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Purification and properties of an extracellular α-amylase from *Thermus* sp.

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Abstract. An extracellular α-amylase from an extreme thermophile, *Thermus* sp., was highly purified by affinity absorption on starch granules. SDS-PAGE showed a single band for the purified enzyme, with an apparent molecular weight of 59000. The optimum pH and temperature for the enzyme action on starch was 5.5–6.5 and 70°C, respectively. The enzyme randomly attacked the bonds in the inner region of the starch and produced various maltooligosaccharides. The minimum length of maltooligosaccharide cleaved by this enzyme was maltohexaose. The enzyme activity was strongly inhibited by the addition of Cu^{2+} and Fe^{2+} ions. The enzyme belonged to the EDTA-sensitive α-amylase group, but its activity was not stimulated by the presence of Ca^{2+} ions.

Keywords: Purified; Thermostable extracellular α-amylase; *Thermus* sp.

Introduction

α-Amylase (EC3.2.1.1, 1,4-α-D-glucan glucanohydrolase, endoamylase) hydrolyzes starch, glycogen, and related polysaccharides by randomly cleaving internal α-1,4-glucosidic linkages. It is widely distributed in various bacteria, fungi, plants, and animals and has a major role in the utilization of polysaccharides. α-Amylase is an important industrial enzyme. As well as being used as an additive in detergents, it can be used for such things as the removal of starch sizing from textiles, the liquefaction of starch, and the proper formation of dextrin in baking. The thermostability of the α-amylase must be matched to the application. For example, thermostable α-amylases are used for the liquefaction of starch at high temperature and thermolabile α-amylases are used for the saccharification of starch in baking . We have recently developed an enzymatic method that uses thermostable α-amylase in combination with β-amylase and debranching enzymes (isoamylase and/or pullulanase) to produce high-maltose syrup and high protein rice flour simultaneously (Shaw and Sheu, 1992; Shaw, 1994; Shaw et al., 1989).

Thermus is a Gram negative extreme thermophile that produces several thermostable enzymes, such as Taq DNA polymerase (Chien et al., 1976) , pullulanase (Plant et al., 1986; Nakamura et al., 1987), lipase (Phutrakul et al., 1993), and protease (Matsuzawa, 1983), but no report on the production and characterization of α-amylase from *Thermus* has been published. In this paper, we describe the purification and characterization of α-amylase from a *Thermus* sp. strain isolated from a hot-spring in northern Taiwan.

Materials and Methods

Screening, Isolation and Identification of Microbial Strains

The sediment samples for screening were obtained from hot-spring sources in Yangmin Shan National Park in northern Taiwan. The organism was enriched on a screening agar plate containing (grams per liter): 2 g $(NH_4)_2 SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.7 g K_2HPO_4, 0.3 g KH_2PO_4, 10 g corn starch, and 17 g agar in distilled water. Incubation at 50–65°C was carried out for 24–48 h, after which the plates were stained with Gram's iodine solution (0.1% I_2 and 1% KI) and the colonies with the largest halo-forming zone were isolated for further investigation. Microbiological properties of the isolated strain were determined according to the methods described in *Bergey's Manual of Systematic Bacteriology* (Sneath et al., 1986).

Amylase Assay

α-Amylase activity was assayed by measuring the reducing sugar released during the reaction, using starch as the substrate, according to the Somogyi-Nelson method. (Nelson, 1944). The reaction mixture contained 50 μl of 1.1% soluble starch (Merck 1252, 0250, MW 6500–8000) in 2 mM imidazole-HCl buffer (pH 7.0) and 250 μl of enzyme solution. The reaction was stopped by adding 100 μl dinitrosalicylic acid solution (100 ml of solution contained 1 g 3,5-dinitrosalicylic acid, 30 g potassium sodium tartarate, and 20 ml 2 N NaOH) after incubation at various temperatures for 1 h. The reaction mixture was then

heated in boiling water for 5 min and the absorbance at 540 nm was measured after cooling in ice and diluting with 1 ml distilled water. Another α-amylase assay, based on Fuwa's colorimetric method of iodine-starch color reaction (Fuwa, 1954), was also used. Fifty microliters of α-amylase solution in 2 mM imidazole HCl buffer (pH7.0) was mixed with 100 μl of prewarmed 1.1% soluble-starch solution and incubated at 60°C for 1 h. The reaction was stopped by adding 250 μl of stop solution (0.5 N acetic acid : 0.5 N HCl = 5:1). A 100 μl aliquote of the reaction mixture was then mixed with 1 ml of iodine reagent (0.01% iodine and 0.1% KI). The absorbance at 660 nm was measured after incubating at room temperature for 20 min. One unit of amylase activity was defined as the amount of enzyme which decreased the absorbance at 660 nm by 1.0 in 10 min.

Preparation of α-Amylase

The isolate was cultured in a 2 l conical flask containing 400 ml of the enzyme production medium (1% soluble starch, 0.3% yeast extract, and 0.3% tryptone) on a rotary shaker (200 rpm) at 60–65°C. After a 2-day cultivation, cells were removed by 10,000-*g* centrifugation at 4°C for 20 min and the extracellular α-amylase in the supernatant liquid was partially purified by 50%–80% ammonium sulfate fractionation and the hydrophobic interaction column of the Fast Protein Liquid Chromatography System (FPLC-HIC) from Pharmacia Co. The enzymatically-active fractions were finally purified by corn starch-affinity absorption. The purified enzyme was eluted from 1% corn starch with water after shaking at 60°C for 15 min.

Analysis of Polysaccharide Hydrolysis Products

The purified α-amylase from *Thermus* sp. was mixed with various lengths of polysaccharides as substrates, and was incubated at the optimum pH (pH 7.0) and temperature (50°C) for various times. Products in the hydrolysate were identified and quantified by High-Performance Liquid Chromatography (HPLC) using Dionex anion exchange column Carbopac PA1 (4 × 250 mm). Authentic sugars (up to seven glucose units) purchased from Sigma were used as the standards.

Other Analytical Procedures

Protein content was determined by spectrophotometric measurement using the formula : mg protein $ml^{-1} = 1.45\ A_{280} - 0.74\ A_{260}$ (Groves et al., 1968). SDS-PAGE was performed as described by Laemmli (1970). After the electrophoresis, some gels were stained for protein with 0.4% Coomassie Brilliant Blue R-250, and the other gels were stained for enzyme activity by the iodine-stain reaction as follows—gels were dipped in 3% soluble starch at 30°C for 10 min, washed with distilled water, dipped in a solution of 0.1% I_2 and 1% KI, and fixed with 3% acetic acid solution. A clear-zone-forming band indicated the presence of the active enzyme.

Results

Taxonomic Assessment of the Isolated Strain

The characteristics of the isolated strain are summarized in Table 1. A strain of *Thermus* sp. was confirmed according to *Bergey's Manual of Systematic Bacteriology* (Sneath et al., 1986).

Identification of Amylolytic Enzymes

Table 2 shows the products of the enzymatic hydrolysis, by purified α-amylase, of soluble amylose and of various maltooligosaccharides. HPLC analysis of the hydrolysates indicated that glucose, maltose, and other maltooligosaccharides were produced. The minimum length of sugar cleaved by this enzyme was maltohexaose. There was no cleavage of α-1,4-glucosidic bonds between maltose and maltopentaose (data not shown).

Purification of the Enzyme

α-Amylase from *Thermus* sp. was purified to an electrophoretically homogeneous state in the presence of SDS. The purification scheme is summarized in Table 3. The purity of the enzyme was more than 360-fold greater than that of the crude enzyme in the supernatant liquid of the cell culture. Specific activity of the purified enzyme was 367 units mg^{-1}.

Properties of the Enzyme

Influence of pH and temperature—The optimum pH was determined to be in the range 5.5–6.5, (Figure 1a). The enzyme was stable at pHs from 4 to 10 on standing for 2 h at 37°C in the presence of substrate. It was stable

Table 1. Morphological and physiological characteristics of the isolated strain.

Morphology:	Rods occurring as single cells, non-motile, Gram negative non sporulating
Growth:	
Agar	Abundant, pale-yellow colonies
Broth	Good growth, no sediment
Aerobic	Abundant growth
Anaerobic	No growth
NaCl broth	No growth in 3%
pH 5.7	No growth
Temperature	Growth at 72°C, optimum 65°C, range 37–72°C
pH	Optimum 7.0, range 5–8.5
Catalase	Positive
Milk	No coagulation or peptonization
Nitrate	Reduced to nitrite
Starch	Hydrolysed
Production of:	
Indole	Produced
Utilization of:	
Carbohydrates	Acid without gas from glucose, L-arabinose, xylose
Citrate	Not utilized
Mannitol	Not utilized

Table 2. Hydrolysis of amylose and maltooligosaccharides by *Thermus* sp. α-amylase[a].

Substrate	Reaction time (h)	Degradation products (mg ml^{-1})						
		G1	G2	G3	G4	G5	G6	G7
Amylose	5	2.39	23.7	36.24	11.18	12.02	18.81	14.75
	10	6.89	47.35	71.27	21.60	23.55	38.57	26.65
Maltoheptaose	5	0.212	0.984	0.027	0.046	0.874	-0.115	
	24	0.669	2.240	0.084	0.182	1.977	0.403	
Maltohexaose	5	0.072	0.028	0	0.030	0.253		
	24	0.283	0.074	0.027	0.068	0.484		

[a] Hydrolysis was performed in 25 mM sodium phosphate buffer (pH 7.0–8.0) containing 10 mM substrate and incubated at 60°C for various times. Hydrolysates were analyzed by HPLC.

Table 3. Purification of α-amylase from *Thermus* sp.

Purification step	Total Vol. (ml)	Activity (unit/ml)	Total activity (unit)	Protein (mg/ml)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Culture supernatant liquid	4000	3.5	14000	3.82	15290.8	0.92	100
Ammonium sulfate fractionation	10.03	314.8	3157.44	35.75	358.55	8.81	23
FPLC-Hydrophobic Interaction chromatography	90.27	18.88	1704.3	0.172	15.57	109.47	12
Corn starch affinity adsorption	166.71	2.22	370.1	0.006	0.59	367.16	2.6

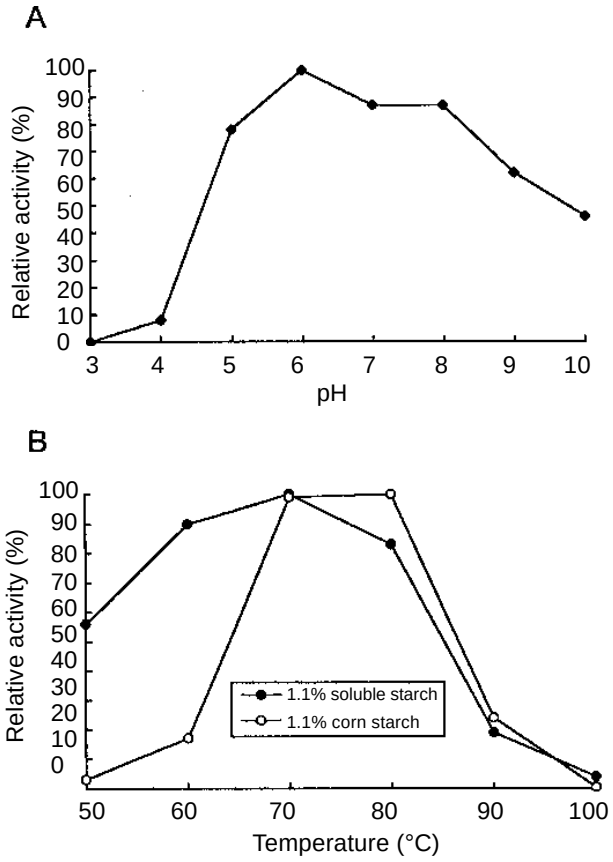

Figure 1. A) Influence of pH on the activity of α-amylase. The enzymatic activity was measured using soluble starch as a substrate in Good's buffer—a wide pH-range buffer (pH 3–10)—as described in *Materials and Methods*. **B**) Influence of temperature on the activity of α-amylase with 1.1% soluble starch or 1.1% corn starch, with 2.0 mM imidazole-HCl buffer (pH 7.0) as the substrate.

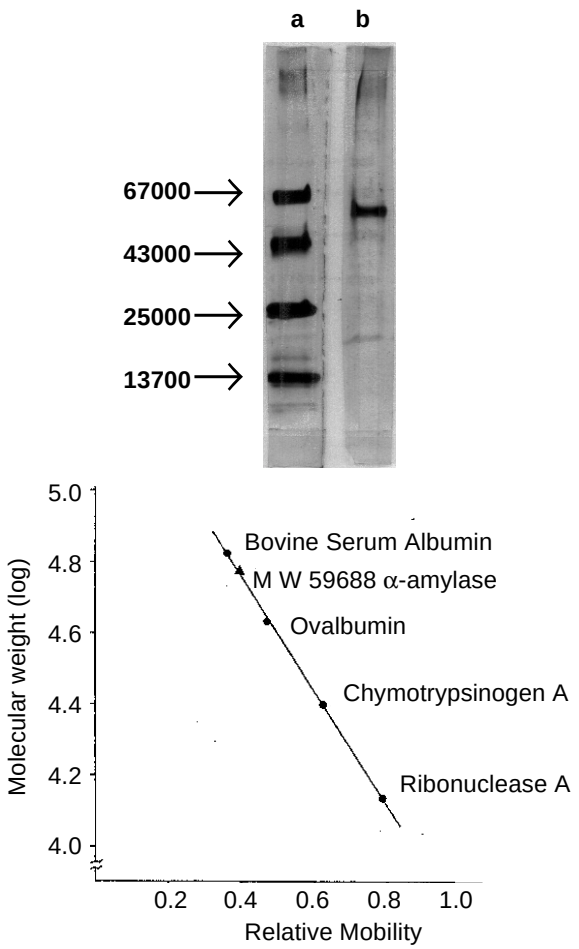

Figure 2. Determination of molecular weight by SDS-PAGE. **a**) molecular weight markers: Bovine Serum Albumin, 67000; Ovalbumin, 43000; Chymotrypsinogen A, 25000; Ribonuclease A, 13700; **b**) α-amylase from *Thermus* sp.

only between pH 5–8 at temperatures higher than 50°C. The optimum temperature for the enzyme was determined to be 70°C, using soluble starch and corn starch as the substrate (Figure 1b).

Molecular weight—The apparent molecular weight of the enzyme subunit that is enzymatically active in the presence of SDS (data not shown) was estimated to be 59000 (Figure 2) by the method of Weber and Osborn (1969).

Table 4. Influence of various metal ions on the activity concentration of metal ions and EDTA, 5 mM. All the metal ions were added as chloride salts.

Metal ions		Relative activity (%)
None		100
Na^+	1 mM	102
	5 mM	103
	10 mM	104
Ca^{2+}	1 mM	91
	10 mM	92
Co^{2+}	1 mM	96
	5 mM	85
	10 mM	79
Mg^{2+}	1 mM	94
	5 mM	80
	10 mM	74
Fe^{2+}	1 mM	93
	5 mM	19
	10 mM	14
Cu^{2+}	1 mM	75
	5 mM	6
	10 mM	3
EDTA	0.05 mM	26
	0.1 mM	14
	0.5 mM	2
	1.0 mM	2

Table 5. Influence of protein denaturants on enzyme activity. The enzyme was incubated with each denaturant, at the final concentrations shown in the table, in 50 mM phosphate buffer (pH 7) at 28ºC for 30 min before the assay. The denaturant-treated enzyme (0.02 ml) was added to 0.2 ml of 1.1% soluble starch and the residual activity was assayed at pH 7 using Fuwa's method.

Protein denaturants		Relative activity (%)
None		100
Urea	2 M	100
	4 M	107
	6 M	109
	8 M	111
SDS	20 mM	65
	40 mM	47
	60 mM	39
	80 mM	42
	100 mM	37
Gn-HCl	2 M	33
	4 M	20
	6 M	15

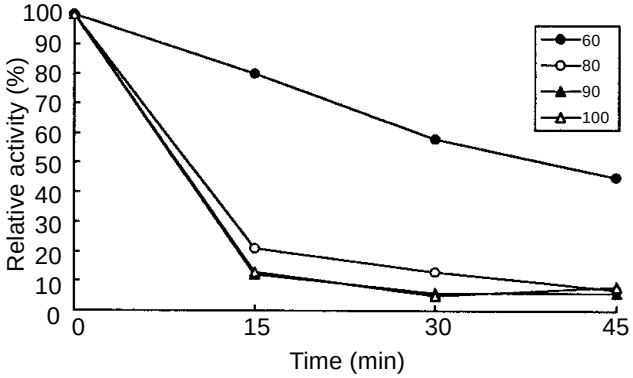

Figure 3. Thermostability of α-amylase. The enzyme solution in the absence of substrate was incubated at various temperatures in 25 mM sodium phosphate buffer (pH 7.0) for 0, 15, 30, and 45 min, and the residual soluble starch-digesting activity was assayed as described in *Materials and Methods.*

Influence of metal ions and protein denaturants on enzyme activity—The metal ions, except Ca^{2+} and Na^+, inhibited the enzyme activity. The inhibition by Cu^{2+} and Fe^{2+} was prominent—the enzyme activities decreased to about 4% and 20% of that of the control, respectively (Table 4). The enzyme was very sensitive to EDTA inhibition, but stimulation of activity by Ca^{2+} was not observed (Table 4). Protein denaturants, such as SDS and Guanidine-HCl (Gn-HCl), decreased the activity of the enzyme. In contrast, urea had no significant influence on the enzyme, even at 8 M (Table 5).

Influence of temperature on enzyme stability—To study the thermostability of the α-amylase, the enzyme was incubated at various temperatures and the residual activity was measured. Figure 3 shows the influence of temperature on the enzyme at pH 7. The enzyme showed relatively high thermostability and retained about 80% of its original activity after heating at 60°C for 15 min.

Discussion

Although a lot of information is available on the characterization of α-amylase from mesophiles and thermophiles (Amylase Research Society of Japan, 1988), only a few extracellular enzymes produced from Gram-negative thermophiles such a *Thermus* have been reported. Our study indicates the secretion of an extracellular, thermostable α-amylase by a starch-degradable thermophilic soil bacterium that was characterized as a *Thermus* sp.

The α-amylase purified from the culture supernatant liquid, of which the initial main hydrolysis product with starch was maltotriose, was more stable than the α-amylase from mesophilic microorganisms such as *Bacillus amyloliquefaciens*, but less stable than those from *Clostridium thermohydrosulfuricum*, *Bacillus licheniformis*, and *Pyrococcus* sp. (Melasniemi, 1987; Gerhartz, 1990; Brown et al., 1990; Koch et al., 1990, 1991). The thermostability of the α-amylase from *Thermus* sp. is comparable to that of those from *Bacillus subtilis* (Gerhartz, 1990) and

rice grain (Shaw and Chuang, 1982; Shaw and Ou-Lee, 1984). The enzyme has features such as sensitivity to EDTA inhibition, no Ca^{2+} activation of the enzymatic activity, and resistance to urea denaturation.

A thermophilic substitute for the mesophilic α-amylase used in the cake baking industry can be advantageous, because the enzyme activity of the thermophile ceases as the temperature approaches and passes through the temperature range of gelatinization (70–80°C). This provides a means to control dextrin formation, which is important in bread baking—the limited formation of dextrin contributes to the browning of the crust and adds flavor to the bread. The broad range of pH stability and moderate thermostability makes this enzyme a useful additive to liquid detergents which must function in hard and warm water.

Much work has been done on the characterization of intracellular and cell-associated enzymes of *Thermus* sp. strains (Plant et al., 1986; Feeze and Brock, 1970; Fujita et al., 1976; Nojma et al., 1979; Taguchi et al., 1982), little information is available on the secretion of enzymes outside of the cells, except for the protease produced by *T. caldophilus* (Taguchi et al., 1983) and *T. aquaticus* (Matsuzawa et al., 1983). It has also been reported that an extracellular, thermostable pullulanase is produced by *Thermus* sp. (Nakamura et al., 1987). The results of this study, in correlation with data on other enzymes produced by this genus, suggests that *Thermus* sp. is one of the better microbial producers of those extracellular, thermostable enzymes that are useful in many industrial fields. The secretion signal and the mechanism of secretion in this α-amylase are under investigation.

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高溫厭氣菌 *Thermus* sp. 胞外 α-澱粉酶的純化及性質

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α-澱粉酵從高溫厭氣菌 *Thermus* sp. 細胞體外，藉澱粉粒親和性吸附之純化方式，得到高純度的 α-澱粉酶。其分子量約 59 Kd 。 澱粉水解最適 pH 及溫度分別爲 5.5-6.5 及 70°C。此酵素以不規則內切方式水解澱粉，麥芽寡糖爲其產物。麥芽六糖爲最小能被此酵素水解的作用基質。 Cu^{2+} 及 Fe^{2+} 對酵素活性有明顯的抑制。此酵素屬 EDTA 敏感者，但 Ca^{2+} 並不增加其酵素活性。

關鍵詞：熱穩定 α- 澱粉酶；純化；酵素性質。