Preparation and Some Properties of a Novel Maltotetraose-Forming Enzyme of *Pseudomonas saccharophila*

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It was found that *Pseudomonas saccharophila* produced a maltotetraose-forming enzyme. The enzyme was purified by ammonium sulfate fractionation and column chromatography on DEAE-Toyopearl 650M and Toyopearl HW-55s.

The activity recovery was 19% at the final step of the purification. The purified enzyme was homogeneous electrophoretically and its molecular weight was 62,000. The optimum pH and temperature were 6.7 and 55°C, respectively. The enzyme was stable up to 40°C in the pH range of 5.5 to 10.5 for 1 hr, and thermostable in the presence of 2 mM CaCl₂ up to 45°C.

The isoelectric point of the enzyme was 4.7. The enzyme activity was inhibited by metal ions such as Ag⁺, Hg²⁺, Co²⁺, Cu²⁺, Fe³⁺, Al³⁺, Zn²⁺, and enhanced by Sr²⁺.

The enzyme specifically produced maltotetraose from starch, did not act on glucose, maltose, maltotriose, and maltotetraose.

The enzyme action proceeded from non-reducing ends of the substrates and seemed to be difficult skipping over the branches of amylopectin.

Maltooligosaccharides (G₃-G₆) have been used as reagents for research and as clinical reagents for the determination of serum amylase activity.¹⁾ G₅ has been used as a nutrient for calorie deprivation.²⁾ This oligosaccharide, as well as G₃, G₄ and G₆ is highly soluble in water and produce clear viscous solutions which are palatable. Therefore, these compounds may potentially be of use as superior nutrients for infants and aged persons. The price of pure maltooligosaccharides is, however, extremely high, because of the difficulties encountered in producing them in a pure state. Birch and Etheridge separated a low molecular weight fraction (LMF) containing G₁-G₆ and a high molecular weight fraction (HMF) containing polymers higher than G₇ by ethanol fractionation.³⁾ Both

fractions were used in feeding experiments with rats. Upwards trends of most tissue lipids and weight gains were observed to correlate with the increasing molecular weight fraction in the order of G₁ < LMF < HMF.

Martin and Young reported that G₃ was highly digestible and usable as a nutrient in comparison to G₁.⁴⁾

Recently in Japan, physiologically active (or bio-active) food is being developed at a rapid pace. In the course of the development it is known that maltooligosaccharides such as G₃, G₄, G₅, and G₆ have antibiotic properties against plant disease bacteria such as *Erwinia carotovora*⁵⁾ and they also act as specific growth depressors of detrimental bacteria such as *Clostridium perfringens* in the intestines.⁶⁾

Several reports have been published on the production of maltooligosaccharides such as G₂, G₃, G₄, G₅, etc., from starch. G₂ production from

Symbols, G₁, G₂, G₃, G₄, G₅, G₆...G₁₀ are glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose...maltodecaose.

starch has been widely studied using plant β -amylases. As for G_3 - G_6 , G_3 -forming amylases were discovered in *Streptomyces griseus* NA-4687⁷⁾ and *Bacillus subtilis*,⁸⁾ and *Bacillus circulans*⁹⁾ (its enzyme also forms G_5), and G_5 -forming amylases in *Bacillus licheniformis*¹⁰⁾ and *Pseudomonas* species.¹¹⁾ Yoshigi *et al.* found that G_5 was accumulated in the culture broth of *Bacillus cereus* NY-14.¹²⁾ G_6 -forming amylases were discovered in *Aerobacter aerogenes* (*Klebsiella pneumoniae*),¹³⁾ *Bacillus circulans*,^{14,15)} and *Bacillus subtilis*.¹⁶⁾

It was reported that a G_4 -forming amylase was produced by *Pseudomonas stutzeri*.¹⁷⁾ The objective of this study was finding other bacteria which produce more stable G_4 -forming amylase than that of *Pseudomonas stutzeri*.

Pseudomonas saccharophila was found to produce that enzyme.

MATERIALS AND METHODS

Materials. Soluble starch GR was purchased from Merck. Reduced soluble starch was prepared by reducing soluble starch with sodium borohydride (pure grade, Wako Pure Chemical Ind., Ltd.), by a modification of the method of Kainuma *et al.*^{11,18)} All other chemicals used were commercially available and of chemically pure grade.

Purification of the enzyme. *Pseudomonas saccharophila* (IAM 1504) was cultured, using a rotary shaker, in a liquid medium containing 1.0% soluble starch, 1% polypeptone (Daigo-eiyokagaku, Osaka, Japan), 0.1% KH_2PO_4 , 0.28% K_2HPO_4 , pH 7.0 in an Erlenmeyer flask for 48 hr at 30°C. The cells were removed by centrifugation at 10,000 rpm for 15 min at 4°C. The supernatant was fractionated by ammonium sulfate precipitation. The fraction, which precipitated between 0.3 to 0.5 ammonium sulfate saturation, was collected and dissolved in a 10 mM phosphate buffer solution (pH 7.0).

The enzyme solution was dialyzed against the same buffer solution at 4°C for 24 hr. After removal of insoluble materials by centrifugation, the dialyzed enzyme was chromatographed on a DEAE-Toyopearl 650M (Tosoh Co., Ltd.) column (26.4 × 420 mm), which was eluted

using a 10 mM phosphate buffer solution (pH 7.0) and then using a linear gradient of 0 (1 l) to 0.5 M sodium chloride (1 l) in the same buffer solution. Elution was performed at the rate of 200 ml/hr.

The concentrated enzyme fraction was re-chromatographed on a Toyopearl 650M column (1.6 × 100 mm) using a linear gradient of 0 (200 ml) to 0.3 M sodium chloride (200 ml) at a flow rate of 40 ml/hr.

The concentrated enzyme from the 2nd chromatography was chromatographed twice using Toyopearl HW-55s column (1.6 × 100 mm) at a flow rate of 9.5 ml/hr with a 10 mM phosphate buffer solution (pH 7.0). Pure fractions, which were checked by disc gel electrophoresis, were collected to obtain the pure enzyme solution (75 IU/ml in 10 mM phosphate buffer, pH 7.0).

Determination of the G_4 -forming enzyme activity. Half a milliliter of a 2% reduced starch solution, 0.4 ml of 0.1 M phosphate buffer (pH 7.0) and 0.1 ml of the enzyme solution were mixed, followed by incubation at 30°C. An aliquot of the reaction mixture was periodically removed and the reducing sugar formed was determined by the Somogyi-Nelson method.¹⁹⁾ One unit was defined as the amount of 1 μ mol G_4 liberated under the above conditions.

Paper chromatography and autoradiography. Ten to fifty μ l of the reaction mixture was spotted on a sheet of filter paper (Toyo Roshi No. 50). The paper was then irrigated twice in the ascending mode with a solvent system of 1-butanol : pyridine : water (6 : 4 : 4, v/v at 60°C. After the irrigation, the chromatogram was treated with glucoamylase²⁰⁾ and then stained by the silver nitrate dip method.²¹⁾ In order to examine the action of the enzyme on maltooligosaccharides, two-dimensional paper chromatography was performed by the method of Kainuma and French,²⁰⁾ using 50 μ l each of a standard series of maltooligosaccharides.

A standard series of maltooligosaccharides was prepared by mixing 1 g of α -cyclodextrin, 1 g of maltose and 150 THU of cyclodextrin glucanotransferase (macrans enzyme), adjusting the po to 6.0 (no addition of buffer solution), followed by reaction at 40°C for 17 hr. The

reaction mixture (100 ml) was stood in a boiling bath for 10 min to inactivate the enzyme and then insoluble material was removed by centrifugation. The supernatant was used as the standard.

A two-dimensional autoradiogram was prepared in the same manner except that a ^{14}C reducing end-labelled maltooligosaccharide series²²⁾ was applied and irrigation was performed 3 times. The dried chromatogram was put into contact with a sheet of X-ray film (Fuji X-ray film, RX, 25.4×30.5 cm), followed by exposure for 2 weeks.

Electrophoresis and molecular weight determination. Polyacrylamide gel electrophoresis (PAGE) in the absence of SDS was performed by using 7.5% gel. SDS-PAGE was performed by the method of Weber and Osborn,²³⁾ using 5% gel and a series of proteins of known molecular weights (Seikagaku Kogyo Co., Ltd., Japan; SDS-PAGE Marker I, thermostable RNA polymerase B). The isoelectric point of the enzyme was measured by polyacrylamide gel electrofocusing as described by Wrigley²⁴⁾ using Ampholine, with a pH range of 3.5 to 10, as a carrier.

Measurement of mutarotation of the G_4 formed. The reaction was started by adding 200 μl of the purified enzyme (3 IU) to 4 ml of 1% soluble starch in 0.1 M phosphate buffer (pH 7.0) in a jacketed cell (10×50 mm) at 40°C, with an automatic digital polarimeter (Nihon Bunko Co., Ltd.; DIP-181), and the change in optical rotation was recorded. After 60 min, 5 mg of sodium carbonate was added and the mutarotation was measured.

Determination of the optimum reaction conditions and enzyme stability. To determine the optimum pH of the reaction, the reducing value was determined after 10 min incubation at 40°C. The reaction mixture consisted of 0.5 ml of a 2% reduced soluble starch solution, 0.4 ml of a 0.1 M buffer solution of different pH values and 0.1 ml of the enzyme solution (0.3 IU/ml in water).

For determination of the pH stability, 0.1 ml of the enzyme solution was mixed with 0.9 ml of a 10 mM buffer solution of various pH values (acetate buffer for pH 3.5–6, phosphate buffer for pH 6–8, and sodium carbonate/bicarbonate

buffer for pH 9–11), followed by standing for 60 min at 30°C. After this treatment, 0.5 ml of 2% reduced soluble starch, 0.4 ml of 0.1 M phosphate buffer (pH 7.0) and 0.1 ml of the treated enzyme solution were mixed and incubated for 10 min at 40°C, and then the remaining activity was determined.

The optimum temperature of the reaction was determined by incubating 0.1 ml of the enzyme solution with 0.4 ml of a 0.1 M phosphate buffer solution (pH 7.0) and 0.5 ml of a 2% reduced soluble starch solution for 10 min at various temperatures.

For determination of the thermostability, 0.1 ml of the enzyme solution (0.3 IU) was incubated with 0.4 ml of a 0.1 M acetate buffer solution (pH 6.0) at various temperatures for 30 min, and then 0.5 ml of a 2% reduced soluble starch solution was added and the reducing sugar formed was determined after incubation for a further 10 min at 40°C.

To determine the effect of calcium ions on the thermostability, the enzyme in 0.1 M acetate buffer (pH 6.0) was incubated for 10 min at various temperatures in the presence and absence of 2 mM calcium chloride, and then the remaining activity was determined.

Effects of various metal ions and chemicals. To determine the effects of various metal ions and chemicals, 0.1 ml of the dialyzed enzyme solution (0.3 IU) was mixed with 0.4 ml of a 0.1 M acetate buffer solution containing various metal ions or chemicals and, after standing for 30 min at 30°C, 0.5 ml of a 2% reduced soluble starch solution was added to react for 10 min at 40°C. The final concentration of the various metal ions and chemicals was 1 mM.

Relative rate of the enzyme reaction on various substrates. A reaction mixture, consisting of 0.5 ml of a 2% substrate solution, 0.4 ml of a 0.1 M phosphate buffer solution and 0.1 ml of the enzyme solution (0.3 IU), was incubated for 10 min at 40°C and then the reducing sugar formed was determined.

RESULTS

Purification of the enzyme. At the ammonium sulfate fractionation step, the recovery of G_4 -forming enzyme activity was 95%. Seventy

Fig. 1. First DEAE-Toyopearl 650M column chromatography for the G_4 -forming enzyme purification.

The fractions having the activity (5 ml/fraction) in tube numbers 127–142 were collected, dialyzed and concentrated to 15 ml for the second column chromatography.

percent of the enzyme was recovered from a DEAE-Toyopearl 650M column at the concentration of 0.1–0.2 M sodium chloride (Fig. 1). Subsequently, on the rechromatography on a DEAE-Toyopearl 650M column, only one symmetrical protein peak associated with the enzyme activity was obtained. The enzyme was further purified by gel filtration on Toyopearl HW-55s twice (Fig. 2). At this stage, the enzyme preparation gave a single band on disc gel electrophoresis (ref. Fig. 11). A summary of the purification is given in Table 1. At the final stage, the enzyme was purified about 150-fold and had a specific activity of 434 IU/mg. The activity recovery was 19%.

Properties of the enzyme. Figure 3 shows the optimum pH and pH stability of the enzyme. The optimum pH of the enzyme was 6.7, and the enzyme retained more than 90% of its activity between pH 5.5 and 10.5.

Figure 4 shows the optimum reaction temperature and thermostability of the enzyme. The optimum temperature of the enzyme was 55°C. As to thermostability, the enzyme was stable up to 40°C, and in the presence of 2 mM calcium chloride the stability was increased by 5°C (Fig. 5).

Figure 6 shows molecular weight determination of the enzyme by SDS-PAGE. The electrophoresis was carried out using thermostable

Fig. 2. The second Toyopearl HW-55s column chromatography for the G_4 -forming enzyme purification.

The fractions having the activity (9.5 ml/fraction) in tube numbers 30–33 were collected and concentrated, and the concentrated enzyme solution was applied on the second gel filtration column.

RNA polymerase B as a standard, which has five subunits (M. W.: α , 39,000; α' , 42,000; χ , 100,000; β , 140,000; β' , 180,000).

The molecular weight of the enzyme was estimated on SDS-PAGE to be 62,000. The pure enzyme preparation gave a single protein band which exhibited amylase activity, on the zymogram. The isoelectric point was 4.7. The

Table 1. Purification scheme of the G₄-forming enzyme.

Step	Volume (ml)	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg protein)	Recovery (%)
Culture sup	810	10686	3504	3.05	100
(NH ₄) ₂ SO ₄ ppt ^{a)}	50	10149	186	54.6	95
DEAE-Toyopearl 650M (1st)	70	7530	21.9	344	70
DEAE-Toyopearl 650M (2nd)	24	7088	18.0	394	66
Toyopearl HW-55s (1st)	12	3787	8.50	446	35
Toyopearl HW-55s (2nd)	27	2039	4.70	434	19

^{a)} 0.3–0.5 saturated (NH₄)₂SO₄.

Fig. 3. Optimum pH and pH stability of the G₄-forming enzyme.

●—●, optimum pH ; ○—○, pH stability. pH 3.5–6, acetate buffer ; pH 6–8, phosphate buffer ; pH 9–11, carbonate/bicarbonate buffer.

molecular weight of the enzyme was also estimated as 61,000 by using gel column chromatography of Toyopearl HW-55s.

Table 2 shows the effects of various metal ions and chemicals on the enzyme activity. The enzyme action was slightly enhanced by Sr²⁺, but was not affected by Ca²⁺ and Rb⁺ at 1 mM. On the other hand, the activity was inhibited by Mn²⁺, Zn²⁺, Al³⁺, Fe³⁺, Cu²⁺, Co²⁺, Ag⁺ and Hg²⁺ in this order. At 1 mM, iodoacetamide, SDS and L-cysteine did not affect the activity but *p*-chloromercuribenzoic acid considerably affected it.

Hydrolysis of various substrates. The mutarotation of the product from soluble starch during the enzyme action was examined. Fig-

Fig. 4. Optimum temperature of the G₄-forming enzyme.

ure 7 shows the rate of the change in optical rotation with time. A decrease in the optical rotation after the addition of sodium carbonate was observed. This suggests that the hydrolysis product is of the α -configuration. The relative initial rate of the enzyme reaction on various substrates was determined and the results are presented in Table 3. Soluble starch, waxy starch (amylopectin), reduced soluble starch, and oyster glycogen were rapidly hydrolyzed, but pullulan, α - and β -cyclodextrins were not hydrolyzed at all.

Figure 8 shows the action of the enzyme on soluble starch. The enzyme specifically produced G₄ from soluble starch during the reaction, but it was quite difficult for the action to proceed over the branching points of branched substrates such as amylopectin, because that β -limit dextrin was resistant to the action as

Fig. 5. Effect of Ca^{2+} on the thermostability of the G_4 -forming enzyme.

●-●, without Ca^{2+} ; ○-○, with Ca^{2+} .

Fig. 6. Molecular weight determination of the G_4 -forming enzyme by SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis was carried out using thermostable RNA polymerase B as a standard (M. W. : α , 39,000 ; α , 42,000 ; χ , 100,000 ; β , 140,000 ; β' , 180,000).

in Table 3.

Figure 9 shows a two-dimensional paper chromatogram illustrating the action of the enzyme on maltooligosaccharides. G_1 , G_2 , G_3 , and G_4 were not converted to any other saccharide.

Through the action, $\text{G}_1 + \text{G}_4$ were formed from G_5 , and $\text{G}_2 + \text{G}_4$ from G_6 , $\text{G}_3 + \text{G}_4$ from

Table 2. Effects of metal ions and chemicals on the enzyme activity.

Material (1 mM)	Residual activity (%)
None	100
SrCl_2	114
MgCl_2	108
Li_2SO_4	105
CaCl_2	99
RbCl	99
MnSO_4	58
ZnSO_4	29
$\text{Al}_2(\text{SO}_4)_3$	19
FeCl_3	5
CuSO_4	3
CoCl_2	1
AgNO_3	0
HgCl_2	0
L-Cysteine	93
$\text{CH}_2\text{I}(\text{CONH}_2)$	89
SDS	89
<i>p</i> -CMB (0.4 mM) ^{a)}	37

^{a)} *p*-Chloromercuribenzoic acid.

Fig. 7. Mutarotation of the product of the G_4 -forming enzyme action on soluble starch.

Table 3. Relative reaction rate of the enzyme reaction on various substrates.

Substrate (final concentration 1%)	Relative reaction rate (%)
Soluble starch	100
Waxy starch (amylopectin)	95
Reduced soluble starch	90
Glycogen (oyster)	67
β -Limit dextrin (glycogen)	0
Pullulan	0
α -Cyclodextrin	0
β -Cyclodextrin	0

Fig. 8. Paper chromatogram showing the time course of G_4 -forming enzyme action on soluble starch.

A mixture composed of final 1% concentration of soluble starch, enzyme concentration 5 IU/g substrate and final 10 mM phosphate buffer (pH 7.0) was reacted at 30°C, and periodically sampled (15 μ l) to spot on the paper.

Fig. 9. Two-dimensional paper chromatogram showing the action pattern of the G_4 -forming enzyme on maltooligosaccharides.

R_1 and R_2 denote reference series of maltooligosaccharides for the first and second directions of the chromatogram. S is the application point of maltooligosaccharide series. The amount applied was 50 μ l of a standard maltooligosaccharide series solution (sugar concentration, 2%). After irrigation in the first direction, the lower side of the chromatogram containing the R_1 channel was cut off for reference. The remaining S channel was uniformly sprayed with 10 ml of a dilute enzyme solution (100 μ l of the original enzyme solution, 75 IU/ml+10 ml of water). After allowing the enzyme action to proceed on the paper in a moist chamber at 45°C for 1 hr, the chromatogram was dried in an oven (100°C) for 15 min, R_2 was applied and then the chromatogram was irrigated again in the second direction.

Fig. 10. Two-dimensional autoradiogram showing the action pattern of the G_4 -forming enzyme on maltooligosaccharides.

The experimental conditions were the same as in Fig. 9, except that applied sample was 100 μ l of a ^{14}C -labelled maltooligosaccharide series.

Fig. 11. Comparison of *Pseudomonas saccharophila* and *stutzeri* G_4 -forming enzyme on disc gel electrophoresis.

I, *Pseudomonas saccharophila* G_4 -forming enzyme; II, *Pseudomonas stutzeri* G_4 -forming enzyme. (1), Polyacrylamide disc gel electrophoresis (stained with Coomassie blue); (2), Zymogram (stained with an iodine solution).

Table 4. Comparison of G₄-forming enzyme from *Ps. saccharophila* and *Ps. stutzeri*.

	<i>Ps. saccharophila</i>	<i>Ps. stutzeri</i>
Action pattern	Exo	Exo
Anomer configuration	α	α
Optimum pH	6.7	6.5
pH stability	5.5~10.5	6.5~11
Optimum temperature	50~55°C	45°C
Thermostability		
without Ca ²⁺	<40°C	<30°C
with Ca ²⁺	<45°C	—
Molecular weight	62,000	57,000
Isoelectric point	4.7	5.3
Inactivated by	Hg ²⁺ , Ag ⁺ , Co ²⁺ , Cu ²⁺ , Fe ³⁺	Hg ²⁺ , Cu ²⁺

G₇, and G₄+G₄ from G₈, respectively.

Figure 10 shows a two-dimensional autoradiogram illustrating the action of the enzyme on maltooligosaccharides which were ¹⁴C labelled at each reducing end.

A radioactive fragment of G₁ was formed from G₅, and G₂ from G₆, G₃ from G₇, G₄ from G₈, G₁ from G₉ and so on through the action of the enzyme on the paper.

From the results, the action of the G₄-forming enzyme can proceed from the non-reducing end of the substrates.

DISCUSSION

The G₄-forming enzyme hydrolyzes substrates, seems to hardly skip over the branch-points of branched substrates such as amylopectin and glycogen, to specifically produce G₄ during the reaction.

It will be useful to determine the degradation limit of a branched substrate with the enzyme and the length of the branches on the stem of substrates. The details of the action of the enzyme on branched substrates will be reported elsewhere.

As for the production of G₄, in the case of using amylopectin, addition of pullulanase or other kinds of debranching enzymes is necessary to enhance the yield. On the contrary, without debranching enzyme, it will be quite effective to separate G₄ from the reaction mixture of amylopectin by precipitating with

organic solvents such as ethanol, acetone and so on and by use of ultra filtration membrane or a membrane reactor, because the reaction mixture contains G₄ and large molecular weight of dextrans.

The action of the novel G₄-forming enzyme (*saccharophila* enzyme) is quite similar to that of the previously reported G₄-forming enzyme of *Pseudomonas stutzeri*,¹⁷⁾ but it is considerably different from that of *stutzeri* enzyme in the following respects: *saccharophila* enzyme is more stable than *stutzeri* enzyme on pH and temperature; molecular weight of *saccharophila* enzyme is larger than that of *stutzeri* enzyme; and their isoelectric points are quite different from each other. Consequently, the mobility on disc gel electrophoresis is different as shown in Fig. 11. The properties of both enzymes are summarized in Table 4.

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シュードモナス・サッカロフィラの新規マルトテトラオース生成酵素の調製とその性質

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Pseudomonas saccharophila がマルトテトラオース生成酵素を生産することを見出した。本酵素を硫酸分画, DEAE-Toyopearl 650M と Toyopearl HW-55s のカラムクロマトにより精製した結果, 電気泳動的に単一のピークを示す最終精製段階では 19% の回収率であった。本酵素の分子量は 62,000, 至適 pH および至適温度はおおの 6.7 と 55°C, 安定 pH 領域は 5.5~10.5, 40°C まで安定であり, CaCl₂ の添加により 45°C にまで温度安定性は増大した。また, 等電点は 4.7 であり, 本酵素活性は Ag⁺, Hg²⁺, Co²⁺, Cu²⁺, Fe³⁺, Al³⁺, Zn²⁺ などの金属イオンにより阻害を受け, Sr²⁺ の添加により上昇した。

本酵素は澱粉からマルトテトラオースを特異的に生成し, グルコース, マルトース, マルトトリオース, マルトテトラオースには作用せず, 反応は基質の非還元末端から進行した。また, アミロペクチンの分岐点を越えての反応は困難であると推測された。