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Purification and Characterization of Maltotriose-producing Amylases from an Alkaliphilic *Nocardiopsis* sp. TOA-1

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Abstract: An alkaliphilic actinomycete, *Nocardiopsis* sp. strain TOA-1, produced extracelluar maltotriose-producing amylases. Two amylases (AmyA-1 and AmyA-2) were purified to homogeneity by three steps of chromatography. The molecular masses of AmyA-1 and AmyA-2 were estimated to be 56 and 60 kDa, respectively. Optimal pH and temperature of both AmyA-1 and AmyA-2 were pH 9.5 and 65°C. These enzymes were stable at pH 7 and even at 13. AmyA-1 and AmyA-2 produced only maltotriose from starch, amylose, amylopectin, glycogen and γ-cyclodextrin at an early stage of reaction and small amounts of glucose and maltose were also produced upon prolonged incubation. The activities of AmyA-1 and AmyA-2 were significantly inhibited by Fe^{2+}, Fe^{3+} and *N*-bromosuccinimide. Substrate specificities were slightly different between AmyA-1 and AmyA-2.

Key words: maltotriose-producing amylase, alkaline enzyme, alkaliphilic, *Nocardiopsis* sp.

Amylases are classified into α-amylase, β-amylase, glucoamylase, and isoamylase according to the action on starch and other related substrates. Since the first discovery of maltotetraose-producing α-amylase,[1] several malto-oligosaccharide-producing amylases have been reported from various microorganisms.[2–8]

Recently, novel functions of malto-oligosaccharides have become an increased concern. Maltotriose shows only ca. 25% of sweetness over glucose; however, it is attracting attention in the food industry for its superior characteristics such as absorbancy and water-holding capacity. To date there are only four reports on maltotriose-producing amylases. A maltotriose-producing amylase was first discovered by Wako *et al.* as an exo-type amylase from *Streptomyces griseus*.[9] Endo-type maltotriose-producing amylases were discovered from *Bacillus subtilis*,[10] *Microbacterium imperiale*[11] and *Nataronococcus* sp.[12]

We have isolated an alkaliphilic *Nocardiopsis* sp. strain TOA-1, that produces a variety of extracellular alkaline enzymes, where an alkaline protease[13,14] and a pectate lyase[15] were purified and characterized. Here we report the purification and some properties of two maltotriose-producing amylases from alkaliphilic *Nocardiopsis* sp. TOA-1.

MATERIAL AND METHODS

Materials. Soluble starch was purchased from Merck. Corn starch, potato starch, amylopectin, glycogen, cyclodextrins (CDs), and malto-oligosaccharides ranging from maltose to maltoheptaose were purchased from Wako Pure Chemical. Pullulan and amylose (DP 10) were purchased from Nacalai tesque.

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Bacterial strains and culture conditions. *Nocardiopsis* sp. TOA-1 was originally isolated from the tile-joint of a bathroom. Strain TOA-1 was grown in a maltose-bonito medium containing 2.0% maltose and 1.0% bonito extract in the presence of 1% Na_2CO_3. The cultivation was carried out in a 5 L jar fermenter at 30°C with agitation (200 rpm) for 72 h.

Enzyme assay. Amylase activity was assayed by measuring the reducing sugar released from soluble starch as a substrate. The reaction mixture contained 50 mM Glycine/NaOH buffer (pH 10.0), 0.5% (w/v) gelatinized-soluble starch and a suitable amount of enzyme. The reaction was initiated by the addition of the enzyme solution. After incubation at 30°C for 10 min, the amount of reducing sugar released was measured by the dinitrosalicylic acid (DNS) method[16] using glucose as a standard. One unit of amylase activity was defined as the amount of enzyme required for the production of 1 μmol glucose per min.

Purification of alkaline amylases. Proteins in the culture broth of strain TOA-1 were precipitated by adding ammonium sulphate to get 80% saturation and the resultant precipitate was dialyzed against 20 mM Tris/HCl (pH 8.0, buffer A), and used as a crude enzyme. The crude enzyme solution was applied to a DEAE-Toyopearl 650M column (2.5×5.0 cm) that had been equilibrated with buffer A. After the column was washed with the same buffer, proteins were eluted using a 100-mL gradient of 0 to 1 M NaCl in buffer A. Fractions showing amylase activity were collected, followed by addition of ammnonium sulphate to set the final concentration of 30%. The resultant proteins were applied to a columun of Butyl-Toyopearl 650M (1.5×10.0 cm) that had been equilibrated with buffer A containing 30% ammnonium sulphate. After the column was washed with the same buffer, proteins

were eluted using a 100-mL gradient of 30% to 0% $(NH_4)_2SO_4$ in buffer A. Fractions with amylase activity were collected and applied to a Superdex 75 column (2.6 cm×60.0 cm) that had been equilibrated with buffer A containing 0.15 M of NaCl. Fractions showing amylase activity were collected and used as a purified enzyme.

Protein analysis. Protein content was determined by the Lowry method using bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done as described by Laemmli[17] and protein was stained with Coomassie Brilliant Blue R-250.

Analysis of hydrolysis products. Sugar products were identified by thin layer chromatograpy (TLC) with silica gel 60 (Merck). TLC was developed by the ascending chromatography technique in a solvent system of *n*-butanol/ethanol/water (2:1:1) at room temperature. Sugars were detected by spraying 10% sulfuric acid in methanol, then heating at 110°C for 10 min.

RESULTS

Purfication and molecular mass of AmyA-1 and AmyA-2. Two types of amylase were purified from cultures of strain TOA-1 by precipitation with ammonium sulphate and the subsequent three steps of the column chromatographic procedure (Table 1). Chromatography on a Butyl-Toyopearl column allowed separation of two amylase fractions (Fig. 1A). SDS-PAGE analysis revealed that AmyA-1 and AmyA-2 gave single proteins with a molecular mass of 56 and 60 kDa, respectively (Fig. 1B). AymA-1 and AmyA-2 were purified to 739- and 796-fold, respectively. Specific activities of AmyA-1 and AmyA-2 were 17.0 and 18.3 U/mg, respectively.

Effect of pH. The activity of AmyA-1 and AmyA-2 was assayed in buffers at various pH ranges. AmyA-1 and AmyA-2 exhibited the same optimal pH of 9.5 (Fig. 2A). To examine pH stability, AmyA-1 and AmyA-2 were incubated in buffers at various pH values for 10 min at 30°C and the remaining activity was determined. Both amylases were stable at pHs ranging from 8.0 to 13.0 (Fig. 2B).

Effect of temperature. The activity of amylase was measured at pH 10.0 for 10 min at various temperatures. The optimal temperatures of both AmyA-1 and AmyA-2 were 65°C (Fig. 2C). To examine the thermostability, these enzymes were incubated in 50 mM Glycine/NaOH buffer (pH 10.0) for 10 min at various temperatures and the remaining activity was measured. AmyA-1 and AmyA-2 activities were stable up to 45°C (Fig. 2D). Above this temperature, the activities decreased gradually. The thermal stabilities were enhanced in the presence of Ca^{2+} (Data not shown).

Effect of metal and chemical reagents. AmyA-1 and AmyA-2 were assayed in the presence of various metal ions at 1 mM or in the presence of chemical reagents. Fe^{2+} and Fe^{3+} significantly inhibited the activity of both enzymes (Table 2). As for Pb^{2+}, Cu^{2+} and Al^{3+} ions, both enzymes were inhibited to the extent of 40–70%. Iodoacetic acid and phenylmethysulfonyl fluoride did not inhibit at 1 mM, while *N*-bromosuccinimide inhibited 89% for AmyA-1 and 94% for AmyA-2, respectively. SDS

Table 1. Purification of maltotriose-producing amylases from *Nocardiopsis* sp. TOA-1.

Step	Amylase type	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture fluid		382	16700	0.023	1.0	100
$(NH_4)_2SO_4$ ppt.		230	489	0.470	20.4	60
DEAE-Toyopearl		176	53	3.32	144	46
Butyl-Toyopearl	AmyA-1	83	7.8	10.6	461	22
	AmyA-2	59	4.3	13.7	596	15
Superdex 75	AmyA-1	80	4.7	17.0	739	21
	AmyA-2	55	3.0	18.3	796	14

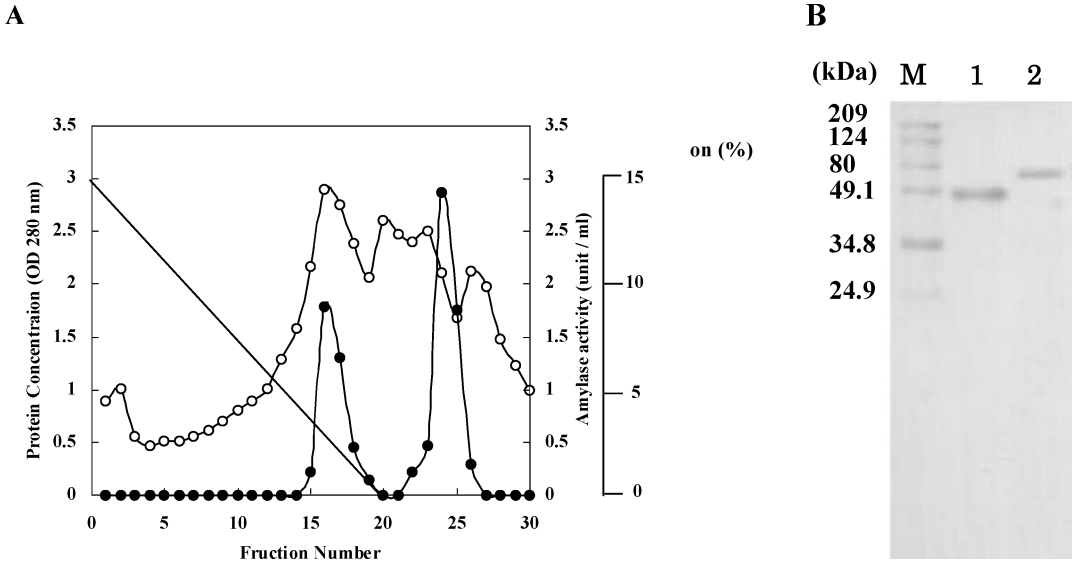

Fig. 1. Chromatogram on Butyl-Toyopearl and SDS-PAGE of purified maltotriose-producing amylase 1 (AmyA-1) and amylase 2 (AmyA-2).

(A) Chromatogram on Butyl-Toyopearl. ○, amylase activity; ●, protein concentration (A_{280}); ——, Ammonium sulfate concentration. (B) SDS-PAGE. Lane M, molecular mass marker protein; Lane 1, AmyA-1; Lane 2, AmyA-2.

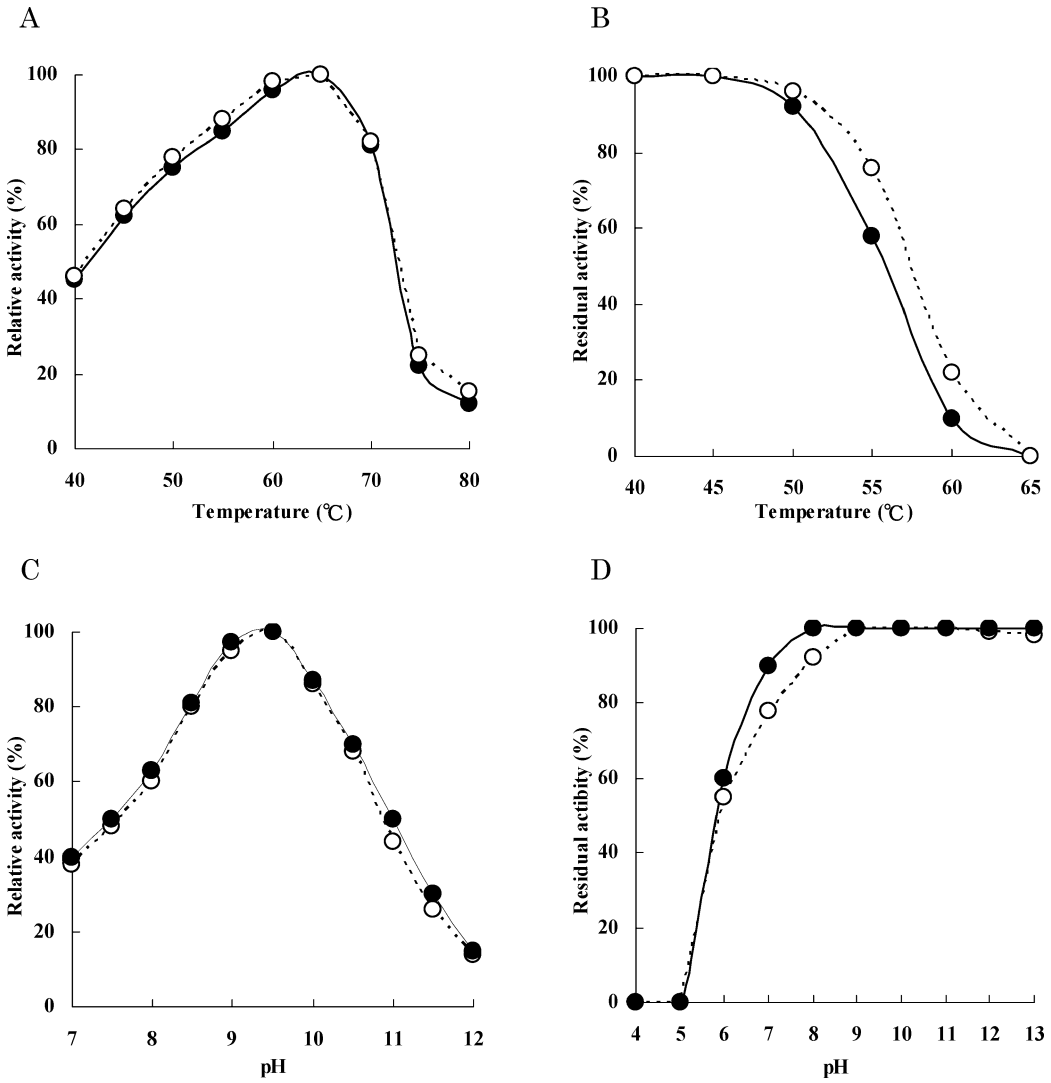

Fig. 2. Effects of temperature and pH on activity and stability of AmyA-1 (●) and AmyA-2 (○).

(A) Effects of temperature on enzyme activity. The enzymatic activity toward soluble starch was assayed at temperatures from 40°C to 80°C, at pH 10.0. (B) Effects of temperatures on enzyme stability. The enzymes in 20 mM Tris/HCl buffer (pH 8.0) were incubated at temperatures from 40°C to 65°C. After 10 min of incubation, the amylase activities were assayed at 30°C, pH 10.0. (C) Effect of pH on enzyme activity. The enzymatic activities toward soluble starch were assayed at pHs from 7.0 to 12.0; D: Effect of pH on enzyme activity. The reaction mixtures were incubated at pH ranging from 4.0 to 13.0, at 30°C for 10 min and the amylase activities were assayed at 30°C, pH 10.0. The buffers used: acetate, pH 4.0–5.0; phosphate, pH 6.0; Tris/HCl, pH, 7.0–8.0; glycine/NaOH, pH 9.0–12.0; KCl/NaOH, pH 13.0.

Table 2. The effects of metals on the activity of AmyA-1 and AmyA-2.

Metal	Residual activity (%)	
	AmyA-1	AmyA-2
No addition	100	100
$BaCl_2$	99	89
$NiCl_2$	77	84
$FeCl_2$	4	8
$CoCl_2$	86	88
$MnCl_2$	70	76
$PbCl_2$	30	46
$CuCl_2$	56	27
$HgCl_2$	33	21
$MgCl_2$	93	86
$AlCl_3$	53	54
$FeCl_3$	2	4

Each enzymatic activity was assayed after the enzyme had been treated with each metal ion at 30°C for 10 min and pH 10.0 in 10 mM Glycine/NaOH buffer. The values shown are the percentages of the activity without additives, which is taken as 100%.

(0.1%) inhibited 31% for AmyA-1 and AmyA-2. No inhibitory effect by EDTA was observed for either amylase (Data not shown).

Substrate specificity. AmyA-1 and AmyA-2 hydrolyzed soluble starch, amylase, amylopectin, and glycogen (Table 3). Neither enzyme hydrolyzed pullulan, α-cyclodextrin, or β-cyclodextrin. However, γ-cyclodextrin was a substrate for both amylases. AmyA-2 showed higher activities towards all substrates tested except for amylose and γ-cyclodextrin than AmyA-1 did.

Action pattern. AmyA-1 and AmyA-2 hydrolyzed soluble starch to maltotriose (G3) predominantly. A small amount of maltose (G2) and glucose (G1) were also produced upon incubation (Fig. 3A, B). The major products from other substrates were also G3 (not shown). Figures 3C and 3D show the hydrolysis products by AmyA-1 and AmyA-2 for malto-oligosaccharides ranging from G2 to maltoheptaose (G7). Maltotetraose (G4), maltohexaose (G6) and G7 were predominantly hydrolyzed to G3. Maltopentaose (G5) was hydrolyzed to G3 and G2. G2 and

G3 were not hydrolyzed at all. In no case was an elongation of malto-oligosaccharaises observed, indicating that AmyA-1 and AmyA-2 have no transglycosylation activity.

DISCUSSION

We have purified and characterized two alkaline maltotriose-producing amylases (AmyA-1 and AmyA-2) from an alkalophilic *Nocardiopsis* sp. TOA-1. These two amylases were very similar in terms of enzyme properties and mode of hydrolysis toward substrates. AmyA-1 and AmyA-2 hydrolyze starches, amylose, amylopectin, glycogen and γ-cyclodextrin to maltotriose as a main product. Based on the hydrolysis of γ-cyclodextrin and rapid decrease of iodine staining capacity (data not shown), AmyA-1 and AmyA-2 are estimated to be end-acting amylases. There are four reports on maltotriose-producing amylases, which are from *S. griseus*,[9] *B. subtilis*,[10] *M. imperilale*[11] and an archaebacterium *Natronococcus* sp. strain Ah-36.[12] Among these amylases, *S. griseus* amylase is exo-acting. The optimal pH of *S. griseus* amylase, *B. subtilis* amylase and *M. imperiale* amylase are 5.6–6.0, 6.0–7.0 and 6.5–7.0, respectively. A haloalkaliphilic amylase from archaebacterium *Natronococcus* sp. strain Ah-36 requires a high salt concentration of 2.5 M NaCl for its activity and stability. An alkaline amylase from alkaliphilic *Bacillus* sp. strain KSM-K38 produces maltohexaose as the major hydrolysis product other than maltotriose.[18] Thus, AmyA-1 and AmyA-2 are novel alkaline and endo-type maltotriose-producing amylases.

AmyA-1 and AmyA-2 are very similar in their enzymatic properties such as optimal pH and temperature, susceptibility to metal, and hydrolysis mode for substrates.

Table 3. Relative activity of AmyA-1 and AmyA-2 toward various substrates.

Substrate (0.5%, w/v)	Relative activity (%)	
	AmyA-1	AmyA-2
Soluble starch	100	100
Corn starch	73	100
Potato starch	69	86
Amylose	94	77
Amylopectin	61	95
Glycogen	21	62
Pullulan	0	0
α-cyclodextrin	0	0
β-cyclodextrin	0	0
γ-cyclodextrin	48	35

Assays were performed at 30°C and pH 10.0 in 50 mM Glycine/NaOH buffer with under the standard conditions. The values shown are the percentages of the activity obtained using soluble starch as substrate, which is taken as 100%.

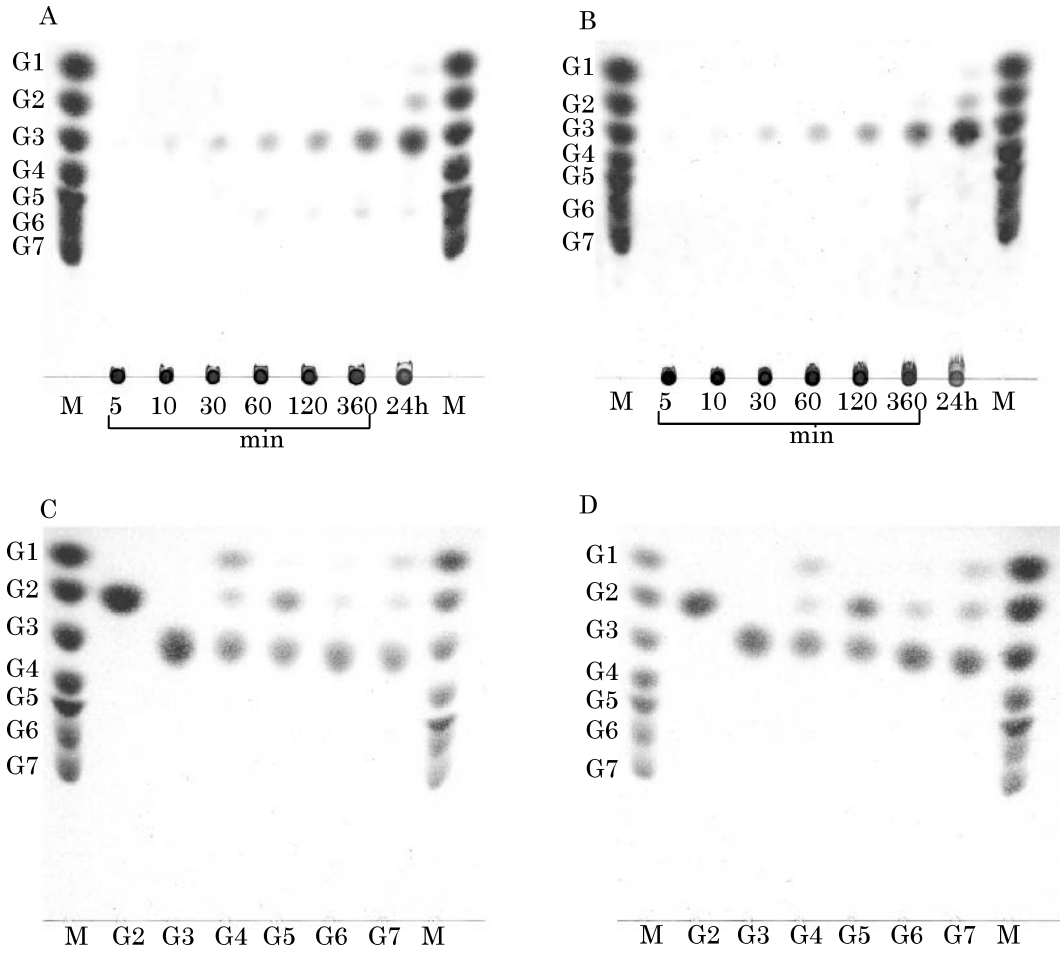

Fig. 3. Thin-layer chromatography of the products of hydrolysis of various saccharides by the AmyA-1 and AmyA-2.

(A) and (B) Chromatograms of the products obtained from soluble starch (0.5% [wt/vol]) by AmyA-1 (A) and AmyA-2 (B). The reaction was done at 30°C and at pH 10.0 in 50 mM Glycine/NaOH buffer. Samples were taken at the indicated intervals and boiled for 5 min to terminate the reaction. (C) and (D) Chromatograms of the products obtained from maltooligosaccharides G2 to G7 (0.25% [wt/vol]) by AmyA-1 (C) and AmyA-2 (D); the reaction was done at 30°C and at pH 10.0 in 50 mM Glycine/NaOH buffer for 24 h. G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose; M, standard markers.

However, their substrate specificity is slightly different. AmyA-2 shows higher activity toward various substrates than AmyA-1. It is likely that AmyA-1 is derived from AmyA-2 by the action of proteases in the culture filtrate.

The optimal pH and optimal temperature of AmyA-1 and AmyA-2 are similar to those of *Natronococcus* sp. strain Ah-36 amylase. As in the case of *Natronococcus* sp. amylase, AmyA-1 and AmyA-2 were inhibited by *N*-bromosuccinimide, suggesting that tryptophan residue(s) are involved in the catalytic activities.[19] The molecular mass of AmyA-1 (56 kDa) and AmyA-2 (60 kDa) are different from that of *Natronococcus* sp. amylase (74 kDa). Furthermore, transglycosylation activity is observed in *Natronococcus* sp. amylase but not in AmyA-1 or AmyA-2. Gene cloning and further characterization of AmyA-1 and AmyA-2 are currently underway.

REFERENCES

1) J.F. Robyt and R.J. Ackerman: Isolation, purification, and characterization of a maltotetraose-producing amylase from *Pseudomonas stutzeni. Arch. Biochem. Biophys*., **145**, 105−114 (1971).
2) T. Hayashi, T. Akiba and K. Horikoshi: Properties of new alkaline meltohexaose-forming amylases. *Appl. Microbiol. Biotechnol*., **28**, 281−285 (1988).
3) T. Hayashi, T. Akiba and K. Horikoshi: Production and purification of new maltohexaose-forming amylases from alkalophilic *Bacillus* sp. H-167. *Agric. Biol. Chem*., **52**, 443−448 (1988).
4) K. Kainuma, K. Wako, S. Kobayashi, A. Nogami and S. Suzuki: Purification and some properties of a novel maltohexaose-producing exo-amylase from *Aerobacter aerogenes. Biochim. Biophys. Acta*, **410**, 333−346 (1975).
5) Y. Takasaki: Studies on enzymatic production of oligosaccharides. 1. Production of maltohexaose by α-amylase from *Bacillus circulans* G-6. *Agric. Biol. Chem*., **46**, 1539−1547 (1982).
6) Y. Takasaki, H. Shinohara, M. Tsuruhisa, S. Hayashi and K. Imada: Studies on enzymatic production of oligosaccharides. 7. Maltotetraose-producing amylase from *Bacillus* sp. MG-4. *Agric. Biol. Chem*., **55**, 1715−1720 (1991).
7) S. Kobayashi, H. Okemoto, K. Hara and H. Hashimoto: Preparation and some properties of a novel maltopentaose-forming enzyme of a *Pseudomonas* species. *Agric. Biol. Chem*., **54**, 147−156 (1990).
8) T.U. Kim, B.G. Gu, J.Y. Jeong, S.M. Byun and Y.C. Shin: Purification and characterization of a maltotetraose-forming alkaline α-Amylase from an alkalophilic *Bacillus* Strain, GM8901. *Appl. Environ. Microbiol*., **61**, 3105−3112 (1995).
9) K.L. Wako, C. Takahashi, S. Hashimoto and J. Kanaeda: Studies on maltotriose- and maltose-forming amylases from *Streptomyces. J. Jpn. Soc. Starch Sci*., **25**, 155−161 (1978).
10) Y. Takasaki: Studies on enzymatic production of oligosaccharides. 3. An amylase producing maltotriose from *Bacillus subtilis. Agric. Biol. Chem*., **49**, 1091−1097 (1985).
11) Y. Takasaki, M. Kitajima, T. Tsuruta, M. Nonoguchi, S. Hayashi and K. Imada: Studies on enzymatic production of oligosaccharides. 6. Maltotriose-producing amylase from *Microbacterium impenmale. Agric. Biol. Chem*., **55**, 687−692 (1991).
12) T. Kobayashi, H. Kanai, T. Hayashi, T. Akiba, R. Akaboshi and K. Horikoshi: Haloalkaliphilic maltotriose-forming α-amylase from the archaebacterium *Natronococcus* sp. strain Ah-36. *J. Bacteriol*., **174**, 3439−3444 (1992).
13) S. Mitsuiki, M. Sakai, Y. Moriyama, M. Goto and K. Furukawa: Purification and some properties of a keratinolytic enzyme from an alkaliphilic *Nocardiopsis* sp. TOA-1. *Biosci. Biotechnol. Biochem*., **66**, 164−167 (2002).
14) S. Mitsuiki, M. Ichikawa, T. Oka, M. Sakai, Y. Moriyama, Y. Sameshima, M. Goto and K. Furukawa: Molecular characterization of a keratinolytic enzyme from an alkaliphilic *Nocardiopsis* sp. TOA-1. *Enzyme Microb. Technol*., **34**, 482−489 (2004).
15) S. Mitsuiki, H. Eguchi, Y. Hara, M. Sakai, Y. Moriyama, M. Goto and K. Furukawa: Purification and some properties of pectate lyase from alkaliphilic *Nocardiopsis* sp. TOA-1. *J. Appl. Glycosci*., **49**, 497−500 (2002).
16) G.L. Miller: Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem*., **31**, 426−428 (1959).
17) U.K. Laemmli: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680−685 (1970).
18) H. Hagiwara, K. Igarashi, Y. Hayashi, K. Endo, K. Ikawa-Kitayama, K. Ozaki, S. Kawai and S. Ito: Novel α-amylase that is highly resistant to chelating reagents and chemical oxidants from the alkaliphilic *Bacillus* isolate KSM-K38. *Appl. Environ. Microbiol*., **67**, 1744−1750 (2001).
19) T.F. Spande and B. Witkop: Determination of the tryptophan content of proteins with *N*-bromosuccinimide. *Methods Enzymol*., **11**, 498−506 (1967).

好アルカリ性 *Nocardiopsis* sp. TOA-1 株の産生するマルトリオース生成アミラーゼの精製と諸性質

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好アルカリ性放線菌 *Nocardiopsis* sp. TOA-1 の培養液より，硫安分画後，DEAE-Toyopearl，Butyl-Toyopearl および Superdex75 カラムクロマトグラフィーを用いて，マルトトリオース生成アミラーゼの精製を行った結果，分子量の異なる二つの酵素を得た（AmyA-1，AmyA-2）（Fig. 1）．SDS-PAGE により，AmyA-1 および AmyA-2 の分子質量をそれぞれ 56，60 kDa と推定した（Fig. 1）．AmyA-1 および AmyA-2 の至適 pH および至適温度はともに pH 9.5，65℃ であり（Fig. 2），安定 pH および安定温度もともに pH 7−13，45℃ 以下であった．AmyA-1 および AmyA-2 は，可溶性デンプン，アミロース，アミロペクチン，グリコーゲンおよび γ-シクロデキストリンを分解し，反応初期にはマルトトリオースのみを生成すること，また最終生成物にはマルトトリオースの他に少量のグルコースおよびマルトースが存在することが明らかとなった（Fig. 3）．さらに，AmyA-1 および AmyA-2 の酵素活性は，Fe^{2+}，Fe^{3+}（Table 2）および *N*-bromosuccinimide により顕著に阻害されるなど，両酵素の諸性質は極めて類似していた．しかしながら，基質特異性については両酵素においてわずかに異なっていた（Table 3）．