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Properties of the enzyme expressed by the *Pseudomonas saccharophila* maltotetraohydrolase gene (*mta*) in *Escherichia coli*

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ABSTRACT

The maltotetraohydrolase gene (*mta*) from *Pseudomonas saccharophila* was expressed in *Escherichia coli* JM109. Maltotetraohydrolase was produced mostly (~90%) in the periplasmic space. The amino-terminal amino acid sequence and molecular weight of the recombinant enzyme were identical with those of the native enzyme, and there was no significant difference in the substrate specificity and modes of action. This system for maltotetraohydrolase expression is useful for studies of the structure and function of the enzyme.

INTRODUCTION

Amylases can be classified as endo-type, such as alpha-amylase (EC 3.2.1.1), which hydrolyses D-glucans endo-wise to produce malto-oligosaccharides, and exo-type, like beta-amylase (EC 3.2.1.2) and glucoamylase (EC 3.2.1.3). Amylases[1–3], which form malto-oligosaccharides from the non-reducing ends of D-glucans, belong to the latter group.

Maltotetraohydrolases (EC 3.2.1.60, G_4-forming amylases)** are found in the bacteria *P. stutzeri*[2] and *P. saccharophila*[4], their enzymic properties are very similar[4–6], and their genes have been cloned[7,8]. The nucleotide sequences indicate a high degree of similarity in the primary structures. It is reasonable to consider these enzymes as essentially identical.

Since G_4-forming amylase produces only maltotetraose (G_4) from various D-glucans, it is of interest to study the molecular basis for this specificity. In order to achieve this goal, a protein expression system is required and we now report on the properties of the enzyme expressed by the G_4-forming amylase gene (*mta*) in *E. coli*.

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** G_n refers to malto-oligosaccharides with *n* glucose residues.

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EXPERIMENTAL

Measurements of enzyme activity. — The periplasm fraction of *E. coli* was prepared by the spheroplast-formation method[9]. The native and recombinant G_4-forming amylases were purified as described[4]. The activity of each enzyme was measured in a mixture (0.5 mL) consisting of 10mM potassium phosphate (pH 7.0), 2.5% of reduced soluble starch, and an appropriate amount of the enzyme. Each mixture was incubated for 20 min at 30°, and the reducing power generated was measured by the Somogyi–Nelson method[10]. One unit of activity is defined as the amount of enzyme which catalyses the generation of reducing power equivalent to 1 μmol of maltose per min.

SDS–PAGE and Western blot analysis. — Proteins were subjected to SDS–PAGE (polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate) as described by Laemmli[11], and transferred electrophoretically to Immobilon-P membranes (Millipore), using a Sartorius Semi-Dry Electroblotter. The blot was probed by rabbit anti-G_4-forming amylase antibodies. Goat anti-rabbit IgG horseradish peroxidase conjugate (Jackson Immunoresearch Laboratory) was used to detect positive bands, according to the method described[12].

T.l.c. — Silica Gel 60 F_{254} (Merck) was used with development for 90 min at 60°, using 1-butanol–1-propanol–water (3:5:4), and detection by charring with sulfuric acid.

RESULTS

A 2.8-kbp DNA fragment was prepared from a recombinant plasmid (pGF11) which contained the *P. saccharophila mta* gene[13] by restriction-enzyme digestion with *Hin*dIII and *Sac*I. After blunt-end treatment, the fragment was inserted into the *Hin*cII site of a plasmid vector pUC19 (see ref. 14). The resulting plasmid (pGF45) was transfected into *E. coli* JM109.

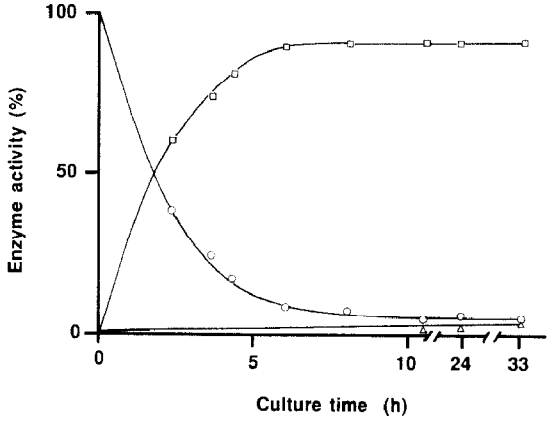

Fig. 1. Distribution of the recombinant G_4-forming amylase expressed in *E. coli*. A suspension (1 mL) of cells was inoculated in LB medium (100 mL; 1% tryptone, 0.5% yeast extract, 0.5% NaCl) and incubated at 37° with constant shaking. The activity of the enzyme was measured in extracts from the periplasmic (□) and cytoplasmic fractions (○), and culture medium (△).

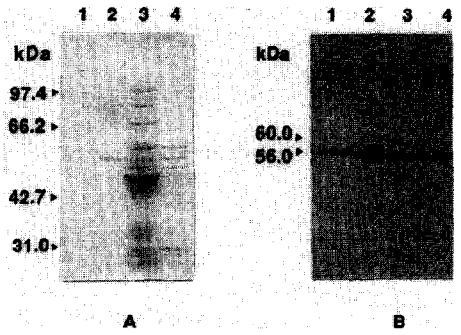

Fig. 2. SDS–PAGE and Western blot analyses of the native and recombinant G_4-forming amylases. Proteins (50 μL) were treated for 3 min at 100° with 20mM Tris/HCl (50 μL, pH 6.8), 2% of SDS, 2% of 2-mercaptoethanol, 16% of glycerol, and 0.04% of Bromophenol Blue, and then applied to 10% polyacrylamide gels. After electrophoresis, the gel was either stained with Coomassie Brilliant Blue (A), or transferred to an Immobilon-P membrane and reacted with anti-G_4-forming amylase antibodies (B). Lane 1, purified G_4-forming amylase from *P. saccharophila*; lane 2, purified recombinant enzyme; lane 3, crude extract from the whole cells of *E. coli*; lane 4, crude extract from the periplasmic space.

When the transfected cells were incubated for 2 h, ~60% of the total G_4-forming amylase activity was secreted into the periplasmic space of the cells (Fig. 1). Production of the enzyme increased during the logarithmic phase of growth, and then remained at a constant level (~90%) in the stationary growth phase. Only 4% of the total enzyme activity was found in the culture medium after incubation for 33 h.

In order to assess the molecular weight of the G_4-forming amylase expressed, crude extracts from the whole-cell and periplasm fractions were analysed by SDS–PAGE and Western blotting (Fig. 2, lanes 3 and 4). The molecular weight of the protein in a band that reacted with anti-G_4-forming amylase antibodies was 56 000 for each extract. The native enzyme from *P. saccharophila* had the same molecular weight (lane 1). Slightly immuno-reactive bands, the ratio of migration of which indicated a molecular weight of 60 000, were detected in the preparation of the purified recombinant enzyme (lane 2) and crude extracts from the whole-cell and periplasm fractions (lanes 3 and 4). The molecular weights of the proteins in these bands were consistent with that of the G_4-forming amylase precursor[8]. The presence of the precursor protein in the recombinant enzyme, purified from the periplasm, may be explained by contamination of the precursor from the cytoplasm fraction which had been broken during the preparation of the enzyme. However, the protein band corresponding to the molecular weight of 60 000 was not detected when 4 μg of the purified recombinant enzyme was analysed by SDS–PAGE (lane 2 in Fig. 2A). Moreover, the amino-terminal amino-acid sequence of the recombinant enzyme was Asp–Gln–Ala–Gly–Lys– as in the native enzyme[8], and there was no significant difference in the amino acid compositions (Table I). Thus, the recombinant G_4-forming amylase secreted into the periplasmic space of *E. coli* is processed correctly to the mature form.

Effects of temperature. — The temperature dependence of G_4-forming amylase activity in potassium phosphate buffer (pH 7.0) is shown in Fig. 3A. The optimum

TABLE I

Amino acid compositions of the native and recombinant G_4-forming amylases

Amino acid	*Residue/molecule (mol%)*		
	Recombinant enzyme	*Native enzyme*	*Putative from gene*
Asx	74 (13.9)	69 (13.1)	75 (14.1)
Thr	18 (3.4)	19 (3.5)	17 (3.2)
Ser	39 (7.3)	47 (8.8)	46 (8.7)
Glx	45 (8.4)	47 (8.8)	43 (8.1)
Pro	27 (5.0)	24 (4.5)	23 (4.3)
Gly	69 (13.0)	72 (13.5)	71 (13.4)
Ala	48 (9.0)	46 (8.6)	45 (8.5)
Val	38 (7.2)	35 (6.6)	38 (7.2)
Cys	5 (1.0)	6 (1.2)	6 (1.1)
Met	7 (1.4)	7 (1.4)	7 (1.3)
Ile	17 (3.2)	18 (3.3)	17 (3.2)
Leu	29 (5.5)	27 (5.1)	26 (4.9)
Tyr	19 (3.5)	20 (3.7)	20 (3.8)
Phe	20 (3.7)	20 (3.7)	20 (3.8)
Lys	14 (2.6)	12 (2.3)	12 (2.3)
His	14 (2.6)	10 (1.9)	11 (2.1)
Arg	26 (4.9)	30 (5.7)	30 (5.7)
Trp	n.d.[a]	n.d.	23 (4.3)

[a] Not determined.

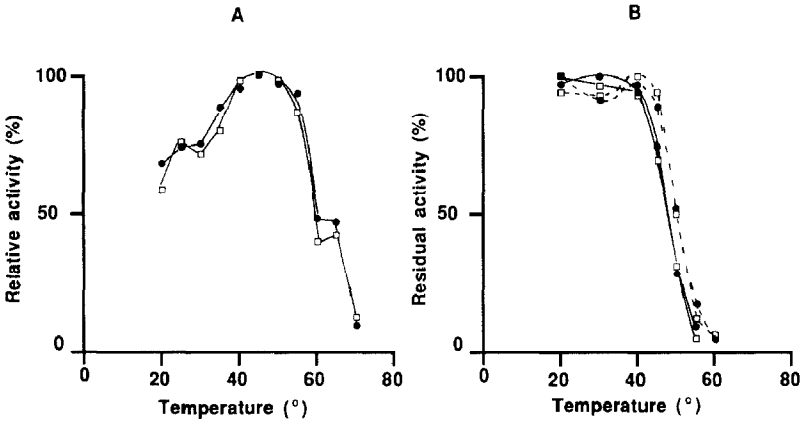

Fig. 3. Effects of temperature on the activity of G_4-forming amylase. A, Optimum temperature. Solutions of the native (□) and recombinant enzymes (●) in 10mM acetate buffer (pH 6.0) were incubated for 10 min with soluble starch, and the reducing power developed was measured by the Somogyi–Nelson method[10]. B, Thermal stability. A solution of each enzyme as in A was kept at the temperatures indicated for 30 min, and the remaining enzyme activity was measured as described in the Experimental section. Broken lines show the thermal stability of the enzyme in the presence of 2mM $CaCl_2$.

temperature was 45° for both the native and recombinant enzymes, which were stable on heating in 10mM acetate buffer (pH 6.0) for 30 min at <40°, but sensitive in the range 45–55° (Fig. 3B). Incubation at 60° caused almost complete inactivation. When the mixture contained 2mM $CaCl_2$, the thermal stabilities of both enzymes increased by 2–3°.

Effects of pH. — The effects of pH on the activity of the native and recombinant enzymes were similar (Fig. 4). Maximum activity was observed at pH 6.5 (Fig. 4A), which was reduced by ~ 50% at pH 4.0 and 9.0. The enzymes were stable after treatment for 30 min at 37° in the pH range 6.0–10.5 (Fig. 4B).

Effects of EDTA and metal ions. — There was no significant inhibition or activation of the G_4-forming amylase by EDTA, and mM $MgCl_2$, Li_2SO_4, $CaCl_2$, $CoSO_4$, and $MnCl_2$ (Table II). The enzyme was inhibited by $ZnSO_4$, $Pb(OAc)_2$, $CuSO_4$, $AgNO_3$, and $FeCl_3$. There was no significant difference between the native and recombinant enzymes.

Substrate specificities. — The native and recombinant enzymes were able to utilise soluble starch, amylopectin, and amylose as substrates (Table III). The rates of hydrolysis for glycogens and beta-limit dextrins (especially that from rabbit-liver glycogen) were relatively low. Neither enzyme acted on pullulan and cyclomaltohexaose (α-cyclodextrin).

Modes of action. — When the native and recombinant enzymes were incubated with soluble starch and malto-oligosaccharides (G_5–G_8), t.l.c. showed that only G_4 was produced from soluble starch, G_1 and G_4, G_2 and G_4, and G_3 and G_4 were produced from G_5, G_6, and G_7, respectively, and only G_4 was formed from G_8.

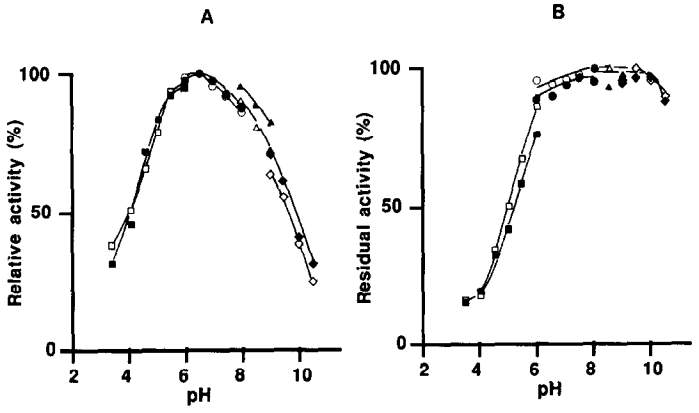

Fig. 4. Effects of pH on G_4-forming amylase activity. A, Optimum pH. The native (□) and recombinant enzymes (●) were reacted with soluble starch in 10mM buffers with various pH. B, pH Stability. The enzymes were treated for 30 min in 10mM buffers with various pHs. The residual enzyme activity was measured as described in Fig. 3 after the addition of the same volume of a solution containing 30mM phosphate buffer (pH 7.0) and 2% soluble starch. The buffers used were sodium acetate at pH 4.0–6.0 (□, ■), potassium phosphate at pH 6.0–8.0 (○, ●), Tris/HCl at pH 8.0–9.0 (△, ▲), and sodium carbonate at pH 9.0–10.5 (□, ◆). Open and closed markers represent the native and recombinant enzymes, respectively.

TABLE II

Effects of metal ions on G_4-forming amylase activity

EDTA and metal ion (1mM each)	*Residual activity (%)*[a]	
	Recombinant enzyme	*Native enzyme*
None	100	100
EDTA	132	110
$MgCl_2$	105	99
Li_2SO_4	101	98
$CaCl_2$	83	95
$CoSO_4$	75	84
$MnCl_2$	75	80
$ZnSO_4$	16	16
$Pb(OAc)_2$	11	15
$CuSO_4$	8	7
$AgNO_3$	6	5
$FeCl_3$	5	3

[a] Mean values of three separate experiments.

TABLE III

Substrate specificities of the native and recombinant G_4-forming amylases

Substrate	*Relative activity (%)*[a]	
	Recombinant enzyme	*Native enzyme*
Soluble starch	100	100
Amylopectin	97	93
Amylose	91	96
Reduced soluble starch	87	89
Glycogen (oyster)	75	77
Glycogen (rabbit-liver)	75	73
beta-Limit dextrin (WMS)[b]	62	63
beta-Limit dextrin (RLG)[c]	29	29
Pullulan	0	0
Cyclomaltohexaose (αCD)	0	0

[a] Mean values of three separate experiments. [b] Waxy-maize starch. [c] Rabbit-liver glycogen.

DISCUSSION

In prokaryotes, almost all of the proteins secreted are synthesised initially as precursors with an amino-terminal signal sequence. Sequence analysis of the known prokaryotic signal peptides reveals three well-conserved features[15]. In spite of the similarities among the signal sequences, expression of the genes for prokaryotic proteins in *E. coli* is different among such genes. Thus, the gene for *P. areuginosa* phospholipase C, an excretion enzyme, is expressed mostly only in the *E. coli* cytoplasm[16]. *Streptococ-*

cus equisimilis streptokinase is also produced by transfection of this gene into *E. coli*. However, the protein produced is located to extents of 17, 30, and 52% in the medium, periplasm, and cytoplasm, respectively[17]. In the present study, the recombinant G_4-forming amylase is produced mostly (~90%) in the periplasmic space during the late logarithmic and stationary phases of growth (Fig. 1). The molecular weight and amino-terminal sequence of the recombinant enzyme agree well with those of the native enzyme from *P. saccharophila* (Fig. 2). Thus, the signal sequence of the recombinant enzyme is recognised and removed correctly by *E. coli* signal peptidase.

Various enzymic properties of the recombinant G_4-forming amylase, including the substrate specificity and mode of action (Figs. 3 and 4, and Tables II and III), are almost identical to those of the native enzyme, and no significant difference is found in the amino acid compositions (Table I). Thus, it is concluded that the recombinant G_4-forming amylase is the same as the native enzyme. The expression system for the *mta* gene in *E. coli* should promote further understanding of the relationship between the structure and function of G_4-forming amylase.

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