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β-Amylase System Capable of Hydrolyzing Raw Starch Granules from *Bacillus polymyxa* No. 26 and Bacterial Identification

SOHN, CHEON-BAE*, MYUNG-HEE KIM, JUNG-SURL, BAE[1] AND CHEORL-HO KIM[2]

Department of Food and Nutrition, Chungnam National University, 220 Kung-dong, Yusung-ku, Taejon 305-764, Korea
[1]Department of Food and Nutrition, Joongkyung Technical Junior College 155-3, Jayang-dong, Dong-ku, Taejon 300-100, Korea
[2]Genome Research Program, Genetic Resources Center, Genetic Engineering Research Institute, KIST, P.O. Box 17, Taedok Science Town, Taejon 305-606, Korea

A soil bacterium which produces raw starch-digesting β-amylase in culture medium, has been screened from soils. One strain, isolated and identified as *Bacillus polymyxa* No. 26, was selected as a β-amylase producing bacterium. Morphological and biological characteristics of the strain were found to be similar to those of a strain belonging to *B. polymyxa*. The electron microscopic observations of the bacterial vegetative cells and sporulated cells were extensively done to know the corelation between the enzyme synthesis and sporulation. When the bacterium was cultured on the appropriate media (3% dextrin, 0.3% beef extract,0.5% polypeptone, 1% yeast extract and 0.3% NaCl at pH 7.0 for 4 days) raw starch-digestible β-amylase was produced extracellularly. This strain produced 130 units of β-amylase per ml in a culture medium containing 3% dextrin at 30°C. This value is compared to those of other β-amylase-producing strains. The optimum pH and temperature for crude enzymes were pH 6.5 to 7.0 and 50°C, respectively. The enzymes were stable between pH 5.5 and 9.0 for 30 min at 45°C.

β-1,4-glucan maltohydrolase(β-amylase) has been found in higher plants such as cereal grains, sweet potato, soy bean, wheat, and barley. In the last decades, however, some reports have shown the presence of β-amylase in microorganisms. Even though there are reports on microbial β-amylase very little is known about the properties of bacterial β-amylase. Robyt *et al.* reported that an amylase from *Bacillus polymyxa* liberated maltose from starch, though its catalytic mechanism seemed to be different from those of plant β-amylases and α-amylases (8). Recently, extracellular amylases from *Bacillus megaterium* and *Bacillus cereus* have been identified as β-amylases based upon the result of measurements of the change in optical rotation of maltose released (2, 10). It has been shown that bacterial β-amylases are adsorbed onto raw starch, and degrade it to some extent, even though the raw-starch-degrading capacity cannot be compared to other raw starch digesting α-amylases. The hydrolysis extents of bacterial β-amylases are much lower than those of α-amylase or glucoamylase (9). However, the hydrolysis of raw starch by bacterial β-amylase may be enhanced by pre-treatment of the raw starch in a heating system, or by co-treatment with raw starch-digesting α-amylases. An RSDA(raw starch-digesting α-amylase) of *Bacillus circulans* F-2 has been known by Kim *et al.* (5, 6). The enzyme consists of a distinct regional domain structure in its polypeptide for raw substrate recognition (4). Prior to this report, there are no papers describing the domain structure of β-amylases which can digest raw starch granules.

In addition to our special concern with β-amylases, we have been interested in the screening of β-amylase producing microorganisms. A new bacterium, *B. polymyxa* No. 26, has been isolated as a potent β-amylase-producer. In this report, we describe isolation and identification of this β-amylase producing strain. The general properties of the strain are described based upon electron microscopic observation and the raw starch-digesting enzyme system is discussed.

*Corresponding author
Key words: β-Amylase, raw starch, spore, maltose, *Bacillus polymyxa*

MATERIALS AND METHODS

Materials

Soluble starch from E. Merck A.G., F.R.G. was reduced with sodium borohydride and used as a substrate for β-amylase assay. Raw corn starch, raw sweet potato starch and raw potato starch were purchased from Wako Pure Chemicals Co. Japan.

Screening and Isolation of β-Amylase-Producing Microorganisms

Screening of β-amylase producing microorganisms was carried out with an induction medium consisting of 2% corn starch, 0.3% beef extract, 0.5% peptone, 1% yeast extract, 0.3% NaCl(pH 7.0) at 30℃ for 2 to 3 days. Plates were exposed to KI-I_2(iodine) solution and colonies which showed white halos were collected. The isolates were aerobically cultured in test tubes each containing 2.0% soluble starch as a carbon sources, and β-amylase activity in the culture supernatant was assayed.

Taxonomical Tests of Strain No. 26.

Taxonomical studies were carried out according to the Manual of Microbiological Methods (1). The strain was classified as endospore-forming rod strain, *Bacillus polymyxa* No. 26, according to Bergey's Mannual of Determinative Bacteriology (11). The morphological aspects and sporulated characteristics were studied using a scaning electron microscope.

Assays of β-Amylase, α-Amylase and Raw Starch Degrading Activity

β-amylase activity was assayed by measuring the amount of reducing sugar released from the digestion reaction of soluble starch using the DNS-method (6). The substrate used was 1.0% soluble starch (Wako Pure Chemicals Co.) dissolved in 50 mM phosphate buffer (pH 6.0). Fifty μl of enzyme solution were added to 0.5 ml of substrate. After incubation for 10 min at 40℃, the reaction mixture was stopped by adding 0.5 ml of DNS reagent and then, heated at 100℃ for 5 min and cooled and added 4 ml of distilled water. The optical density of the solution was measured at 535 nm. α-Amylase and raw starch-digesting enzyme activities were assayed with soluble starch or raw corn starch as substrates using the colorimetric method of Iodine-starch interaction described by Kim *et al.* (1990) (3, 6). One unit of the enzyme was defined as the amount of enzyme which liberated 1 μmol of maltose per min.

Analytical Methods

Protein content was determined by the method of Lowry (7), with bovine serum albumin as a standard. Protein content in the culture medium was routinely followed by absorbance at 280 nm. Oligosaccharide patterns produced by enzyme action were examined by thin-layer chromatography (TLC). TLC was done on a Whatman K5F silica gel plate with a solvent system of n-propanol/n-butanol/water (5:3:4, v/v/v). Sugars on the plates were detected by the silver nitrate dip method following glucoamylase treatment (6).

Digestion of Starch Granules

The assay method described by Kim C-H *et al.* (6) was used to analyze digestion of starch granules. Crude enzyme (0.5 units) was incubated with 10 mg of starch granules at 40℃. When the enzyme reaction was finished, the supernatant of the reaction mixture was assayed for reduced sugar by DNS-method. The supernatant was spotted on TLC for development.

RESULTS AND DISCUSSION

Screening and Taxonomical Study of β-Amylase Producing Strain

From the screeing method described in Materials and Methods, one strain, No. 26, showed large halos on the starch-agar plate with high β-amylase activity. This strain was selected as the best β-amylase producing microorganism.

The morphological, cultural and physiological characteristics of the strain were tested. Strain No. 26 was rod shaped (2−6×0.6−0.8 μm), gram positive, formed en-

Table 1. Morphological, cultural and biological characteristics of the isolated strain No. 26.

A. Morphological characterisitics
Form: rod
Size: 2−6×0.6−0.8 μm
Motility: motile
Gram reaction: positive
Spore: oval, central or subterminal
B. Cultural and biological characteristics
pH of growth: 4.5~10.0
Growth at 45℃: negative
Growth in 7% NaCl: negative
Utilization of citrate: negative
Anaerobic growth in glucose broth: positive
Catalase: positive
Carbohydrates, acid and gas from: positive (glucose, arabinose, mannitol, xylose)
VP test: positive
Starch hydrolysis: positive
Casein hydrolysis: positive
Nitrate reduction: negative
Urease: negative
Indole: negative
Gelatin hydrolysis: positive

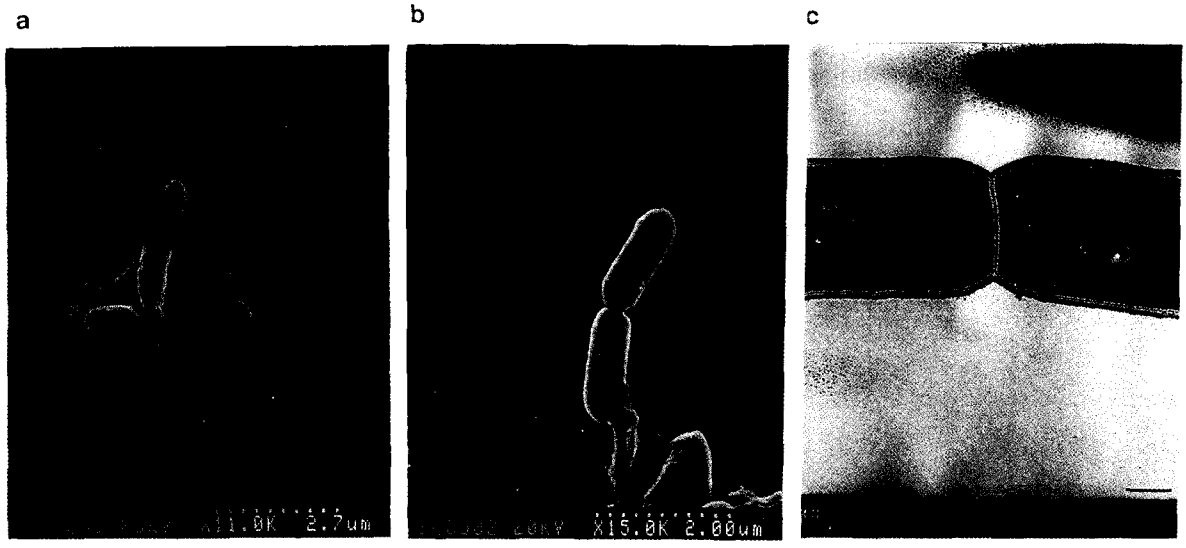

Fig. 1. Scanning electron micrograph of vegetative cells of *Bacillus polymyxa* No. 26.
(a) low magnification(length of dot line=2.7 μm); (b) higher magnification(length of dot line=2.0 μm); (c) transmission electron micrograph (0.25 μm).

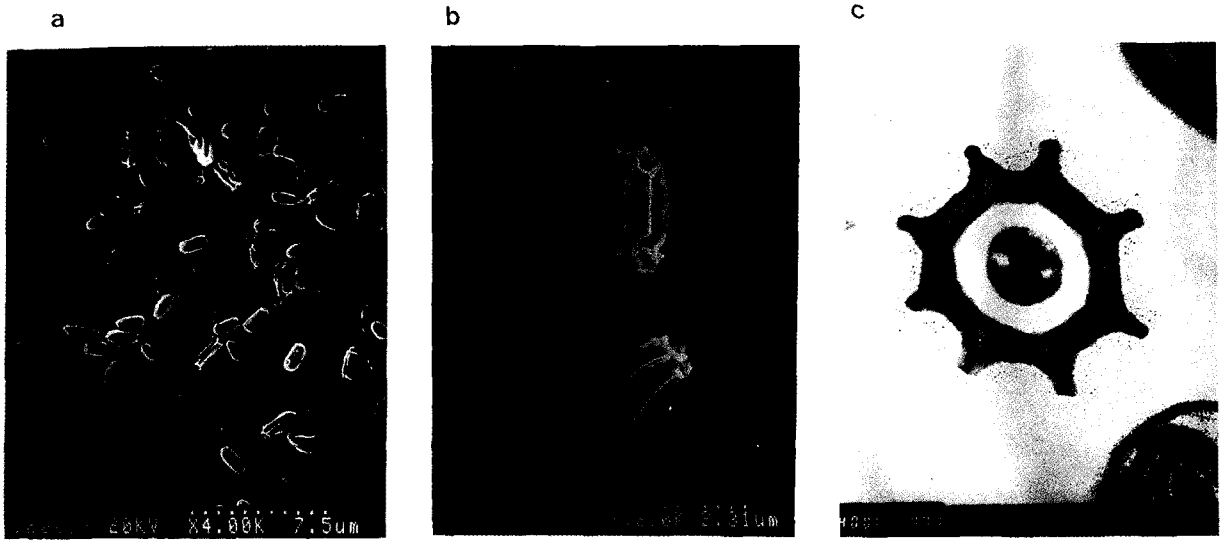

Fig. 2. Electron micrograph of sporulated cells of *Bacillus polymyxa* No. 26.
(a) low magnification(length of dot line=7.5 μm); (b) higher magnification(length of dot line=2.31 μm); (c) transmission electron micrograph (0.25 μm).

dospores, catalase positive, showed no growth in 7% NaCl, and produced gas and acid from glucose, arabinose and xylose. Details of these summarized results are shown in Table 1 and Fig. 1 and 2. Strain No. 26 was identified as *Bacillus polymyxa* from Bergey's manual of Determinative Bacteriology.

Production of β-Amylase in a Culture System

Experiments were carried out in a shaking flask. The strain produced about 130 units of β-amylase per ml in a medium consisting of 3% dextrin, 0.3% beef extract, 0.5% bacto peptone, 1.0% yeast extract, and 0.3% NaCl at an initial pH of 7.0, at 30℃ for 4 days. Starch and dextrin were inducible for the enzyme production of β-amylase, but mono- or oligosaccharides such as glucose and galactose were not. The time course of β-amylase production under optimum conditions is shown in

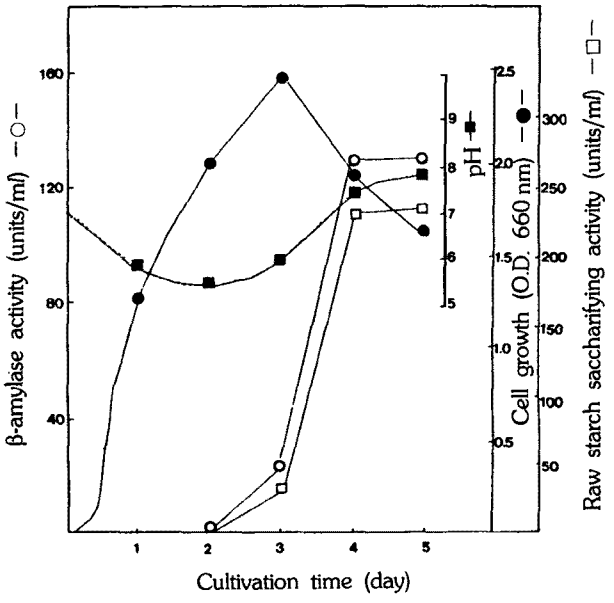

Fig. 3. Maximum β-amylase production on the induction medium.
The induction medium for β-amylase production was described in Material and Methods.

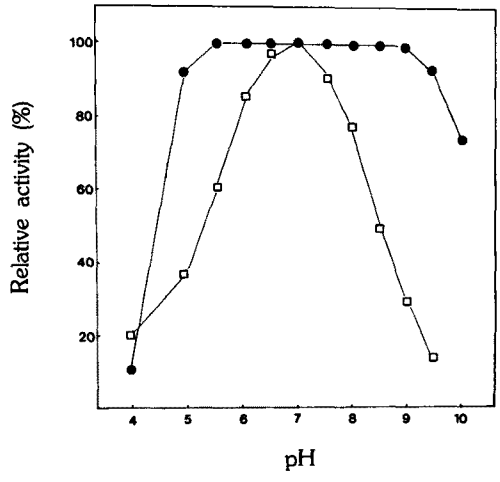

Fig. 4. Effect of pH on the crude β-amylase activity and stability.
The following buffers were used: 50 mM sodium acetate buffer (pH 3.5 to 5.5); 50 mM sodium phosphate buffer (pH 6.0 to 7.5); 50 mM Tris-HCl buffer (pH 8.0 to 9.5) and 50 mM Glycine-NaOH buffer (pH 10.0 to 11.0). To test pH stability, properly diluted enzyme solution was preincubated at each pH for 30 min (4℃) and enzymatic activity was then checked. ●, pH stability; □, optimum pH.

Fig. 3. Onset of β-amylase activity began when bacterial growth was in the log phase. Then, fully grown cells started to lysis. After 72 to 96 hours cultivation β-amylase production reached a maximum and activity was 130 units/ml broth.

It is known that *B. polymyxa* produces β-amylase with some polysaccharide-hydrolyzing activities. When the enzymatic characters of the β-amylases of other *B. polymyxa* strains were investigated no differences in the properties were observed(data not shown). The large production of total extracellular β-amylase in strain No. 26 was the only difference.

Effect of pH and Temperature on Enzyme Activity and Stability

The culture filtrate containing β-amylase was most active at pH 6.5 to 7.0 and was stable over a pH range of 5.5 to 9.0 for 30 min at 40℃, as shown in Fig. 4. The pH activity curve and the pH stability of the β-amylase were essentially the same as those of β-amylases produced by other *Bacillus* species (2, 8). The crude enzyme was most active at 50℃ and was stable below 45℃, as shown in Fig. 5. The optimum activity and the stability of crude β-amylase were essentially the same as those of β-amylases produced by other bacterial β-amylase systems with a like pH optima (9, 10).

Digestion of Raw Starches by Crude Enzyme

The action of the enzyme on corn, sweet potato and potato starch granules was investigated and compared

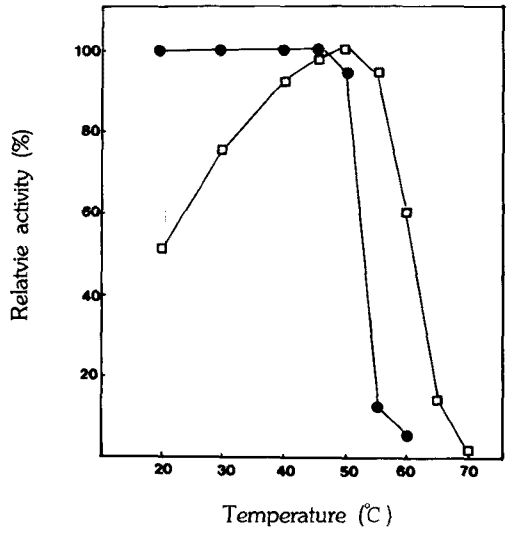

Fig. 5. Effect of temperature on crude β-amylase activity and stability.
Enzyme activity was assayed at various temperatures at standard conditions (pH 6.0). Symbols are same as Figure 4 (●, temperature stability; □, optimum temperature).

with the action on soluble starch. The enzyme hydrolysed raw corn starch, raw sweet potato starch and raw potato starch with respective hydrolysis yields of 50, 25 and 8% for 3 hrs at 40℃. In contrast to raw starch, soluble starch was perfectly digested by the enzyme so-

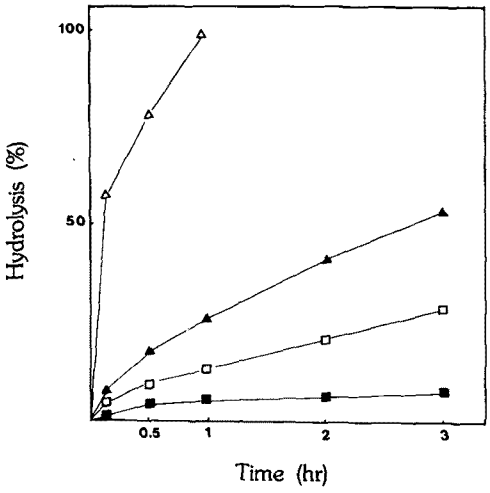

Fig. 6. Hydrolysis of various starches by culture filtrate of *Bacillus polymyxa* No. 26.
Enzymatic hydrolysis of various raw substrates and soluble substrate was performed using the method. □, raw sweet potato starch; ■, raw potato starch; △, soluble starch; ▲, raw corn starch.

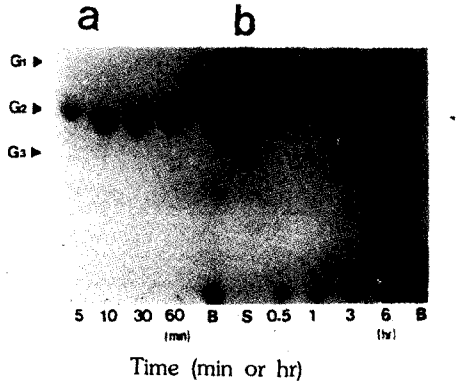

Fig. 7. Thin-layer chromatographic analysis of starch substrate-hydrolyzates by *Bacillus polymyxa* No. 26 culture filtrate.
An oligosaccharide analysis on silica plate (Whatman K5F silica gel, USA) was carried out. S denotes standard oligosaccharide molecules of glucose (G1), maltose (G2) and maltotriose (G3). B shows non-digested substrates as a control. a, soluble starch-hydrolysate; b, raw corn starch-hydrolysate.

lution in 1 hr. These results clearly indicated that the enzyme can be used in a raw corn starch saccharification process in the fermentation industry. The enzyme solution hydrolyzed a one-fourth amount of raw corn starch in 30 min (Fig. 6). As a powerful raw starch digesting enzyme the extracelluar enzyme of *B. circulans* F-2 strain has been regarded as the best(Kim *et al.*, 1990) (6). However, the enzyme of *B. circulans* is an exo-wise type of α-amylase. Therefore, β-amylase type which can effectively digest raw starches have long been searched by many researchers. To our best knowledge, a newly isolated *B. polymyxa* No. 26 β-amylase should be a good sample for above purpose.

Action Pattern of β-Amylase on Soluble and Raw Substrates

The action patterns of crude β-amylase on soluble starch and raw starches, such as raw potato starch, raw sweet potato starch and raw corn starch were examined by TLC. The enzyme system produced only maltose in all reaction periods and converted soluble starch into maltose with 100% yield in incubation for 60 min. These results suggest that the enzyme produced by *B. polymyxa* No. 26 is exo-wise, since no maltotetraose or maltohexaose was detected in digestion of substrates (Fig. 7). In order to see whether other types of starch-degrading enzymes are existed in the bacterial enzyme system or not, the enzyme-reaction products were carefully analysed on TLC. However, maltose was a main product through all stages of incubation. The enzyme system of *B. polymyxa* No. 26 contains β-amylase as a main supplier of carbon source.

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