Pseudozyma aphidis I-8株の産生するα－Amylaseの単離精製とその応用

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Purification, Characterization and Application of α-Amylase from *Pseudozyma aphidis* I-8

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Etsuko Hirose,[1] Naho Izawa,[1] Junna Adachi,[1] Shigeharu Mori[2] and Tamio Mase[1,*]

[1]*Department of Human Nutrition, Sugiyama Jogakuen University (17–3, Hoshigaoka-motomachi, Chikusa-ku, Nagoya 464–8662, Japan)*
[2]*Food & Industrial Enzyme Division, Amano Enzyme Inc. (1–2–7, Nishiki, Naka-ku, Nagoya 460–8630, Japan)*

Abstract: A yeast strain I-8 was isolated as an α-amylase producer from digestive juice of *Nepenthes bicalarate*. The yeast was identified as *Pseudozyma aphidis* by the morphological test and comparative 26S rDNA-D1/D2 and ITS-5.8S rDNA gene sequence analysis. The α-amylase was purified from the culture filtrate by $(NH_4)_2SO_4$ precipitation, DEAE-TOYOPEARL 650M, Butyl-TOYOPEARL 650M, Hydroxylapatite and TOYOPEARL HW-55 chromatography. The purified enzyme was shown as a single band and the molecular mass was 55 kDa by SDS-PAGE. The specific activity was 1679 U/mg protein. The optimum temperature and pH were around 60°C and 5.0, respectively. The enzyme was stable in a pH range from 5.0 to 9.0 and at below 60°C. The enzyme hydrolyzed soluble starch and released glucose, maltose and oligosaccharides. Maltooligosaccharides (G3–G5) were also favorable substrate but it showed no activity toward maltose, isomaltose or pullulan. On the hydrolysis of soluble starch, the iodine color of the reaction mixture disappeared at almost 10% of reducing sugar formation and the hydrolysis limit was about 70% of soluble starch. From these results, the α-amylase was recognized as a unique α-amylase. The α-amylase was applied to the bread making process. Addition of the α-amylase to the bread making process presented no effect toward the crumb softness or color but the improved taste of the bread by sensory evaluation.

Key words: ***Pseudozyma aphidis*, α-amylase, maltotriose, bread making**

α-Amylase (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolase) catalyzes the endo-hydrolysis of 1,4-α-D-glycosidic linkage in polysaccharides such as starch and has many applications for food processing such as bread making, high glucose syrup production and so on. Above all, throughout the history of bread making, α-amylase has always played an important role. The first application of α-amylase in bread making was supplementation of malt as α-amylase. The role of α-amylase in bread making is development of the crust color and flavor, increasing of loaf volume, preparation of open crumb structure and extension of shelf life.[1] Later malt was replaced by microbial α-amylase having a more suitable thermal stability for bread making. The beneficial effects of using enzymes in the bread making industry are also obtainable by using chemical agents such as potassium bromate, cystain, azodicarbonamide, *etc*. However, the food market today shows a clear trend toward more natural products, and this has clearly favored the use of enzymes.

The bulk of α-amylases used in bread making industry are extracellular enzymes from fermentation with microorganisms. Microbial α-amylases are produced from high-yield strains by fermentation under controlled conditions in submerged culture or solid culture.

There are many α-amylases from cultures of various microorganisms including fungi,[2,3] bacteria,[4–10] actinomycetes[11–13] and yeast.[14–18] α-Amylase was also classified into many types by its application from hydrolytic action toward starch, for example, the effect of Ca^{2+} and the difficulty of raw-starch digestion.[5,16,19]

From this background, we have screened a yeast produced α-amylase possessing unique properties and investigated its use in the bread making process.

MATERIALS AND METHODS

Chemicals. Soluble starch, glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4) and maltopentaose (G5) were purchased from Wako Pure Chemical Industries, Ltd. The flour mixture named Panmix and dry yeast were purchased from Nisshin Flour Milling Inc. Polypepton-S was purchased from Japan Pharmaceutical Co., Ltd. All other chemicals used were obtained from commercial sources.

Microorganisms. The strain I-8 used in this study was newly isolated from digestive juice in a pitcher of *Nepenthes bicalarate* gathered from Higashiyama Botanical Garden in Nagoya City. The strain I-8 was identified by the morphological characteristics and gene sequence analysis based on the 26S rDNA-D1/D2 and ITS-5.8S rDNA. Genomic DNA was extracted by using the method of Marmur[20] *et al*. The DNAs were amplified by PCR using primer NL1, NL2, NL3 and NL4 for 26S,[21] and primers ITS5 and ITS4 for ITS.[22] The PCR products were subsequently sequenced using ABI PRISM 3130xl Genetic Analyzer System (Applied Biosystems Co., USA). Assembly of base sequence fragments was done using Chro-

* Corresponding author (Tel. +81–52–781–4483, Fax. +81–52–782–7265, E-mail: tmasej28@sugiyama-u.ac.jp).

masPro 1.4 (Technelysium Pty., Ltd., Australia). To analyze the sequences, homology analysis was performed by BLAST search using GenBank/EMBL/DDBJ as the database.[23] Sequences were aligned with the Clustal W program. Phylogenetic tree was constructed from evolutionary distance data calculated by the neighbor-joining method using the Tamura-Nei model.[24]

The stock culture was maintained on agar slants (pH 6.0) containing agar (1.6%) yeast extract (2.0%), glucose (1.0%) and Polypepton-S (2.0%).

Cultivation. Pieces of the colony I-8 strain were picked up from an agar slant and inoculated into 100 mL shaking flasks containing 20 mL of medium. The medium (pH 6.0) consisted of 1% soluble starch, 1% glucose, 1% sucrose, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 1% yeast extract and 1% Polypepton-S. After incubation at 25°C for 20 h, 40 mL of the culture were inoculated into a 3 L jar fermentor containing 2 L of the medium described above. The cultivation was done at 25°C for 72 h, with agitation at 350 rpm and aeration at 1.5 L/min.

Enzyme assay. The standard assay mixture contained 0.9 mL of 1% soluble starch dissolved in 100 mM McIlvaine buffer (pH 5.0) and 0.1 mL of enzyme solution. After incubation for 20 min at 40°C, the reducing sugars released were measured by the method of Somogyi-Nelson.[25] One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar as glucose per min under the above conditions.

The α-amylase assay kit (Kikkoman Co., Ltd.) was used for monitoring enzyme activity in column effluent on α-amylase purification.

Purification of α-amylase. All operations were performed at 4°C.

Step 1. Ammonium sulfate fractionation. After cultivation, the cells were removed by centrifugation and 5500 mL of crude α-amylase solution were got by filtration. The filtrate was concentrated by ultrafiltration using AIV-1010 (Asahi Kasei Co.) with 100% recovery of α-amylase activity. The enzyme solution was re-filtered to remove insolubles. To 630 mL of the filtrate, solid $(NH_4)_2SO_4$ was added up to 80% saturation with stirring and left overnight at 5°C. The resulting precipitates were collected by filtration and dissolved in 20 mM phosphate buffer (pH 6.0). The enzyme solution was dialyzed against the same buffer.

Step 2. DEAE-TOYOPEARL 650M column chromatography. The enzyme solution in step 1 was applied to a DEAE-TOYOPEARL 650M column (4.0 ϕ × 20 cm) equilibrated with 20 mM phosphate buffer (pH 6.0). The column was eluted with the same buffer, and non-adsorbed active fractions (425 mL) were combined. Solid $(NH_4)_2SO_4$ was added into the active fraction.

Step 3. Butyl-TOYOPEARL 650M column chromatography. The enzyme solution in step 2 was applied on a Butyl-TOYOPEARL 650M column (4.0 ϕ × 20 cm) equilibrated with 20 mM phosphate buffer (pH 6.0) with 20% $(NH_4)_2SO_4$. After the column was washed with 50 mL of equilibration buffer, the adsorbed proteins were eluted with 500 mL of a linear gradient of 20–0% $(NH_4)_2SO_4$ in the same buffer. The active fraction was dialyzed against 5 mM phosphate buffer (pH 6.0).

Step 4. Hydroxylapatite column chromatography. The enzyme solution in step 3 was applied on a hydroxylapatite column (2.0 ϕ × 20 cm) equilibrated with 5 mM phosphate buffer (pH 6.0). After the column was washed with 50 mL of equilibration buffer, the adsorbed enzyme was eluted with 300 mL of a linear gradient of the same buffer (5–200 mM).

Step 5. TOYOPEARL HW-55 column chromatography. Two mL of the active fractions obtained from step 4 was subjected to gel filtration on a TOYOPEARL HW-55 column (2.2 ϕ × 92 cm) using 20 mM phosphate buffer (pH 6.0) containing 0.5 M NaCl at a flow rate 20 mL/h. The active fractions were combined and dialyzed against 500 mL of 10 mM phosphate buffer (pH 7.0). The dialyzate was stored at 4°C and used for further experiments as the purified enzyme solution.

Determination of the anomer of D-glucose. The colorimetric system with D-glucose oxidase, peroxidase and mutarotase reported by Okada[27] was used.

Substrate specificity. For the study of hydrolysis of various substrates, the amount of reducing sugar was measured by the method of Somogyi-Nelson with glucose as the standard. The relative activities were defined with percentage toward the value of soluble starch as a substrate.

Hydrolysis of soluble starch by α-Amylase.

Determination of action pattern. The reaction mixture was containing 0.8 mL of 0.5% soluble starch containing 1 mM $CaCl_2$ and 0.2 mL of α-amylase solution possessed several activities (4.5×10^{-3} to 4.5 U/mL). After reaction at 50°C for 30 min, the reducing sugar released was measured with the Somogyi-Nelson method and the iodine blue value was measured at 700 nm. The action pattern of α-amylase was defined as the relationship between the decrease in blue value and increase in hydrolysis rate of soluble starch.

Measurement of hydrolysis rate. The reaction mixture was contained 4.0 mL of 2.5% soluble starch dissolved in McIlvaine buffer (pH 5.0) and 1.0 mL of α-amylase solution (3.45 U). After reaction at 50°C for several times (1, 3, 5 and 7 h), reducing sugars were measured with the Somogyi-Nelson method and the sugar released was assayed by thin layer chromatography (TLC).

TLC. TLC was done using Silicagel 60 plate (Sigma Co.). After development with the mixture of chloroform–methanol–water (30:20:4), the reagent consisting of a mixture of diphenylamine–aniline–acetone–80% phosphoric acid (2:2:100:15) was sprayed and heated at 90°C for 15 min for appearance of sugar spots.

Protein assay. The protein concentration was measured with Bio-Rad protein reagent (Bio-Rad Laboratories, USA) using bovine serum albumin as the standard. The absorbance at 280 nm was used for monitoring protein in the column effluent.

Determination of molecure weight.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done with the Compact PAGE AE-7305 using C-PAGEL (Atto Co., Ltd.). To measure the molecular mass, *Ez* Standard AE-1440 (Atto Co., Ltd) was used as the standard proteins.

Gelfiltration. The molecular mass was also estimated

by gel filtration with TOYOPEARL HW-55 column (1.5 ϕ × 100 cm). After equilibration with 10 mM phosphate buffer containing 0.5 M KCl (pH 7.0), 1 mL of the enzyme solution was applied to the column. A calibration curve was obtained with the standard protein (myokinase, 32.4 kDa; enolase, 67 kDa; lactic dehydrogenase, 142 kDa; glutamate dehydrogenase, 290 kDa).

Bread making.

Bread making procedure. The test was carried out with the no-time straight dough method.

Dough ingredients consisted of flour mixture (320 g), dry yeast (2.7 g) and 200 mL of purified enzyme solution (0, 2, 4 and 8 (× 10^3 U)). The ingredients were mixed and fermented in an automatic breadmaker SD-BT50 (Matsushita Electric Industrial Co., Ltd.) by the standard bread-dough preparation conditions described in the maker's manual. Then, the dough was rested at 28°C for 30 min, and punched lightly seven times. After being divided into portions of 32 g, each portion was rounded by hand, and given an intermediate proof of 30 min. Each portion molded to a round arch type was put into microwave oven RO-EL2 (Mitsubishi Electric Corporation) for a second fermentation (at 38°C for 20 min) and baking (at 200°C for 20 min).

Measurement and evaluation of bread properties. The bread loaf volume was determined by the rapeseed displacement method after holding at 23°C for 1 h. The crumb color of the bread was measured with Hunter color differences by color meter NE 2000 (Nippon Denshoku Industries Co., Ltd.).

The crumb firmness was measured with Texthurometer TDU-1 (YAMADEN Co., Ltd.) by measuring the compression force for bread slices (30 × 30 × 13 mm) prepared by Ultrasonic wave (YAMADEN Co., Ltd.). The crumb firmness was defined with the relative rate (%) toward firmness of standard bread (no enzymes were added).

The sensory characteristics of the breads were evaluated according to ranking method by fifteen women students aged 22 of Sugiyama Jogakuen University as a panel.

The color, flavor, smoothness, hardness, elasticity and taste by discrimination test and total acceptance by palatability test were compared; an order rating of 1, 2, 3 or 4 was given to each sample, with 1 being the most or best, 2 being the second-most/best, *etc*. The total sums of order were analyzed by means of Kramer's test.[27]

RESULTS

Identification of an isolated strain I-8.

The strain, designated I-8, was subjected to a polyphasic taxonomic investigation. For the morphological test, strain I-8 was grown on yeast extract-malt extract agar (Becton, Dickinson and Campany, USA) for 1 to 3 weeks at 25°C aerobically. The cells formed yeast-like colonies fringed with pseudohyphae at the margin. The cells were elongated and produced fusiform blastconidia by budding on short sterigmalike stalks. The colour became pink with age but no sexual structure was observed in the culture. The characteristic colony and blastoconidium morphology suggested that strain I-8 belongs to the genus *Pseudozyma*.[28,29]

Comparative 26S rDNA-D1/D2 gene sequence analysis also indicated that it formed a distinct phylogenetic lineage within the genus *Pseudozyma*. Additionally, the ITS-5.8S rDNA sequence of yeast I-8 by BLAST searching based on GenBank/EMBL/DDBJ showed 100% similarity with *Pseudozyma aphidis* CBS517.83^T (Fig. 1).

Purification of the enzyme.

The enzyme was purified by $(NH_4)_2SO_4$ fractionation and consecutive column chromatography. The results of purification are summarized in Table 1. The enzyme subjected purification procedure was shown as a single band on SDS-PAGE and single peak on gel filtration. The final preparation of the α-amylase was purified 4.3-fold on the

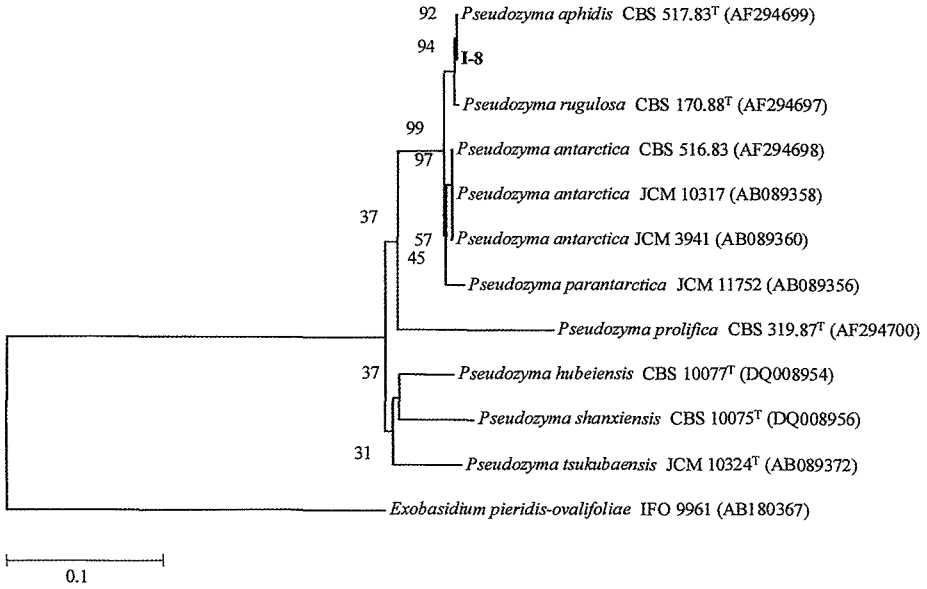

Fig. 1. Phylogenetic analysis of strain I-8, isolated from the digestive juice of *N. bicalarate* based on IST-5.8S rDNA.

Genomic DNA was extracted and amplified by PCR. The PCR products were sequenced using an ABI PRISM 3130xl genetic analyzer. The homology analysis was performed by BLAST search. The phylogenetic tree was constructed from evolutionary distance date calculated by the neighbor-joining method using the Tamura-Nei model.

Table 1. Summary of purification of α-amylase from *P. aphidis* I-8.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Culture filtrate	1868	727,150	389.1	100
$(NH_4)_2SO_4$	730.3	562,810	770.6	77.4
DEAE-TOYOPEARL	484.8	427,560	881.8	58.8
Butyl-TOYOPEARL	181.0	213,050	1177	29.3
Hydroxylapatite	97.0	132,340	1364	18.2
TOYOPEARL-HW-55	51.9	87,258	1679	12.0

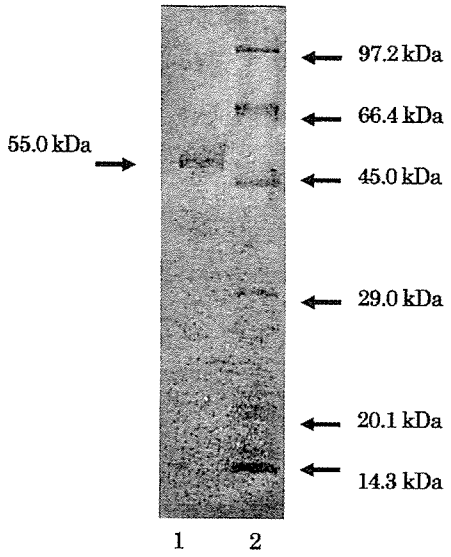

Fig. 2. SDS-PAGE of the purified α-amylase.

Lane 1, purified α-amylase; lane 2, molecular mass markers; phosphorylase b (97.2 kDa). Bovin serum albumin (66.4 kDa), ovalbumin (45.0 kDa), carbonic anhydrase II (29.0 kDa), soybean trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa).

basis of the standard activity assay, with a yield of 12.0%. The specific activity toward soluble starch was 1679 U/mg of protein. The purified enzyme was used for subsequent characterization.

Estimation of molecure weight.

SDS-PAGE of purified α-amylase was defined a single band with 55 kDa of molecular mass (Fig. 2). The molecular mass determined by gel filtration with TOYOPEARL HW-55 was also 55 kDa.

Effects of pH and temperature on the activity of the enzyme.

The effects of pH and temperature on the activity of the purified enzyme was studied under the standard assay conditions. The enzyme showed maximum activity at pH 5.0 (Fig. 3) and at 60°C (Fig. 4).

Stabilities of the purified enzyme toward pH and temperature.

Enzyme stability at different pH levels were measured by incubating the enzyme solution at 60°C and several pH for 15 min. After cooling in an ice bath and 20-fold dilution with 50 mM acetate buffer (pH 5.0), the residual activity was measured under the standard assay conditions. The enzyme was stable over a range of pH values between 6.0 and 9.0 (Fig. 5).

Thermal stability was measured by incubating the en-

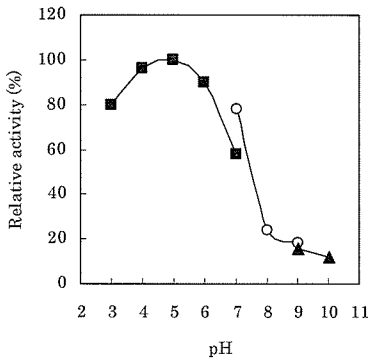

Fig. 3. Effect of pH on the enzyme activity.

The enzyme activities were measured at 40°C and several pH according to the standard assay method. ■, 100 mM McIlvain buffer; ○, 100 mM Tris-HCl buffer; ▲, 100 mM Atkins buffer.

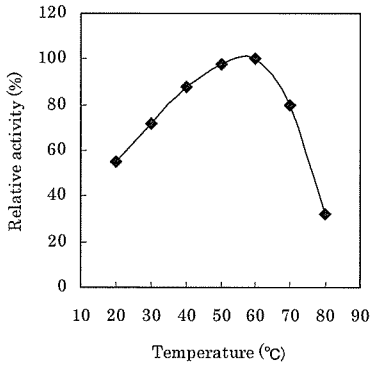

Fig. 4. Effect of temperature on the enzyme activity.

The enzyme activities were measured at pH 5.0 and several temperature according to the standard assay method.

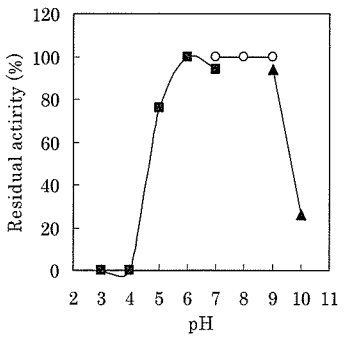

Fig. 5. pH stability of the enzyme.

After the preincubation at several pHs at 60°C for 15 min, the residual activities were measured according to the assay method at pH 5.0 and 40°C. ■, 100 mM McIlvaine buffer; ○, 100 mM Tris-HCl buffer; ▲, 100 mM Atkins buffer.

zyme solution at pH 5.0 and various temperatures for 15 min. After cooling in an ice bath, the residual activity was measured under the standard assay conditions. The enzyme retained its original activity on heating below 60°C (Fig. 6).

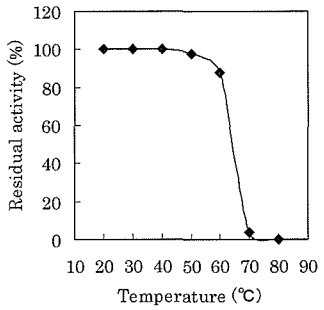

Fig. 6. Thermal stability of the enzyme.

After the preincubation at pH 5.0 and several temperatures for 15 min, the residual activities were measured at pH 5.0 and 40°C according the standard assay method.

Table 2. Effect of metal ions and reagents.

Chemical	Relative activity (%)
None	100
PCMB	88
SDS	86
EDTA	91
Monoiodoacetic acid	95
Al^{3+}	107
Cu^{2+}	98
Ca^{2+}	99
Zn^{2+}	104
Na^{+}	96
Fe^{3+}	95
K^{+}	100
Mg^{2+}	102
Co^{2+}	99
Fe^{2+}	105
Ag^{+}	73

The enzyme activity was assayed by the standard assay method with 1 mM of metal ions or reagents. The relative activity was defined as a percentage of enzyme activity without chemicals.

Table 3. Substrate specificity of the α-amylase.

Substrate	Relative activity (%)
Soluble starch	100
Starch (corn)	50
Amylopectin	82
Amylose	52
Maltopentaose	32
Maltotetraose	40
Maltotriose	20
Maltose	0
Isomaltose	0
Isomaltooligosaccharides	0
Pullulan	0
γ-Cyclodextrin	5
Trehalose	0
Sucrose	0
Lactose	0
Raffinose	0

The enzyme activity was assayed by the standard assay method with indicated substrates. The relative activity was defined as a percentage of enzyme activity toward soluble starch.

Effect of metal ions and chemical reagents on the activity of the enzyme.

The enzyme activity was assayed with the standard assay method with 1 mM of metal ions or reagents (Table 2). The enzymatic activity was weakly inhibited by Ag^{+} (27%), Fe^{3+} (5%), SDS (14%), EDTA (9%), *p*-chloromercuriobenzoic acid (PCMB) (12%) and monoiodoacetic acid (5%), but not affected by Ca^{2+}. The enzymatic activity was moderately stimulated by Al^{3+} (7%), Fe^{2+} (5%) and Zn^{2+} (20%).

Substrate specificity.

The hydrolyzed activities toward various substrates were measured under the standard assay conditions with 1.0% substrates in the reaction mixture. The results are shown in Table 3.

The purified α-amylase in this study exhibited significant starch-hydrolyzing activity. Amylopectin and maltooligosaccharides (G3–G5) were also better substrates, whereas activities toward other maltooligosaccharides such as maltose or isomaltose were not detected. Pullulan containing α-1,6-linkage, trehalose with α-1,1-linkage did not serve as substrate.

Hydrolysis action and limit toward soluble starch.

The blue value disappeared at about 10% hydrolysis rate of soluble starch (Fig. 7) and the limit of hydrolysis

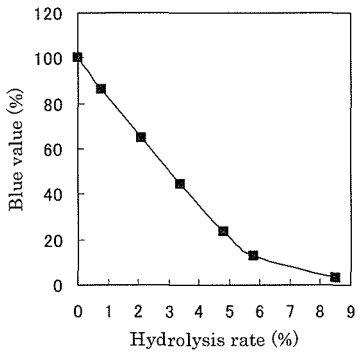

Fig. 7. The relationship between blue value and hydrolysis rate.

The reaction mixture contained 0.8 mL of 0.5% soluble starch with 1 mM $CaCl_2$ and 0.2 mL of enzyme solution (4.5×10^3–4.5 U/mL). After reaction at 50°C and pH 5.0 for 30 min, reducing sugar was measured by the Somogyi-Nelson method and iodine blue value was measured.

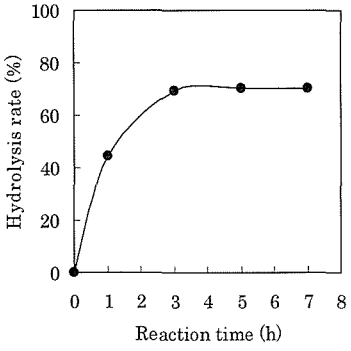

Fig. 8. Hydrolysis of soluble starch.

The reaction mixture contained 4.0 mL of 2.5% soluble starch dissolved in McIlvaine buffer (pH 5.0) and 1.0 mL of enzyme solution (3.45 U). After reaction at 50°C for the indicated times, reducing sugar was measured by the Somogyi-Nelson method.

Table 4. Sensory evaluation of the round arch type bread with α-amylase.

Enzyme (U/320 g flower)	Score for palatability						
	Color	Flavor	Smoothness	Hardness	Elasticity	Taste	Total
0	38	36	39	31	41	45	43
2×10^3	32	31	34	34	35	44	46
4×10^3	42	46	40	40	41	34	32
8×10^3	38	37	42	31	31	27*	29

Fifteen panelists ranked the round arch type breads according to their palatability. Differences among the total scores evaluated by Kramer's test. Significant difference, $^*p < 0.05$.

was almost 70% (Fig. 8). The products from soluble starch on the hydrolysis reaction were also assayed with TLC (data not shown). The product from soluble starch was mainly glucose. A small amount of maltose and oligosaccharide were detected but maltotriose and maltotetraose were not detected at the final stage of the reaction.

Bread making test with α-amylase.

The addition of the α-amylase on the round arch type bread making had no effects on crumb softness or crumb color. In contrast, a little difference was found in increasing the loaf volume of breads (data not shown). The results of sensory characteristics are shown in Table 4. According to sensory evaluation of the breads, the breads' taste was improved by the addition of α-amylase ($p < 0.05$). The smoothness, elasticity and total evaluation of breads with α-amylase were also preferred by panelists.

DISCUSSION

An α-amylase produced by a newly isolated yeast from a digestive juice in a pitcher of *N. bicalarate* was purified and characterized from culture filtrate. This strain, designated I-8, was identified a strain of *P. aphidis* by a morphological test and comparative 26S rDNA-D1/D2 and ITS-5.8S rDNA gene sequence analysis. *P. aphidis* was reported to be isolated from aphid excretions on *Solanum pseudocapsicum* and known to be a microorganism in risk group 1.[30] The enzymes for food processing are required to be registered as food additives under the Ministry of Health, Labor and Welfare in Japan. Therefore, the production of food enzymes must be carried out under strict safety checks. The safety checks are also required on all microorganisms for enzyme production. From the point of safety, strain I-8 is a good producer of food enzyme.

The α-amylase was purified to a single band on SDS-PAGE and its molecular mass was 55 kDa. The specific activity of the purified α-amylase was 1679 U/mg protein toward soluble starch and the structural configuration of the products was the α-form (data not shown). On the hydrolysis of soluble starch, the iodine color of the reaction mixture disappeared at almost 10% of reducing sugar formation and hydrolysis limit was about 70% of soluble starch based on release of reducing sugar. From these results, the purified enzyme is an α-amylase.

Fukumoto *et al*., after studying amylases of 100 strains of *B. subtilis*, have been dividing them into two groups according to the hydrolysis limit of soluble starch; one hydrolyzes about 30 to 40%, and the other, about 50 to 60%. The names of liquefying α-amylase for the former, and saccharifying α-amylase for the latter are proposed.[9] The percentage of the hydrolysis rate of starch at the point of iodine color disappearance is 22 to 30% in the case of saccharifying α-amylase, 10 to 15% about liquefying α-amylase.[9,31] From these points, the hydrolytic mode of the purified α-amylase was a type of liquefying α-amylase with saccharifying action.

Regarding substrate specificity, amylolytic substrates tested were favorable substrate including maltotriose (G3) and γ-cyclodextrin (γ-CD) except for maltose (G2). The definition of α-amylase in regard to hydrolytic action is endohydrolysis of 1,4-α-glycosidic linkages in polysaccharides containing three or more 1,4-α-glycosidic linked D-glucose units. Okada *et al.*[32] investigated the action patterns of ten kinds of α-amylases from animals, funji, bacteria and plants by the oligosaccharides mapping method. The results showed that G3 was hard for α-amylases to hydrolyzed compared with maltotetraose or higher molecular oligosaccharides. In contrast, there are several α-amylases reported to hydrolyze G3 easily. Suganuma *et al*.[33] reported that the Taka-amylase A (TAA) from *Aspergillus oryzae* can hydrolyse G3. The α-amylase of a yeast, *Saccharomycopsis* sp. (former name *Endomycopsis* sp.), hydrolyzed G2 and G3 strongly.[32,34] Other yeast α-amylase derived from *Pichia burtonii*[15] and *Cryptococcus* sp.[16] did not hydrolyze G3. On the long term hydrolysis of soluble starch by this purified α-amylase, the main product detected as a colored spot on a TLC plate was glucose (G1) and minor spots of G2 and oligosaccharides were detected, but no reverse compound spots were formed. The purified α-amylase can hydrolyze G3 but not G2. So, it is natural that G2 is formed on a level with G1. The result that G1 had a much greater yield than G2 suggests that this α-amylase has weak ability to hydrolyze G2.

The effect of Ca^{2+} and EDTA on the activity were determined at 1 mM in the reaction mixture. It is generally known that Ca^{2+} promote the activity and thermal stability of α-amylase, but Ca^{2+} (1 mM) did not promote the activity of the purified α-amylase. Inhibition of EDTA on the activity was also little. It is reported that the saccharifying type α-amylase is not affected by EDTA.[9]

On the study of *Pyrococcus furiosus* α-amylase, 95% activity of α-amylase remained after treatment with 1 mM EDTA. But it was proved later that Ca^{2+} was bound tightly to the enzyme and essential for the activity of α-amylase.[35] Therefore, additional study of this α-amylase in regard to Ca^{2+} and EDTA is necessary.

Today, enzymes play an important role in the bread making field. In the history of the use of enzymes in the

bread making industry, amylases have a long history of usage.[36] Amylase can be used as a dough softener that leads to improved machining properties, higher loaf volume and softer bread crumbs.

We are investigating the effect of *P. aphidis* α-amylase on bread making. With the addition of the purified α-amylase, we got the results of improvement of bread taste and elasticity, but not crumb softness or color. This α-amylase can provide glucose or maltose from dough starch, an important source of fermentable sugar and those sugars seem to change to tasty components. From this view point, the α-amylase in this study is another useful enzyme for bread making. α-Amylase in bread making is also used for antistaling, *i.e.* improving the fresh keeping of breads. So, additional studies of the bread making process with this α-amylase are expected.

As the results, we conclude that this α-amylase derived from *P. aphidis* I-8 is similar to TAA and *Saccharomycopsis* α-amylase on the point of G3 substrate specificity, but slightly different from α-amylases reported in its action pattern toward soluble starch.

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Pseudozyma aphidis I-8 株の産生する α-Amylase の単離精製とその応用

廣瀬悦子[1]，伊澤菜穂[1]，安立純奈[1]，森　茂治[2]，間瀬民生[1]
[1] 椙山女学園大学生活科学部管理栄養学科
(464–8662 名古屋市千種区星が丘元町 17–3)
[2] 天野エンザイム株式会社マーケティング本部
(464–8630 名古屋市中区錦 1–2–7)

食虫植物の一種であるウツボカズラ (*Nepenthes bicalarate*) の捕虫袋中の消化液から，α-amylase を産生する酵母を分離した．分離した酵母は形態観察および 26S rDNA-D1/D2 に続く ITS-5.8S　rDNA の遺伝子解析から *Pseudozyma aphidis* I-8 と同定した．本酵母の培養液から各種クロマトグラフィーにより，α-amylase を SDS-PAGE で単一バンドを示すまで精製した．収率は 12% であり，精製酵素の比活性は 1679 U/mg，分子質量 55 kDa，反応最適温度，pH はそれぞれ 60℃ および 5.0 であった．また，Ca^{2+} による酵素活性の上昇は認められなかった．可溶性デンプンを基質とした場合，分解率約 10% でヨード呈色反応が消失し液化型の α-amylase と推定されたが，分解限度は約 70% で糖化型の性質をも示した．基質特異性の解析の結果，本酵素は，maltose や isomaltose，pullulan には作用せず，一般的な α-amylase と同様の基質特異性を示したが，maltotriose にも良く作用した．本酵素を用いた製パン試験では，パンの柔らかさや色調には改善効果が認められなかったが，パネラーによる官能試験においてパンの味に良好な結果が認められた．