containing soybean meal 3%, rice bran 1%, K_2HPO_4 0.2%, $MgSO_4 \cdot 7H_2O$ 0.2%, soluble starch 2%, $CaCl_2$ 1×10^{-3} M, $MnCl_2$ 1×10^{-4} M, $CuSO_4$ 2.5×10^{-5} M, $FeCl_2$ 2.5×10^{-5} M and sodium lauryl sulfate 2.5×10^{-2} %, in a 200 ml Erlenmeyer flask. Cultivation was carried out for 3 days at 30°C on a rotary shaker at 150 rpm. The enzyme was produced extracellularly. Cells were removed by centrifugation (7,000 rpm, 5 min) and the resultant supernatant was used as the material for purification of the enzyme.

Purification of the enzyme. To 500 ml of the supernatant, ammonium sulfate was added to 80% saturation. The resultant precipitate was collected, dissolved in distilled water and then dialyzed against distilled water containing 1 mM L-cysteine.

The dialysate was applied on a DEAE-Sepharose column (2.5 $\times$ 34 cm) equilibrated with 2.5×10^{-2} M Tris-HCl buffer (pH 7.0) containing 1 mM L-cysteine. The column was washed with 700 ml of the same buffer and then 700 ml of the buffer containing 0.2 M KCl, and then eluted by

[†] Studies on Enzymatic Production of Oligosaccharides. Part V.

This paper was presented at the Annual Meeting of the Agricultural Chemical Society of Japan in Tokyo on April 1~4, 1987 (Abstracts of Papers, p. 78).

linearly increasing the concentration of KCl in the buffer from 0.2 to 0.4 M.

The enzyme fractions were collected, concentrated with a membrane filter (Amicon UM 10) and then re-chromatographed on the same column.

The enzyme solution from the previous stage was applied on a Bio-gel A 0.5 m column (1.5 × 86 cm) equilibrated with 2.5×10^{-2} M Tris-HCl buffer (pH 7.0) containing 1 mM L-cysteine and then eluted with the same buffer. The enzyme fractions were collected and then concentrated with the membrane filter.

Molecular weight estimation. The molecular weight of the enzyme was estimated by the Sephadex G-200 (1.5 × 86 cm) filtration method of Andrews⁹. The column was equilibrated with 2.5×10^{-2} M Tris-HCl buffer (pH 7.0) containing 1 mM L-cysteine and 0.1 M KCl. After application of the purified enzyme on the column, it was eluted with the same buffer at the flow rate of 20 ml/hr. The elution of standard proteins was carried out in the same manner.

Assaying of amylase. Amylase activity was measured in a 1 ml reaction mixture containing 0.5 ml of 2% soluble starch, 0.1 M phosphate buffer (pH 7.0) and the enzyme solution. The reaction was carried out for 10 min at 40°C and the reducing sugar formed was determined by the Sömogyi and Nelson method.^{9,10}

One unit of the enzyme was defined as the amount of enzyme that produced reducing sugar corresponding to one μ mol of maltose from soluble starch in one min under the assay conditions.

Paper chromatography. Paper chromatography for detection of sugar in the hydrolysates of substrates was carried out as described in the previous paper.¹¹

Liquid chromatography. The sugar constituents of hydrolysates of starch or related substances with the enzyme were determined by the high-performance liquid chromatography described in the previous paper.¹¹

Gas chromatography. The anomeric form of the maltose produced from soluble starch with the enzyme was determined according to the method of Kimura and

Chiba.¹²

Electrophoresis. Electrophoresis of the purified enzyme was carried out at a constant current of 12 mA per gel for 2.5 hr on a polyacrylamide gel at pH 8.3. After electrophoresis, the gel was stained with amido black.

Materials. Standard proteins (cytochrome *c*, chymotrypsinogen, albumin from hen egg, albumin from bovine serum, aldolase and ferritin for molecular weight determination) were obtained from Böhringer Mannheim GmbH. Pullulan (MW 86,000), amylose (DP 17), amylopectin (corn), glycogen (oyster) and other reagents were from Wako Chemical Ltd., Japan.

Results

I. Purification of the enzyme

Table I shows the results of purification of the maltose-producing amylase from *Bacillus megaterium* G-2. The specific activity of the final purified material was about 73 times that

Fig. 1. Plot of Elution Volume against Logarithm of Molecular Weight of Proteins on Sephadex G-200.

Column, Sephadex G-200 (1.5 × 86 cm); flow rate, 20 ml/hr; fractions, 2 ml.

1, cytochrome *c*; 2, chymotrypsinogen; 3, albumin (bovine serum); 4, aldolase (rabbit muscle); 5, ferritin; □, the enzyme.

Table I. PURIFICATION OF A MALTOSE-PRODUCING AMYLASE FROM *Bacillus megaterium* G-2

Step	Protein (OD _{280nm})	Amylase (units)	Spec. activity (units/OD _{280nm})	Yield (%)
Culture filtrate	42.7	1780	41.7	100
Ammonium sulfate	7.90	1210	153	68.0
DEAE-Sephadex	1.05	1060	1010	59.6
2nd DEAE-Sephadex	0.46	692	1500	38.9
Bio gel A 0.5 m	0.021	64.0	3050	3.6

Fig. 2. Effects of pH on the Activity and Stability of This Amylase.

—○—, pH-activity curve (the reaction was carried out in a mixture containing 5×10^{-2} M acetate or phosphate buffer at various pH, as indicated, 1% soluble starch and 4.8×10^{-2} units of the amylase at 40°C).

—□—, pH-stability curve [a mixture (0.4 ml) containing 0.1 M acetate or phosphate buffer and 3.7×10^{-1} units of the amylase was kept for 3 hr at 30°C . After adjustment of the pH to 7.0, the residual enzyme activity was measured].

Fig. 3. Effects of Temperature on the Activity and Stability of This Amylase.

—○—, temperature-activity curve [the reaction was carried out in a mixture (1 ml) containing 5×10^{-2} M phosphate buffer (pH 7.0), 1% soluble starch and 1.5×10^{-1} units of the amylase for 30 min].

—●—, heat stability [the enzyme solution (1.1×10^{-1} units) in 0.2 ml of 5×10^{-2} M phosphate buffer (pH 7.0) was heated for 10 min at various temperatures, as indicated, and the residual enzyme activity was measured].

Fig. 4. Paper Chromatograms of Hydrolysates of Several Substrates with This Amylase.

A reaction mixture containing 10 mg of a substrate (pullulan, amylose (DP 17), amylopectin, glycogen, soluble starch or α -cyclodextrin), 5×10^{-2} M Tris buffer (pH 7.0), 0.1 units of the amylase, in a total volume of 1.0 ml, was incubated at 50°C .

of the culture filtrate. The molecular weight of the enzyme was determined by the Sephadex G-200 gel filtration method. As shown in Fig. 1, its molecular weight was estimated to be about 60,000.

II. Properties of the enzyme

The enzymatic properties of the maltose-producing amylase from *B. megaterium* G-2 were examined using the partially purified enzyme (Table I, step 4).

Effects of pH and temperature on the enzyme activity. Figure 2 shows the effects of pH on the activity and stability of this enzyme. The optimum pH was around 7.0 and the amylase was stable in the pH range of 5 to 8.

Figure 3 shows the optimum temperature and heat stability of this amylase. The optimum temperature was about 60°C (30 min reaction). Protection of the amylase from heat in the presence of calcium ions was not observed.

Action pattern of the enzyme. Figure 4 (A) shows a paper chromatogram of the products produced through the action of this amylase on soluble starch, amylose (DP=17), amylopectin and glycogen. Maltose was the sole product from soluble starch, amylose, amylopectin and glycogen, but it did not act on pullulan or α -cyclodextrin. The yields of maltose were about 88% from amylose, about 57% from amylopectin, about 21% from glycogen and about 63% from soluble starch, respectively. Figure 4(B) shows the time course of hydrolysis of starch with this amylase. From the initial stage of the reaction, only maltose was produced.

It has been reported that the maltose-producing amylase from *B. stearothermophilus* produces α -maltose from starch. However, this enzyme produces maltotetraose, maltotriose, maltose, and also traces of G₅ and G₆ in the initial stage of hydrolysis of amylopectin, and with the progress of the hydrolysis, maltotetraose and maltotriose disappear, glucose being formed through the splitting of maltotriose into equimolar amounts of maltose and glucose.⁷⁾ Therefore, it can be said that the action

mechanisms of these two amylases are different.

Figure 5 shows the maximum yields of maltose from liquefied starch (DE 5.81) with this amylase and β -amylase from malt. As can be seen in the figure, the yield of maltose with this amylase was about 2% higher than that obtained with the β -amylase. This indicates that this amylase can hydrolyze the α -1,4-glucosidic linkages closer to the branching points of amylopectin than the β -amylase

Fig. 5. Maximum Yield of Maltose from Liquefied Starch with the Enzyme or β -Amylase.

—○—, the enzyme; —●—, malt β -amylase.

A reaction mixture (1.0 ml) containing 10% liquefied starch (DE 5.81), 4×10^{-2} M Tris buffer (pH 7.0) and various amounts of the amylase or malt β -amylase, as indicated, was incubated for 3 days at 60°C.

Table II. SACCHARIFICATION OF LIQUEFIED STARCH BY THIS AMYLASE, β -AMYLASE AND A MIXTURE OF THEM

Enzyme for pre-saccharification	Second enzyme	Sugar Composition (%)			
		G1	G2	G3	Others
β -Amylase	None	0.0	55.5	4.7	39.8
	β -Amylase	0.0	56.7	5.2	38.1
	The amylase	0.0	58.4	5.7	35.9
The amylase	None	0.0	61.2	5.8	33.0
	β -Amylase	0.0	61.2	6.4	32.4
	The amylase	0.0	61.9	6.1	32.0

The first saccharification was carried out in a mixture containing about 10% liquefied starch (DE 1.43), 5 units of the amylase or β -amylase per g substrate and 4×10^{-2} M Tris buffer (pH 7.0) at 50°C. After one day reaction, the same amount of the other enzyme was added to each reaction mixture, as indicated in the table, and then the reaction was continued at 50°C for one more day.

does. This was confirmed by the results of the combined action of this amylase and the β -amylase on liquefied starch. The results ob-

Fig. 6. Mutarotation of the Product with This Amylase. The reaction mixture containing about 4.2% soluble starch, 4.2×10^{-2} M phosphate buffer (pH 7.0) and 29 units of the partially purified amylase (Table I, Step 4), in a total volume of 3.0 ml, was incubated at room temperature (26°C). After 20 min, 0.1 ml of 10% HgCl_2 was added to stop the reaction.

Fig. 7. Determination of the Anomeric form of Maltose Produced by This Amylase by a Gas Chromatographic Method.

A reaction mixture containing 5 mg potato starch, 5×10^{-2} M phosphate buffer (pH 7.0), 1 unit of the purified amylase (Table I, Step 4), in a total volume 0.25 ml, was incubated at 25°C. Aliquots of 50 μl of the reaction mixture were pipetted out after 1, 3 and 5 min reaction, followed by conversion to trimethylsilyl compounds according to the method of Kimura and Chiba.¹²⁾ Column, OV-17; temperature, from 190 to 330°C, at the rate of 4°C/min; carrier, nitrogen gas, at 5 ml/min.

1, sucrose (internal standard); 2, α -maltose; 3, β -maltose.

tained are shown in Table II. After hydrolyzing the liquefied starch with the β -amylase or this amylase, the reaction was continued by adding this amylase or the β -amylase (or without the addition of a second enzyme), respectively, in the manner described in the legend to the table. When this amylase was used, the yield of maltose was higher than that in the case of the β -amylase, and such an increase in maltose was also obtained through the successive action of this amylase on a hydrolysate obtained with the β -amylase. However, such an increase in maltose was not obtained through the successive action of the β -amylase

Table III. INHIBITION OF THE ENZYME BY *p*-CHLOROMERCURIBENZOATE AND MONOIODOACETATE, AND REACTIVATION WITH CYSTEINE

<i>p</i> -CMB (M)	MIA (M)	Cysteine (M)	Relative activity (%)
None	None	None	100
2×10^{-3}	None	None	30.5
2×10^{-3}	None	2×10^{-2}	100
None	2×10^{-3}	None	70.1
None	2×10^{-2}	2×10^{-2}	100

p-CMB, *p*-chloromercuribenzoate; MIA, monoiodoacetate.

After a mixture containing 2×10^{-3} M *p*-chloromercuribenzoate or monoiodoacetate, 5×10^{-2} M Tris buffer (pH 7.0) and 5×10^{-2} units of the enzyme had been incubated for 1 hr at room temperature, 2×10^{-2} M cysteine was added, followed by incubation for a further 1 hr at room temperature.

Table IV. EFFECT OF PULLULANASE ON HYDROLYSIS OF LIQUEFIED STARCH BY THE ENZYME

Pullulanase (units/g)	G1 (%)	G2 (%)	G3 (%)	Others (%)
0	0.0	59.9	0.0	40.1
1	0.0	81.7	4.6	13.7
2	0.0	81.9	4.7	13.4
5	0.0	81.4	7.7	10.9
10	0.0	80.9	9.7	9.4

A reaction mixture containing 100 mg of liquefied starch (DE 1.43), 4×10^{-2} M Tris buffer (pH 7.0), 5 units of the enzyme and pullulanase (from *Klebsiella pneumoniae*), as indicated, in a total volume of 1.0 ml, was incubated for 4 days at 55°C.

on the hydrolysate obtained with this amylase.

Anomeric form of the maltose produced. The anomeric form of the maltose produced by this amylase was examined by means of both polarimetric and gas chromatographic methods.

Figure 6 shows the mutarotation of the hydrolysis product derived from soluble starch with this enzyme. A decrease in the optical rotation was observed after cessation of the reaction. This indicates that the product is of the α -configuration.

The gas chromatographic method used for determining the anomeric form of the product was that of Kimura and Chiba.¹²⁾ The results obtained are shown in Fig. 7 (B). Although a very small amount of β -maltose, which seemed to be due to mutarotation of the α -maltose formed, was detected, the maltose also appeared to be of the α -form.

Inhibitors. The effects of metal ions and sulfhydryl reagents, such as *p*-chloromercuribenzoate and moniodoacetate, on this amylase were examined. The amylase was inhibited by Hg^{2+} , Ag^+ , Cu^{2+} , Ni^{2+} , Fe^{2+} , Zn^{2+} , *p*-chloromercuribenzoate and moniodoacetate, as shown in Table III. These characteristics are similar to those of β -amylases from plants and *Bacillus* sp., and the inhibition by *p*-chloromercuribenzoate and moniodoacetate was reversed with the addition of cysteine (Table III).

Effect of pullulanase on hydrolysis of liquefied starch by this amylase. This amylase could not hydrolyze α -1,6-glucosidic linkages. Therefore, the effect of pullulanase on the saccharification of liquefied starch with this amylase was examined. The results obtained are shown in Table IV. The presence of pullulanase remarkably increased the yield of maltose, similar to in the case of the β -amylase.

Discussion

There are many known amylases that produce maltose from starch. Of them, β -amylase is the sole enzyme which strictly hydrolyzes starch with maltose units, from its non-reducing ends, and produces β -maltose. It was found that a strain of *Bacillus megaterium* G-2, newly isolated from soil and identified by the author, produces a new type of maltose-producing amylase in the culture medium. The enzyme was purified to near homogeneity, as judged on polyacrylamide gel electrophoresis, and its enzymatic properties were examined in comparison with those of β -amylase. Its optimum pH and temperature were around 7.0 and around 60°C, respectively. It thus has a higher optimum pH and a little higher thermostability, compared with those of plants β -amylases, as shown in Table V. This enzyme produced only maltose from starch, amylose,

Table V. COMPARISON OF MALTOSE-PRODUCING AMYLASES FROM PLANTS AND MICROORGANISMS

Properties	β -Amylase				α -Amylase	
	Malt	Soybean	Sweet potato	<i>Bacillus</i>	<i>B. stearothermophilus</i>	<i>B. megaterium</i> G-2
Action	Exo	Exo	Exo	Exo	Exo	Exo
Optimum pH	5.2	5.5~6.0	4~5	7.0	5.3	7.0~7.5
Optimum temp.	55~60	50~60	55~60	50	60	60
Action on starch	β -Maltose	β -Maltose	β -Maltose	β -Maltose	α -Maltose (G1, G3, G5, G6)	α -Maltose
Action on CD	—	—	—	—	+	—
Action on G3	Bad	Bad	Bad	Bad	Good	Bad
Inhibition by <i>p</i> -CMB	+	+	+	+	+	+
Molecular weight ($\times 10^3$)			15.2	3.5 $\pm$ 0.5	70	60

CD, cyclodextrin; G3, maltotriose; *p*-CMB, *p*-chloromercuribenzoate.

amylopectin and glycogen from the initial stage of the reaction, a limit dextrin being finally left in the case of starch. The presence of a debranching enzyme during the saccharification of starch with this enzyme remarkably increased the yield of maltose.

It was found that the maximum yield of maltose from starch with this enzyme was about 2% higher than that with β -amylase, and the maltose produced by this enzyme is of the α -form, as found in both polarimetric and gas chromatographic experiments. From this hydrolysis mechanism for starch and the higher yield of maltose with this enzyme compared with β -amylase, it seemed that this amylase can hydrolyze starch closer to the branching points than β -amylase does.

This enzyme was inhibited by sulfhydryl reagents such as *p*-chloromercuribenzoate, monoiodoacetate and Hg^{2+} , and the inhibition by *p*-chloromercuribenzoate and monoiodoacetate was reversed with the addition of cysteine. This characteristic was similar to that of β -amylase.

Outtrup and Norman⁷⁾ reported that 1) the maltose-producing amylase from *Bacillus stearothermophilus* produced α -maltose by hydrolysing amylose, amylopectin and related glucose polymers through an exo-mechanism, 2) the enzyme was not inhibited by sulfhydryl reagents, 3) the enzyme exhibited an endo-action, hydrolyzing cyclodextrin to maltose

and glucose, and 4) the enzyme completely hydrolyzed maltotriose to maltose and glucose. Characteristics 1) to 4) are completely different from those of β -amylase, and characteristics 2), 3) and 4) are also different from those of the maltose-producing amylase of *B. megaterium* G-2. It can be said that the enzymatic properties of β -amylase, other than anomeric form of the product, are more similar to those of the amylase from *B. megaterium* G-2 than those of the enzyme from *B. stearothermophilus*.

References

- 1) E. Kneen and L. D. Beckord, *Arch. Biochem.*, **10**, 41 (1946).
- 2) D. Rose, *Arch. Biochem.*, **16**, 349 (1948).
- 3) M. Higashihara and S. Okada, *Amylase Symposium* (Japan), **6**, 39 (1971); *Agric. Biol. Chem.*, **38**, 1023 (1974).
- 4) R. Shinke, Y. Kunimi and H. Nishira, *J. Ferment. Technol.*, **53**, 687, 693 (1975).
- 5) Y. Takasaki, *Agric. Biol. Chem.*, **40**, 1515 (1976); **40**, 1523 (1975).
- 6) H. Hidaka, Y. Koaze, K. Yoshida, T. Niwa, T. Shomura and T. Niida, *Starch*, **12**, 413 (1974).
- 7) H. Outtrup and B. E. Norman, *Starch*, **36**, 405 (1984).
- 8) P. Andrews, *Biochem. J.*, **91**, 222 (1964).
- 9) M. Somogyi, *J. Biol. Chem.*, **160**, 61 (1945).
- 10) N. Nelson, *J. Biol. Chem.*, **153**, 357 (1944).
- 11) Y. Takasaki, *Agric. Biol. Chem.*, **46**, 1539 (1982).
- 12) A. Kimura and S. Chiba, *Agric. Biol. Chem.*, **47**, 1747 (1983).