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Thermal Stability and Starch Degradation Profile of α-Amylase from *Streptomyces avermitilis*

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Amylases from *Streptomyces* are useful in the production of maltooligosaccharides, but they have weak thermal stability at temperatures higher than 40 °C. In this study, α-amylase (SAV5981 gene of *Streptomyces avermitilis*) was expressed from *Streptomyces lividans* 1326 and purified by ammonium sulfate fractionation followed by anionic chromatography (Q-HP sepharose). The properties of the purified SAV5981 amylase were determined by the starch-iodine method. The effect of metal ions on amylase activity was investigated. The optimal temperature shifted from 25 to 50 °C with the addition of the Ca^{2+} ion. The thermal stability of SAV5981 was also dramatically enhanced by the addition of 10 mM $CaCl_2$. Improvement of the thermal stability of SAV5981 was examined by CD spectra in the presence and the absence of the Ca^{2+} ion. Thin-layer chromatography (TLC) analysis and HPLC analysis of starch degradation revealed that SAV5981 mainly produced maltose and maltotriose, not glucose. The maltoorigosaccharide-producing amylase examined in this study has the potential in the industrial application of oligosaccharide production.

Key words: maltogenic α-amylase; *Streptomyces avermitilis*; calcium-dependent thermal stability

α-Amylases (EC 3.2.1.1) are amylolytic enzymes that catalyze the hydrolysis of internal α-1,4-glycoside bonds in amylose and related carbohydrates. They are very important, and are widely used in the food, pharmaceutical, and detergent industries.[1–3] Over the past few decades, considerable research has focused on extracellular α-amylase produced by a wide variety of microorganisms, and currently enzymes from fungal and bacterial sources are the industry standard.[3] In particular, maltooligosaccharides-producing amylases are important in the industrial conversion of raw starch into sugars for food, beverages, and fermentation. They are often used in intermediate temperature range (40–60 °C) to prevent contamination or due to the food production process. Amylases from *Streptomyces* species have been reported and highlighted for the production of many products,[4] but most enzymes from *Streptomyces* sp. have weak thermal properties (thermostability and optimal temperature) compared to amylases from other hosts such as *Bacillus* and *Aspergillus* species,[5–7] and they usually show high activity at ambient temperature or below 40 °C, except in a few cases.[8,9]

Streptomyces avermitilis is known to produce many secondary metabolites, and its genome sequence has been determined.[10] It should have many genes, not only metabolite-related genes but also other enzymes that are available for industrial use. Based on the CAZy data base, *S. avermitilis* has 63 genes for amylolytic enzymes. In the present study, putative α-amylase amyA4 from *S. avermitilis* (SAV5981), which is classified into the GH13 family, was cloned and expressed in *Streptomyces lividans* 1326. SAV5981 showed excellent thermostability with the addition of the calcium ion. We also examined the substrate specificity and the starch degradation patterns of SAV5981, and found that it produced mainly maltose from starch.

Materials and Methods

Bacterial strains. S. avermitilis NBRC14893 and *S. lividans* 1326 (NBRC 15676)[11] were purchased from the National Institute of Technology and Evaluation (NITE), Japan. *Escherichia coli* DH5α was used as host strain in DNA manipulation.

Cloning of the SAV5981 gene and plasmid construction. After colony culture of *S. avermitilis* on an inorganic salt starch agar plate, the colony was cultured in 5 mL of TSB medium (17 g/L of pancreatic digest of casein, 3 g/L of papaic digest of soybean meal, 2.5 g/L of glucose, 5.0 g/L of sodium chloride, and 2.5 g/L of dipotassium phosphate) for 3 d at 28 °C with mild agitation at 200 rpm. After collection the cells, the *S. avermitilis* genomic DNA was extracted with a Promega Wizard Genomic DNA purification kit (cat# A1120, Promega, Madison, WI), and the purity was checked by agarose gel electrophoresis.

The gene encoding amyA4 (KEGG Database entry SAV5981, http://www.genome.jp/dbget-bin/www_bget?sma+SAV_5981, 1383 bp, Supplemental information; see *Biosci. Biotechnol. Biochem.* Web site) was amplified from *S. avermitilis* chromosomal DNA by PCR with

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KOD-FX polymerase (Toyobo, Osaka, Japan) with the forward primer 5′-GGAATTCCATATGACTTCGTTCCGTCCCGC-3′ and the reverse primer 5′-CCCAAGCTTCTACCGGCCCAGGTCGAAGA-3′, designed containing *Nde*I and *Hind*III restriction sites (underlined) respectively. The amplified SAV5981 gene was cloned into a pDNCAex1 vector, which was constructed for overexpression in *S. lividans* under the control of a PLD promoter.[12] The nucleotide sequence and the direction were determined with a BigDye terminator cycle sequencing kit and an ABI3130 DNA Sequencer (Applied Biosystems, Foster City, CA). Plasmid was named pDNCAex1-SAV5981. It was used in the transformation for *S. lividans* 1326.

Transformation of S. lividans. *S. lividans* 1326 was used as a host strain for protein expression.[11] The procedure for the preparation of the protoplast of *S. lividans* 1326 followed by transformation with plasmid pDNCAex1-SAV5981 was according to a method reported by Hopwood, *et al.*[13] Selection of transformants was carried out by overlaying autoclaved soft agar, which contained 200 µg/mL of thiostrepton (Sigma, St. Louis, MO) as antibiotic. The selected transformants were stored as 20% glycerol stock. For the glycerol stock, one spore of each transformant selected was inoculated into a test tube containing 5 mL of TSB medium supplemented with 50 µg/mL of thiostrepton, followed by cultivation at 28 °C for 3 d. The amylase-expressing transformants were screened with a 4% starch agar plate based on TSB containing 1% glucose. After 2 d of incubation at 28 °C, the plate was stained with iodine solution (final concentration 0.005% I_2 and 0.05% KI). A colony with a halo circle around it was selected for expression of the amylase. The amylase-expressing strain was stored as 20% glycerol stock.

Overexpression of SAV5981 in S. lividans *and protein purification.* One loop of *S. lividans* glycerol stocks were pre-cultured in 5 mL of TSB medium with 10 µg/mL of thiostrepton for 54 h at 28 °C with shaking at 200 rpm. When the OD of the pre-cultured broths reached approximately 8.5, they were inoculated into 50 mL of TSB medium with 1% glucose and 50 µg/mL of thiostrepton in a 500 mL baffled flask. The cells were grown with orbital shaking at 160 rpm at 28 °C for 27 h, and the pH decreased to less than 6.8. The culture broth was centrifuged at 15,000 × *g* for 20 min at 4 °C to remove the cells. The pH of the supernatant was adjusted to 5.5, and the proteins were precipitated by stepwise ammonium sulfate addition (final concentration, 60% saturation), followed by centrifugation at 16,000 × *g* for 25 min at 4 °C. The pellets were resuspended into 20 mM Tris–HCl at pH 8.0, then they were dialyzed (MWCO, 15 kDa) overnight in the same buffer at 4 °C. The dialyzed enzyme solution was purified with a Q-HP column (1.6 × 5 cm, 5 mL of column volume) that was equilibrated with 20 mM Tris–HCl (pH 8.0), and eluted 20 column volumes with 1 M NaCl in 20 mM Tris–HCl (pH 8.0) with a gradient of 0 to 0.5 M. The eluted fractions were corrected by 2 mL, and enzyme activity was measured. The active fractions were dialyzed (MWCO, 15 kDa) with 50 mM MES buffer at pH 6.5 at 4 °C.

Determination of enzyme activity by the starch-iodine method. Amylase activity was measured by a starch-iodine assay. The reaction mixture was composed of 1 w/v% soluble starch from potato (Nacalai Tesque, Kyoto, Japan), which was used as substrate in a 50 mM MES buffer (pH 6.5). The substrate solution was pre-incubated at 25 °C for 15 min, and then the enzyme was added to the solution at a final concentration of 5.3 µg/mL. The enzymatic reaction was conducted at each temperature for 10 min and then stopped with 0.9 M HCl as final concentration. After the reaction, an iodine solution containing 0.005 w/v% of I_2 and 0.05 w/v% of KI was mixed with the reaction mixture for color development. After 5 min of incubation at room temperature, the absorbance was measured at 660 nm. The amylase activity (DU/mg) was calculated by eq. (1),

$$\text{Activity (DU/mg)} = \frac{A_0 - A_S}{A_0} \times \frac{100}{\text{reaction time [min]} \times \text{enzyme concentration [mg/mL]}}, \quad (1)$$

where DU is the dextrinizing unit, A_0 is the absorbance of the samples without an enzyme, and A_S indicates the absorbance of the sample reacted with an enzyme. One DU was defined as the amount of enzyme required to lessen the color intensity of the starch-iodine complex by 1%. The enzyme concentration was measured by the BCA method.[14]

Effect of the calcium ion on thermal stability. The effect of the calcium ion on the thermal stability of SAV5981 at higher temperatures was investigated at temperatures ranging from 40 to 60 °C. An enzyme solution containing 10 mM of $CaCl_2$, the enzyme (58 µg/mL), and the protease inhibitor in 50 mM MES (pH 6.5) was incubated for 0, 10, and 30 min and for 1, 2, 3, and 5 h at each temperature. To stop the heat treatment at a specified time, the enzyme solution was cooled directly on ice. Residual amylase activity was measured by the starch-iodine method using the treated enzyme, as described above. Then we calculated the half-life of the amylase based on the assumption that the deactivation follows the first-order deactivation model, where deactivation rate constant k_d was determined by liner-regression from the deactivation profile, as follows:

$$\ln \frac{C_N}{C_{N0}} = -k_d \cdot t \quad (2)$$

where $C_N = C_{N0}/2$, the time (t) is defined as the half-life time ($t_{1/2}$), and thus $t_{1/2} = \ln 2/k_d$.

Time-course hydrolyzed product analysis by TLC. The starch substrate (1% w/v) and SAV5981 (145 µg/mL) were incubated at 25 °C for 2 d maximum. Samples were withdrawn at specified incubation durations, and were heated at 95 °C for 2 min to deactivate amylase. Each sample solution (1.5 µl) was spotted on a TLC plate (TLC silica gel 60 F_{254}, Fluka, Germany), and the solvent was dried after 1.5 h of running time. The running solvent was a mixture of n-buthanol:ethanol:water (5:3:2 vol%) with DAP solution as developing reagent. The DAP solution was composed of 1 g of diphenylamine, 1 mL of aniline, and 50 mL of acetone with the addition of 7.5 mL of phosphoric acid prior to use. After it was sprayed with DAP solution, the TLC plate was baked at 100 °C for 30 min to visualize spots. Malto-oligosaccharides (G1 to G6) were used as standard.

Circular dichroism (CD) spectrum. CD spectra were collected with a JASCO J-725K spectropolarimeter (Japan Spectroscopic, Tokyo) equipped with a JASCO PTC-348 temperature controller. CD spectrum measurement was carried out at 222 nm, changing the temperature of the protein solution in the presence and the absence of $CaCl_2$. The CD scan was performed changing the temperature from 20 to 90 °C at a scan speed of 1 °C/min.

Results and Discussion

Purification of amylase SAV5981 expressed from S. lividans.

Recombinant *S. lividans* 1326 with expression vector pDNCAex1-SAV5981 produced SAV5981 amylase in large amount. After the cultivation of recombinant *S. lividans* in TSB medium at 28 °C for 27 h, the culture supernatant was collected, and the secreted amylase was purified by ammonium sulfate fractionation followed by anion-exchange chromatography. Then the purity of SAV5981 was confirmed by SDS–PAGE analysis (Fig. 1). The molecular weight of SAV5981 was approximately 49 kDa, which is consistent with the value estimated by the gene size. Zymogram analysis of the culture supernatant of SAV5981 indicated the same molecular weight (see Supplemental information). The specific activity of the purified amylase was gradually increased through purification steps (Table 1), and

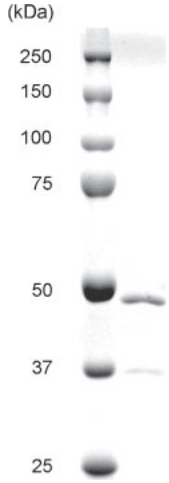

Fig. 1. SDS–PAGE of Purified SAV5981.
Lanes: left, molecular weight marker; right, purified SAV5981.

Table 1. Purification Chart for Amylase Expressed from SAV5981

Purification	Protein concentration (mg/mL)	Total protein (mg)	Specific activity (DU/mg)
Culture supernatant	0.40	80	1202
After ammonium sulfate fractionation	5.9	68	1725
After anion chromatography	0.58	5.8	3200

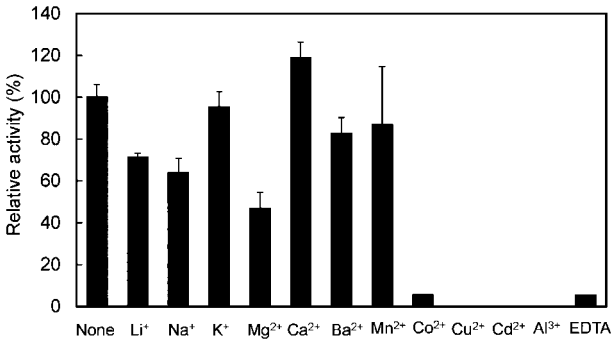

Fig. 2. Effects of Metal Ions and Chemical Reagents on the Activity of SAV5981.
Starch degradation was conducted with 10 mM of each additive.

finally provided a specific activity of 3,200 DU/mg by anion-exchange chromatography.

Effects of metal ions and chemical reagent.

The effects of various metal and chemical reagents on the amylase activity of SAV5981 is shown in Fig. 2. Among alkaline and alkaline earth metal ions, the calcium ion only moderately enhanced activity. Transition metals such as Co^{2+}, Cd^{2+}, Cu^{2+}, and Al^{3+} severely inhibited the activity of SAV5981. Furthermore, chelate agent EDTA, which removes the Ca^{2+} ion from protein molecules, also decreased amylase activity, indicating that Ca^{2+} ion is necessary for the activity of SAV5981. The calcium ion is known to be essential to maintain tertiary structure and to express the catalytic activity of α-amylase.[15] Most α-amylases consist of three domains: A, B and C.[16,17] The calcium binding site belongs to domains A and B, and the active site cleft is located between these two domains. Thus the ionic

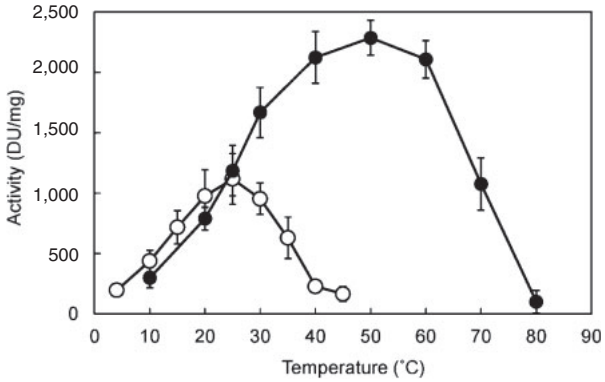

Fig. 3. Amylase Activity of SAV5981 at Various Temperatures.
Symbols: open, without addition of Ca^{2+}; closed: with 10 mM Ca^{2+}.

bridge between the two domains induced by the Ca^{2+} ion appeared to stabilize the active site cleft, resulting in high expression of amylase activity.[18] For the same reason, SAV5981 amylase requires Ca^{2+} to exhibit its catalytic activity, and removal of Ca^{2+} from the protein molecule with EDTA inactivated the enzyme. Further investigation is necessary to determine the effects of metal ion concentration and detailed information as to calcium binding site in SAV5981.

Optimal temperature and thermal stability.

The effect of the calcium ion on the temperature dependency of the activity of SAV5981 was evaluated (Fig. 3). Without addition of the calcium ion, the optimal temperature was 25 °C. Above this temperature, activity decreased gradually and became negligible at 45 °C. Much higher activity was observed at a higher temperature, of approximately 50 °C, when the calcium ion (10 mM) was added to the reaction mixture. This shift in optimal temperature enhanced amylase activity, from 1,000 DU/mg at 25 °C to 2,300 DU/mg at 50 °C. By the addition of the calcium ion, the structural stability of SAV5981 was enhanced, and the reaction rate was increased at higher temperatures.

Next, the thermal stability of SAV5981 was evaluated (Fig. 4). SAV5981 was found to be readily deactivated within 10 min at temperatures above 45 °C and was gradually deactivated in several hours at 40 °C in the absence of the calcium ion (Fig. 4A). On the other hand, the stability of SAV5981 dramatically improved in the presence of $CaCl_2$ (Fig. 4B). For example, the enzyme maintained its original activity for 4 h at 50 °C. Table 2 shows the half-life of the activity of SAV5981 at 40, 55 and 60 °C. The half-life at 40 °C with $CaCl_2$ could not be calculated because there was no activity loss. The half-life increased about 50 times at 55 °C due to the addition of $CaCl_2$. The high activity and long-term thermostability found in the present study should be useful in the starch processing industry in the initial degradation of starch to dextrins.

Conformational stability.

To investigate further the thermal stability of SAV5981 with the addition of $CaCl_2$, we carried out a CD spectra analysis of SAV5981 changing the solution temperature in the presence and the absence of the Ca^{2+} ion (Fig. 5). The denaturation temperature of SAV5981,

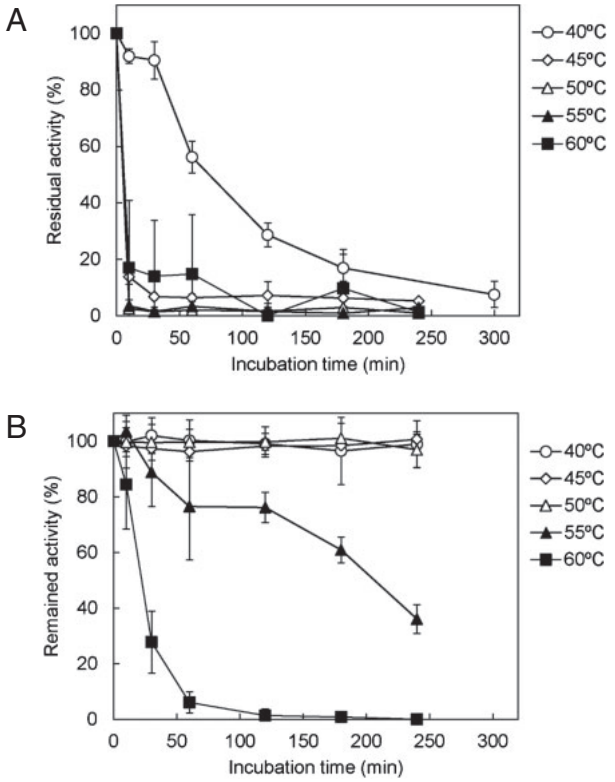

Fig. 4. Thermostability of SAV5981 with (A) and without (B) Addition of 10 mM Ca^{2+} Ion.

Table 2. Half-Life of SAV5981 with and without $CaCl_2$

Temperature (°C)	Half-life (min)	
	without $CaCl_2$	with $CaCl_2$
40	72	—[a]
55	4.3	196
60	3.9	17

[a]The half-life at 40 °C with $CaCl_2$ could not be calculated because no activity loss was observed.

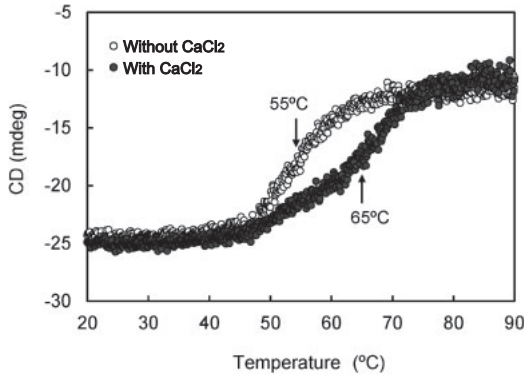

Fig. 5. CD Spectra Profiles at 222 nm at Various Solution Temperatures.

which was defined as the inflection point in the profiles, was found to be 55 °C in the absence of $CaCl_2$. On the other hand, the denaturation temperature shifted to 65 °C with the addition of $CaCl_2$. Since CD scanning at 222 nm was equivalent to analysis of the secondary structures of the proteins, this experiment differed from an examination of the tertiary structure, the addition of a

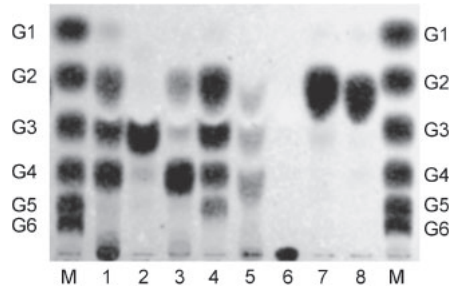

Fig. 6. Degradation of Saccharide Substrates by SAV5981.
Lanes: M, maltooligosaccharide standards; 1, starch; 2, maltotriose; 3, maltotetraose; 4, maltopentaose; 5, amylopectin; 6, pullulan; 7, cellobiose; 8, trehalose. The reaction was performed for 4 h by the addition of 10 mM Ca^{2+} ion.

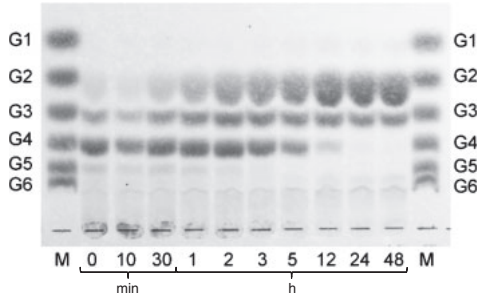

Fig. 7. Degradation Profile of Soluble Starch by SAV5981 with the Addition of 10 mM Ca^{2+} Ion.
Lane M shows maltooligosaccharide standards.

Ca^{2+} ion made the protein molecule more rigid, leading to an increase in the thermal stability of SAV5981.

Substrate specificity and degradation profile of starch. The hydrolysis of various substrates by SAV5981 over 4 h was investigated using soluble starch, maltooligosaccharides (G3, G4, G5), amylopectin, pullulan, cellobiose, and trehalose as substrate with the addition of Ca^{2+} (Fig. 6). Apparent activity occurred for soluble starch and G5, while G4 and amylopectin were slightly degraded by SAV5981. No activity was observed for G3, pullulan, cellobiose, or trehalose. This indicates that SAV5981 shows degradation activity only for relatively long maltooligosaccharides, including starch. Small molecules such as G3 and G4 might not reach the active site or might lack sufficient binding affinity to the subsite of the enzyme.

To determine the degradation mode of SAV5981, the hydrolysis pattern of soluble starch was investigated by varying the reaction time (Fig. 7). At the initial phase of degradation, maltotetraose (G4) and maltotriose (G3) were mainly generated, and finally maltose (G2) and G3 were the primary products after 24 h of reaction time. Glucose (G1) was not detected during the reaction. In hydrolysis by SAV5981, the G4 generated from starch should be sequentially degraded to G2 over several hours, resulting in a decrease in the concentration of G4 and an increase in that of G2. G3 increased in the first 1 h and then the concentration remained constant, indicating that SAV5981 did not react with G3. If amylase hydrolyzes G3, glucose should be observed as well as the formation of G2. It is noteworthy that no G1 was formed during the reaction by SAV5981. There are

reports of maltose-producing amylases from *Streptomyces*, where certain quantities of G1 and other oligosaccharides were produced simultaneously.[8,9,19] SAV5981 might have a characteristic degradation property that differs from other maltose-producing amylases.

Finally, we quantified the formation of maltose (4.49 g/L) and maltotriose (1.77 g/L) from 10.0 g/L of soluble starch by the HPLC system equipped with an evaporative light scattering detector (ELSD).

Conclusion

In this study, amylase SAV5981 from *S. avermitilis* was produced by *S. lividans*, and enzymatic characterization of it was done in detail. Recombinant protein production and purification by conventional column chromatography were achieved, and amylase activity was stabilized by the addition of the calcium ion. The resulting secreted protein has potential for industrial application in oligosaccharide production.

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