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Production and Characterization of Raw Starch Hydrolyzing Enzyme from Bacteria

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세균에 의한 생전분 분해효소의 생성 및 특성

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A bacterium capable of hydrolyzing raw starch was isolated from soil, which was identified as a strain of *Bacillus*. The effects of culture conditions and medium compositions on the enzyme production were investigated. Among tested carbon sources, soluble starch and wheat starch were most effective for the production of the enzyme, and the level of concentration for the optimal enzyme production was 0.5%. For nitrogen sources, polypeptone was best for the enzyme production, with the level of 0.5%. The enzyme was maximally produced by cultivating the organism at medium of initial pH 6.5, and temperature of 35°C. The enzyme was partially purified by Sepharose CL-6B gel filtration and DEAE-Sephacel ion-exchange chromatography. The optimal pH and temperature for the enzyme reaction were 6.5 and 70°C, respectively. The enzyme most stable at pH 8.0, and temperature up to 60°C. In kinetic studies, the k_m values for corn, wheat, rice and potato starch were 1.7, 1.4, 2.5 and 1.0%, respectively.

A number of enzymes can hydrolyze soluble starch into reducing sugars but most of them are unable to degrade raw starch. Starch has to be gelatinized by cooking in conventional saccharification of starch. Therefore the enzymatic hydrolysis of raw starch without cooking is of considerable industrial importance (1). Several microorganisms such as *Aspergillus awamori* (2, 3), *Rhizopus* sp. (4, 5), *Streptococcus bovis* (6), *Bacillus circulans* (7), *Chalara paradoxa* (8), *Aspergillus sp. (9), Streptomyces* sp. (10) and *Corticium rolfsii* (1) have been reported to be producers of microbial amylases capable of digesting raw starch. A search for additional enzyme producers from new sources was needed for effective raw starch hydrolysis. Recently we isolated a bacterium which can digest various raw starches effectively. This paper describes the production and characterization of raw starch hydrolyzing enzyme from the bacterium.

Key words: Production, characterization, raw starch, enzyme, bacteria

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Materials and Methods

Materials

Corn, wheat, potato, rice starch and dinitrosalicylic acid were obtained from Sigma Chemical Co. Soluble starch was purchased from Hayashi Pure Chemical Co. Sepharose CL-6B and DEAE-Sephacel were from Pharmacia Fine Chemicals. All other reagents used were of analytical grade.

Microorganism and cultivation

Organism used was one of our isolates from soil. It was cultivated in a basal medium (1) containing, in g/liter: $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0; $CaCl_2$, 0.3; $MgSO_4 \cdot 7H_2O$, 0.3; urea, 0.3; polypeptone, 10.0;

starch, 20.0; and a mineral solution, 1.0. The mineral solution contained, in g/liter: $FeSO_4 \cdot 7H_2O$, 5.0; $MnSO_4 \cdot H_2O$, 1.6; $ZnSO_4 \cdot 7H_2O$, 1.4; and $CoCl_2$, 2.0. The kinds and concentrations of some components were changed and the effects of the enzyme production was examined.

Enzyme assay

The final concentration of starch was 5% in 0.2 M Na-phosphate buffer (pH 6.5). The enzymatic reaction was initiated by addition of 0.1 m*l* enzyme to the assay mixture and incubated for 2 hrs at 45°C. The total volume of the assay mixture was 1 m*l*. After reaction, it was centrifuged to remove unreacted raw starch, and reducing sugars liberated into the supernatant were measured by dinitrosalicylic acid method (11). One unit of enzyme activity was defined as the amount of enzyme to produce one μ mole of maltose per hr under the defined conditions.

Analysis of hydrolysis products

The product of the amylase reaction from the isolated bacterium was investigated by paper chromatography. The enzyme reaction for product identification was performed in assay mixture (1 m*l*) containing wheat starch (5%), Na-phosphate buffer (0.2 M, pH 6.0) and enzyme solution (0.1 m*l*) for 18 hrs at 45°C. Then 20 μl of reaction mixture, glucose, maltose and starch was spotted separately on Whatman No. 1 paper and they were developed in 65% n-propyl alcohol in H_2O for 4 hrs by ascending method. The spots of reducing sugars were detected by dipping the paper into reducing sugar specific reagent,aniline-diphenylamine (12).

Determination of protein concentration

Protein concentration was measured by the method of Lowry *et al.* (13) using bovine serum albumin as a standard protein.

Results and Discussion

Identification of the isolated organism

The isolated bacterium is aerobic, rod-shaped, Gram positive, and spore former. It also produces catalase. Therefore it seems to be *Bacillus* sp.

Product identification

It was confirmed by paper chromatography that the products of the amylase reaction from the isolated bacterium were maltose, maltotriose, and other oligosaccharides when assayed for 2 hrs, but maltose mainly when assayed for 18 hrs. Therefore, the amylase produced from the organism seems to be α-amylase.

Table 1. Effect of carbon sources on production of raw starch hydrolyzing enzyme

Carbon source	Enzyme activity (unit/m*l*)
Corn starch	26.0
Rice starch	20.5
Potato starch	18.7
Soluble starch	30.5
Wheat starch	30.2
Amylopectin	20.5
Lactose	18.7
Ribose	21.0
Maltose	20.5
Dextrose	17.5
Galactose	8.5
Fructose	17.5
Sucrose	16.5
None	12.3

The concentration of each carbon source was 0.5%. The initial pH of medium was adjusted to 6.5. Cultivation was carried out for 48 hrs at 35°C.

Effect of carbon sources on production of the enzyme

Production of raw starch hydrolyzing enzyme from the isolated *Bacillus* strain was examined by replacing starch in basal medium with various carbon sources. As shown in Table 1, soluble starch and wheat starch showed the highest enzyme productivity. Therefore, soluble starch or wheat starch was used as carbon source for maximum production of the enzyme from the isolated strain. Table 2 demonstrates that the effect of varying concentration of starch on production of the enzyme. The enzyme was maximally produced when 0.5% carbon source was used in the medium. This result seems to be identical with that from fungal enzyme (14).

Effect of nigrogen sources on production of the enzyme

Various nitrogen sources were replaced with polypeptone to investigate the effect of nitrogen

Table 2. Effect of carbon source concentration on production of raw starch hydrolyzing enzyme

Concentration (%)	Reducing sugar (mM)	Enzyme activity (Unit/ml)
0	0.15	18.0
0.1	0.30	20.5
0.5	0.60	27.0
1.0	1.25	24.0
1.5	1.80	17.5
2.0	3.60	4.5

Wheat starch was used as a carbon source. Cultivation was performed for 48 hrs at 35°C. Initial pH of the media was adjusted to 6.5.

Table 3. Effect of nitrogen sources on production of raw starch hydrolyzing enzyme

Nitrogen source	Unit/ml
Asparagine	13.5
Gelatin	11.5
Albumin	13.5
Ammonium sulfate	3.3
Peptone	16.5
Polypeptone	20.5
Yeast extract	19.5
Urea	7.8
Potassium nitrate	5.5
Tyrosine	6.0
Cysteine	4.0
Glycine	5.5
Sodium nitrate	5.5
Ammonium molybdate	4.0
None	0.8

Cultivation was carried out for 48 hrs at 35°C. Initial pH of the medium was adjusted to 6.5. The carbon source used was wheat starch (0.5%), and the concentration of nitrogen sources used was 0.3%.

sources on the enzyme production. As shown in Table 3, polypeptone was most suitable for the enzyme production among tested nitrogen sources. The inorganic nitrogen sources are generally ineffective for the organism to produce the enzyme. The concentration of polypeptone was varied from 0 to 2.0% to examine concentration effect on the enzyme production. The optimal polypeptone concentration for the enzyme production was 0.5% as shown in Table 4.

Table 4. Effect of polypeptone concentration on production of raw starch hydrolyzing enzyme

Concentration (%)	Unit/ml
0	0
0.05	3.3
0.1	8.5
0.3	25.5
0.5	31.5
0.75	25.5
1	25.5
2	22.5

Carbon source used was wheat starch (0.5%). Cultivation was peformed for 48 hrs at 35°C. The initial pH of the medium was 6.5.

Effect of initial pH on production of the enzyme

The initial pH of the medium was adjusted to various pH values from 3.0 to 9.0 and cultivation was carried out for 3 days at 35°C. The medium was composed of 0.5% soluble starch, 0.5% polypeptone and same salts as described in Methods. As shown in Fig. 1, maximal yield of raw starch hydrolyzing activity was achieved when initial pH of the medium was 6.5. In case of *Rhizopus oryzae*, the maximal enzyme production was achieved when initial pH of the medium used was adjusted to 4.0 (13).

Effect of culture temperature on production of the enzyme

For determining the optimal temperature for the production of the enzyme by the isolated *Bacillus*, cultivation was carried out at various temperatures ranging from 25 to 45°C. The optimal temperature for the enzyme production was 35°C as shown in Fig. 2.

Effect of inorganic salts on production of the enzyme

Various metal salts affect the production of the enzyme. As shown in Table 5, Cu^{++}, Zn^{++}, Fe^{++}, and Hg^{++} ions inhibited the production of the enzyme completely.

Enzyme purification

Crude enzyme was loaded to Sepharose CL-6B column (2.5 × 58 cm), which was equilibrated with 10 mM Tris/HCl buffer (pH 8.0). The elution was carried out with the buffer, and the active fractions were pooled (Fig. 3). Then they were loaded to DEAE-Sephacel ion

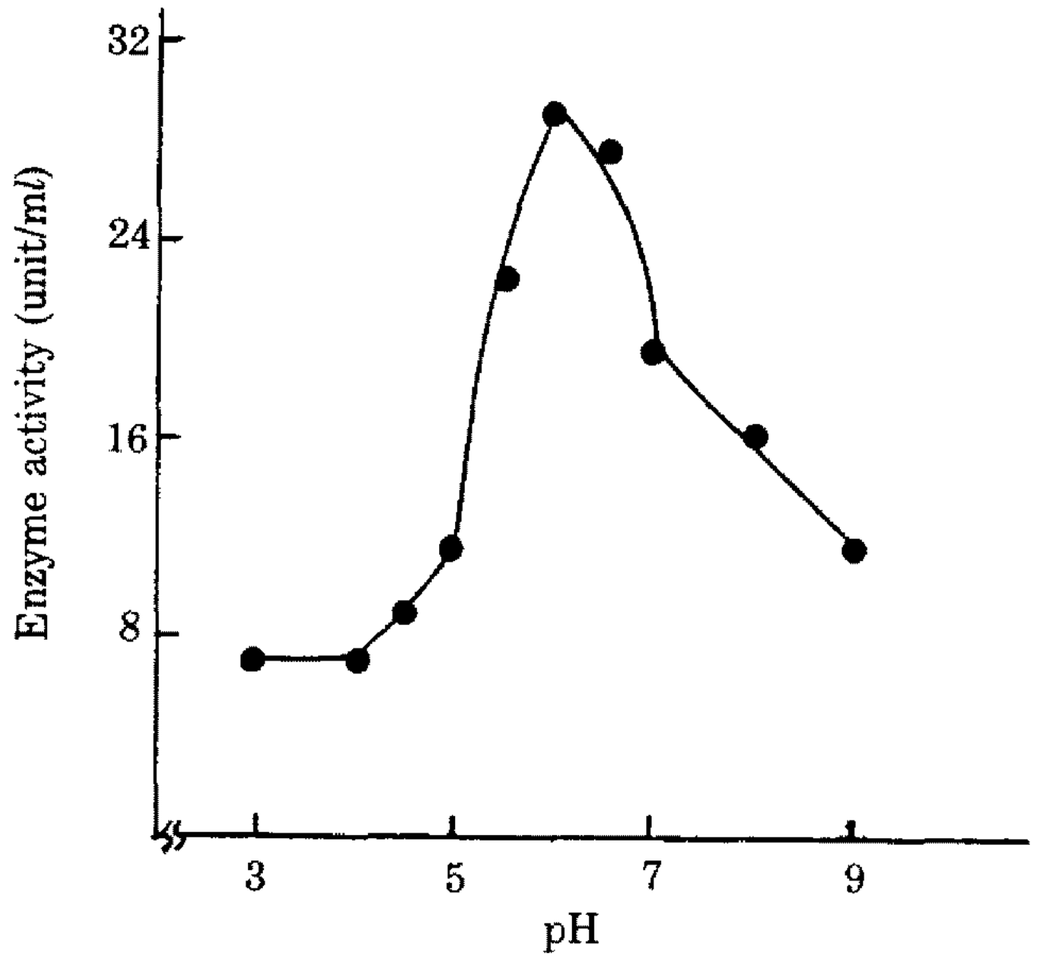

Fig. 1. Effect of initial pH on production of raw starch hydrolyzing enzyme.

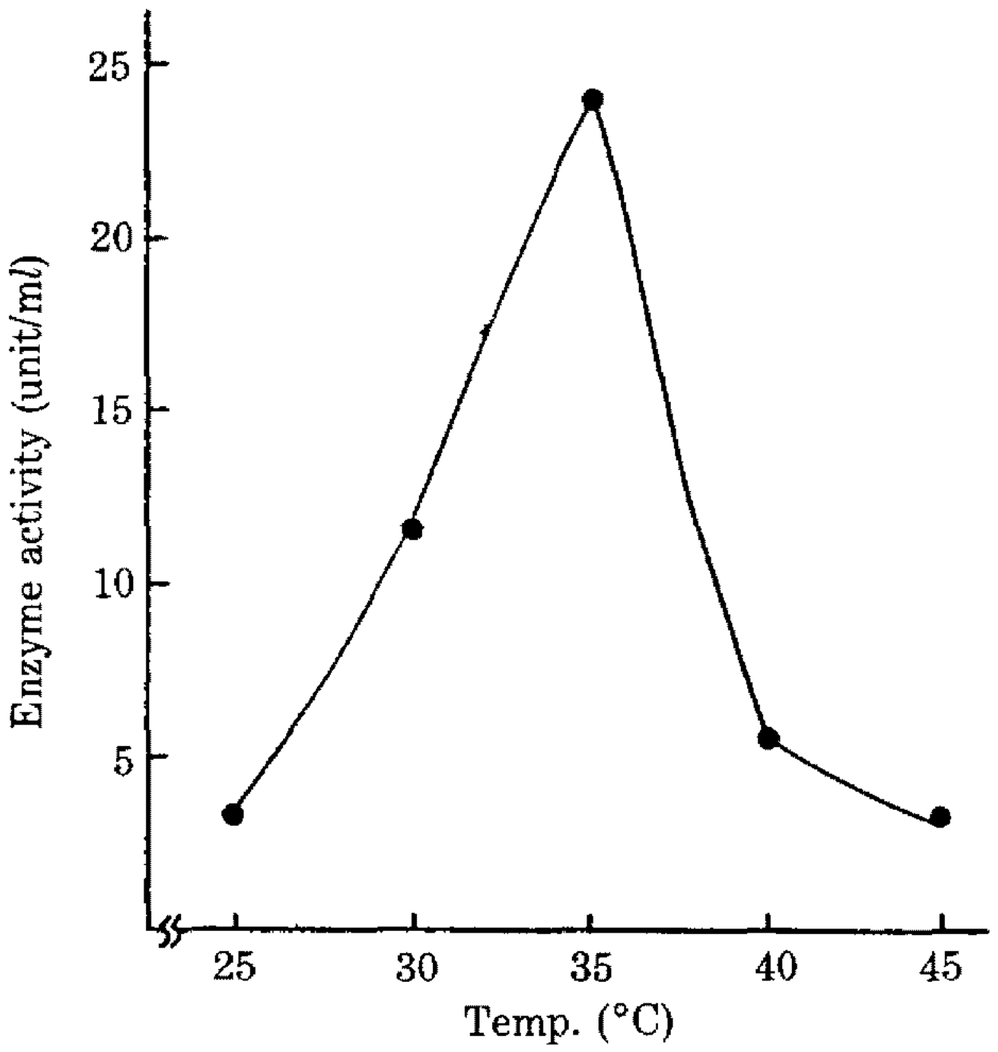

Fig. 2. Effect of temperature on production of raw starch hydrolyzing enzyme.

The cultivation was carried out for 48 hrs, and initial pH of the medium used was 6.5.

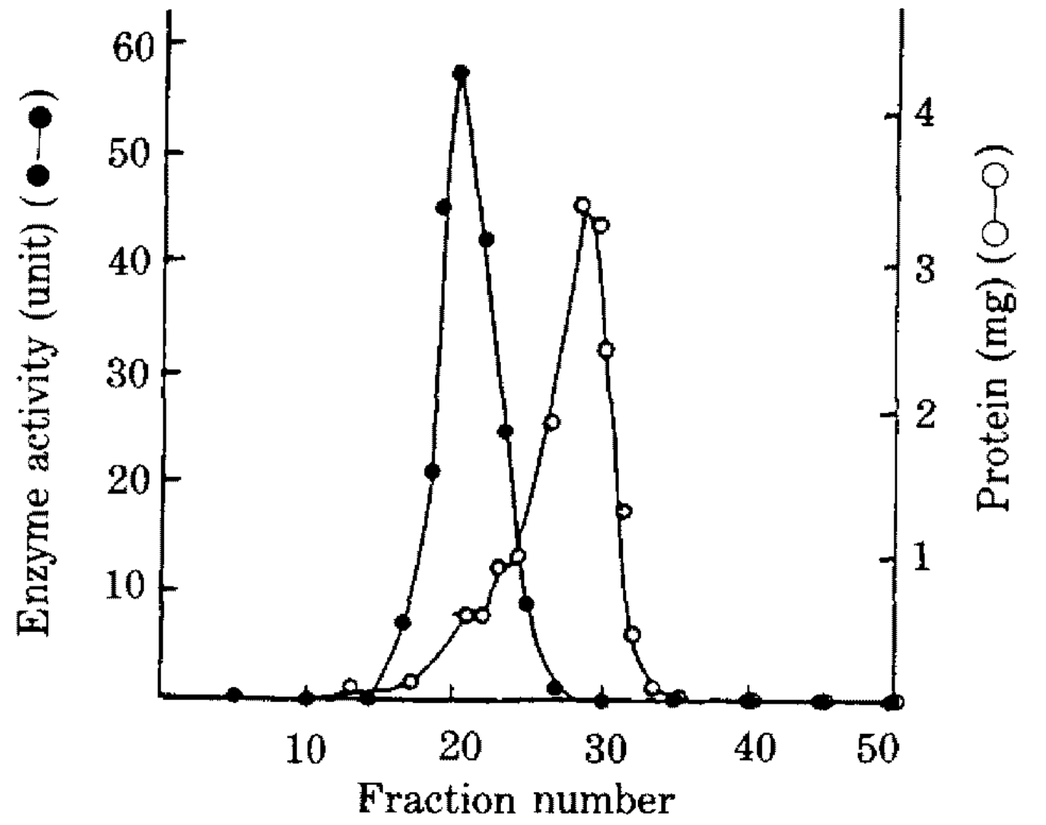

Fig. 3. Sepharose CL-6B gel filtration pattern of raw starch hydrolyzing enzyme.

5 m*l* of crude extract was loaded to the column, and fractions of 5.6 m*l* were collected at a flow rate of 9.5 m*l*/hr. Enzyme activity, (●); protein (○).

Table 5. Effect of added metal salts on production of raw starch hydrolyzing enzyme

Metal salts (1 mM)	Relative activity (%)
Complete	100.0
None	40.1
Calcium chloride	77.3
Cupric acetate	0
Sodium tungstate	54.5
Sodium arsenate	11.1
Zinc sulfate	0
Ammonium molybdate	10.0
Silver nitrate	13.6
Barium hydroxide	27.3
Ferric chloride	13.6
Lithium carbonate	66.8
Lead acetate	36.4
Ferrous sulfate	0
Manganese chloride	72.7
Mercuric chloride	0

The cultivation was carried out for 48 hrs at 35°C. The medium was composed of 0.5% soluble starch, 0.5% polypeptone and each metal salts described above. Complete contained all metal salts of basal medium described in Materials and Methods except carbon and nitrogen sources, and none contains no metals salts.

exchange column (2.5×15 cm), which was preequilibrated with the 10 mM Tris/HCl buffer (pH 8.0). The column was washed with the same buffer and eluted with 300 m*l* of linear gradient of 0-0.5 M NaCl in the buffer. The enzyme was eluted by washing the column with linear gradient of 0-0.5 M NaCl in the equilibration buffer (Fig. 4). The purification procedures of the enzyme is summarized in Table 6. The enzyme was partially purified about 19.2- fold with an overall yield of 57.6%.

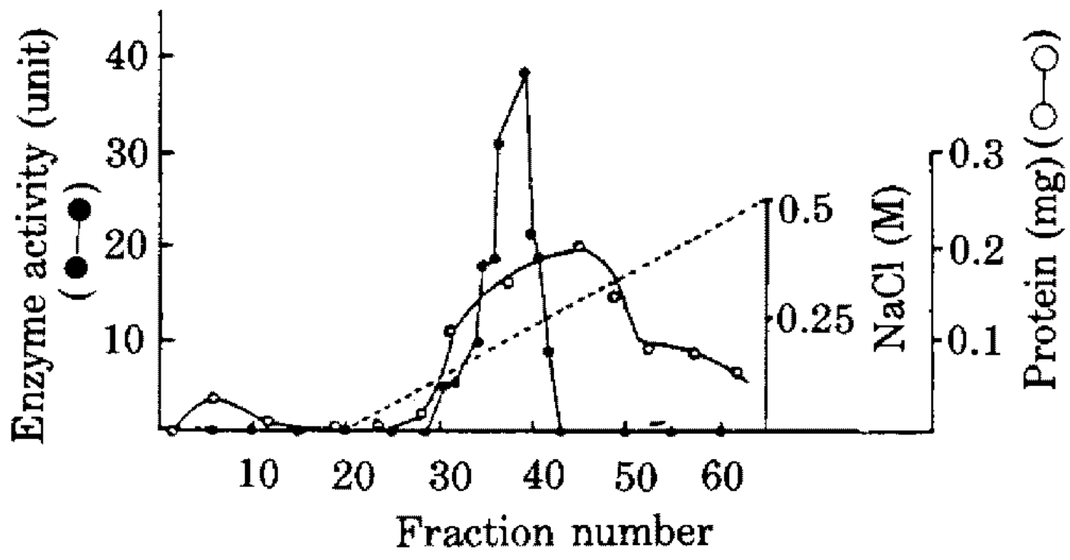

Fig. 4. DEAE-Sephacel ion exchange chromatography of raw starch hydrolyzing enzyme.

The active fractions after Sepharose CL-6B column were pooled and loaded to DEAE-Sephacel ion exchange column. Fractions of 5.6 m*l* were collected. Enzyme activity, (●); protein (○); NaCl conc. (---).

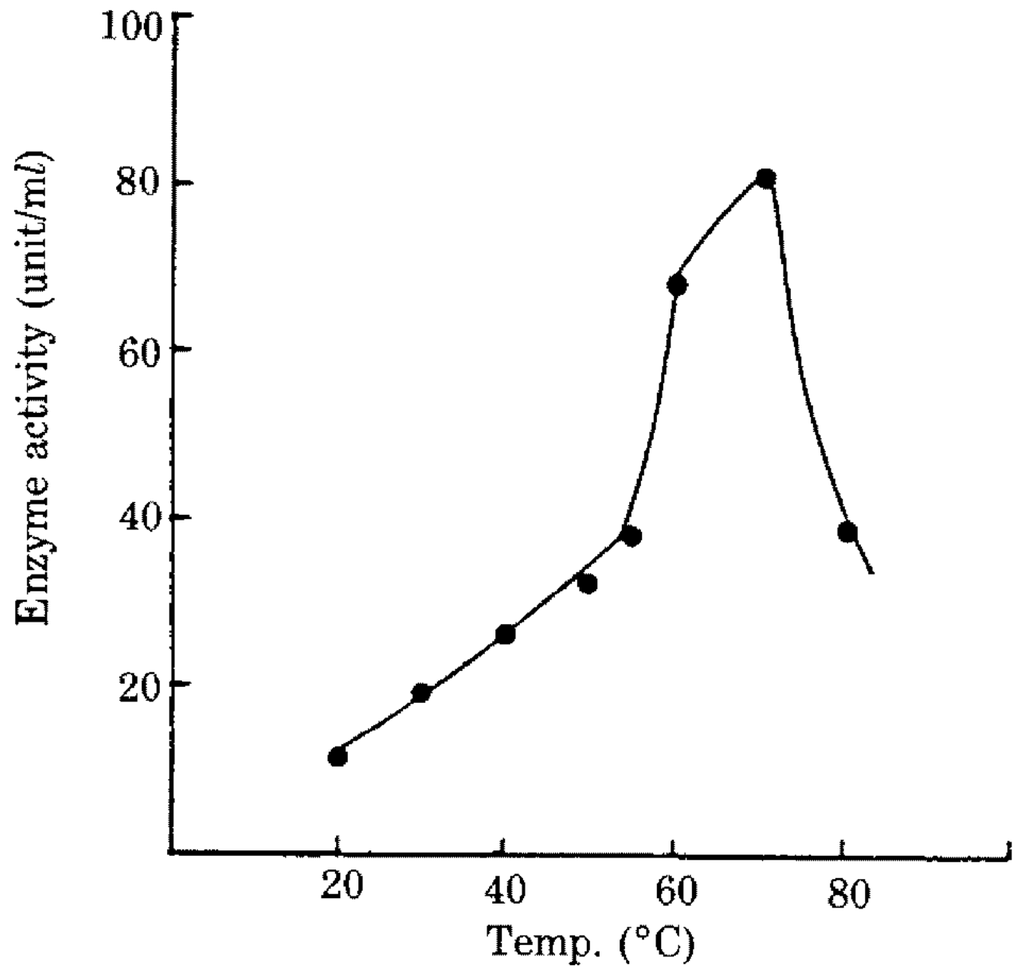

Fig. 5. Effect of temperature on activity of raw starch hydrolyzing enzyme.

Effect of temperature on activity and stability of the enzyme

The effect of temperature on activity of raw starch hydrolyzing enzyme was investigated at various temperatures ranging from 20 to 80°C. The enzyme exhibited maximal activity at 70°C when it was assayed using wheat starch as a substrate. Above that temperature, the enzyme activity decreased, indicating inactivation of the enzyme (Fig. 5). Fig. 6 exhibited the thermoinactivation of the enzyme at various temperatures ranging from 55°C to 80°C in 0.2 M Na-phosphate buffer (pH 7.0). The enzyme was stable below 60°C, but it was completely inactivated at 80°C in 5 min. In case of fungal enzymes, the stability of the enzyme decreased sharply above 50°C (8). Table 7 demonstrates that effect of calcium chloride on thermostability of the enzyme. Calcium chloride protected the enzyme from thermoinactivation drastically. This result coincides well with other amylases reported (15).

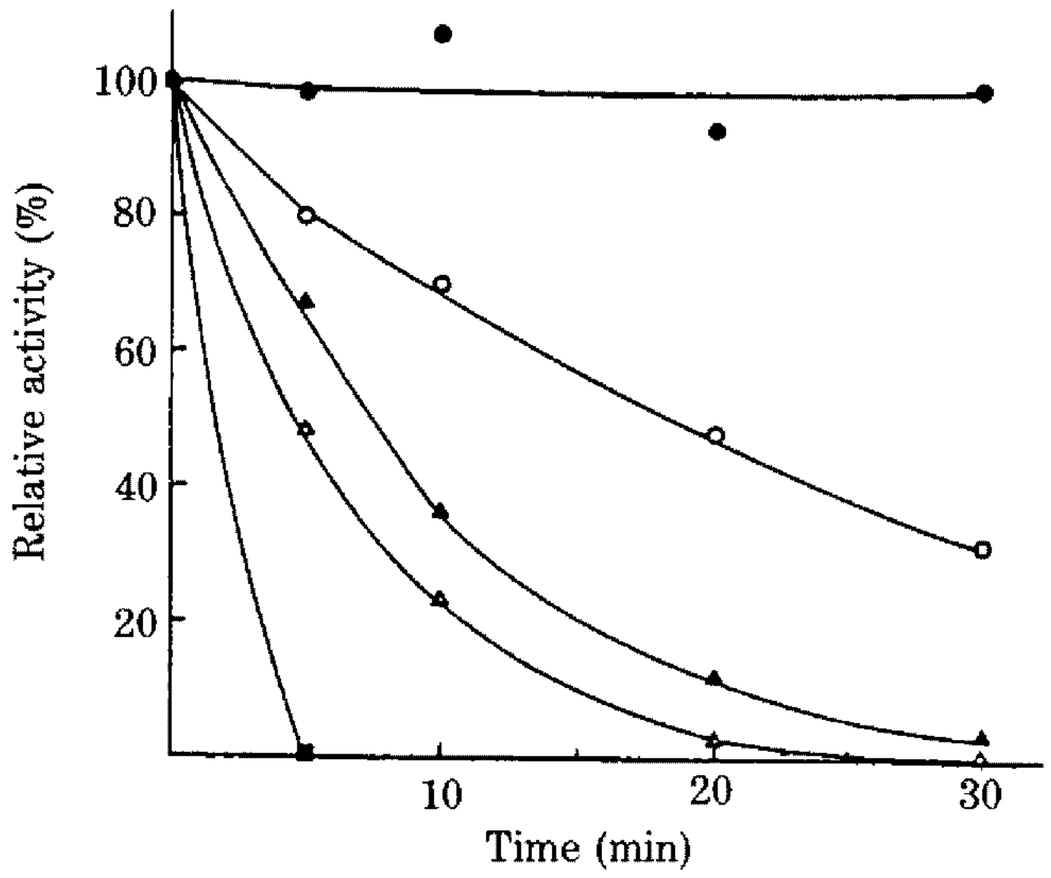

Fig. 6. Thermostability of raw starch hydrolyzing enzyme at various temperatures.

Enzyme solutions were heated at various temperatures. The remaining activities were determined under the standard assay conditions immediately after heating. 55°C (●–●), 60°C (○—○), 65°C (▲—▲), 70°C (△—△), 80°C (■—■).

Effect of pH on activity and stability of the enzyme

The effect of pH on activity of the enzyme was ex-

Table 6. Summary of purification procedure

Fraction	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Yield (%)	Purification fold
Crude extract	225.0	23.0	9.8	100.0	1.0
Sepharose CL-6B gel filtration	176.6	3.5	50.5	78.5	5.2
DEAE-Sephacel ion exchange Chromatography	159.6	0.85	187.8	57.6	19.2

Table 7. Effect of $CaCl_2$ concentration on stability of raw starch hydrolyzing enzyme

Conc. (mM)	Relative activity (%)
0	8.0
0.1	9.3
1.0	8.0
10.0	39.6
100.0	60.6

The enzyme was incubated at 70°C for 5 min, and residual activity was measured under the standard assay conditions. The relative activity of the enzyme was determined as % activity remained after heat treatment compared with the enzyme activity before heat treatment.

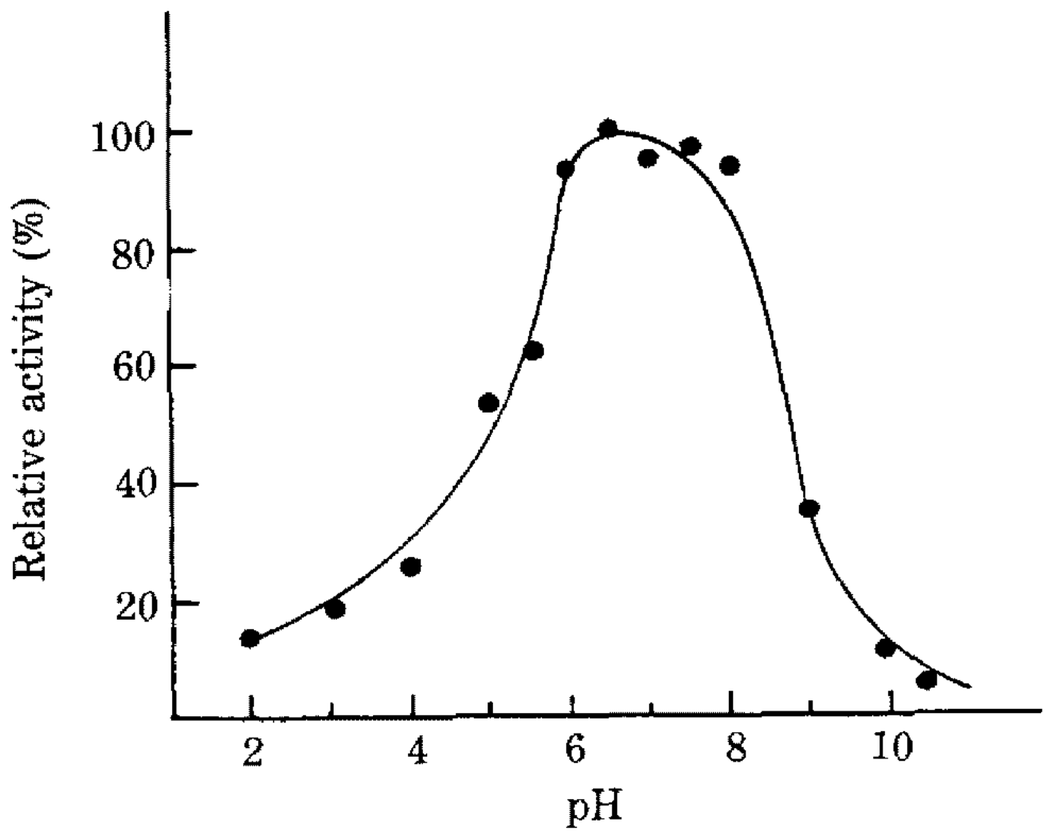

Fig. 7. Effect of pH on activity of raw starch hydrolyzing enzyme.
The relative activity was expressed as % activities at various pH values compared to the enzyme activity at pH 6.5. The buffers (0.2 M) used were as follow: pH 2.0-2.5, glycine; pH 2.5-3.5, citric acid; pH 4.0-5.5, Na-acetate; pH 6.0-7.5, Na-phosphate; pH 8.0-8.5, Tris/HCl; pH 9.0-10.0, boric acid; pH 10.5, carbonic acid.

amined over pH 2.0 to 10.5 (Fig. 7). The enzyme showed maximal activities at neutral pH from 6.0 to 8.0, therefore it seems to be neutral amylase. Most fungal enzymes exhibited the pH optima at acidic pH (8). The pH stability of the enzyme was measured by standard assay conditions after preincubation of the enzyme at various pH values at 70°C for 5 min (Fig. 8). The enzyme was maximally stable at pH 8.0.

Effect of inorganic salts on activity of the enzyme

The effect of various metal salts on enzyme activity was determined by adding 10 mM metal salts to the standard assay mixtures. The enzyme activity was completely inhibited by addition of Ag^+, Fe^{++} and Hg^{++} ions (Table 8). This result was similar to that

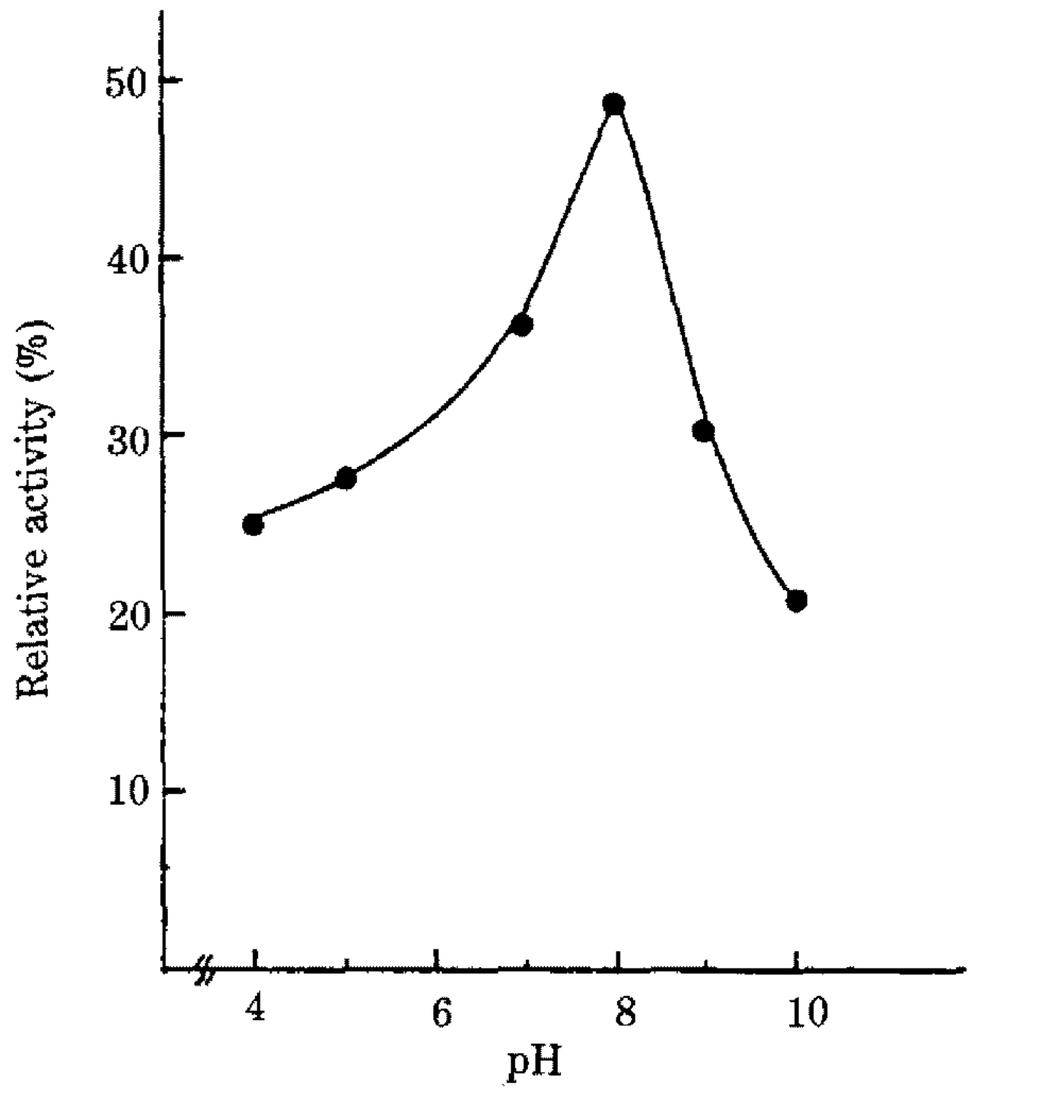

Fig. 8. Effect of pH on stability of raw starch hydrolyzing enzyme.
The enzymes of various pH values were preincubated for 5 min at 70°C. The residual activities were assayed under the standard assay conditions. The relative activity of the enzyme was measured as described in Table 7.

Table 8. Effect of metal salts on relative activity of raw starch hydrolyzing enzyme

Metal salts (10 mM)	Relative activity (%)
None	100
Calcium chloride	77.4
Cupric acetate	40.2
Sodium tungetate	71.8
Sodium arsenate	86.4
Zinc sulfate	47.4
Magnesium chloride	95.0
Silver nitrate	0
Ferric chloride	76.2
Lithium carbonate	77.7
Lead acetate	83.9
Ferrous sulfate	0
Manganese chloride	73.9
Mercuric chloride	0

Table 9. Kinetic parameters using variable substrates

Substrate	V_{max} (unit/ml)	K_m (%)
Corn starch	70.5	1.7
Wheat starch	90.0	1.4
Rice starch	70.5	2.5
Potato starch	21.7	1.0

of the enzyme from *Bacillus circulans* (16).

Kinetic properties

Table 9 shows the effect of varying substrates on kinetic parameters of raw starch hydrolyzing enzyme. From double reciprocal plots for substrates, the K_m values of the enzyme for the substrates were measured at pH 6.5 and 45°C. The enzyme seems to weak in digesting raw potato starch, whcih is often resistant to amylases of microbial, plant and animal origin. However, it seems to hydrolyze wheat starch most among tested raw starches, which is identical with the enzyme from *Bacillus polymyxa* (15).

요 약

토양으로부터 생전분을 분해하는 세균을 분리하였으며, *Bacillus* sp.로 동정하였다. 분리한 세균으로부터 생전분 분해효소의 생성을 위한 최적조건을 검토하였다. 세균은 탄소원으로 wheat 및 soluble starch를, 질소원으로 polypeptone을 사용했을 때 최대 효소생성을 얻을 수 있었다. 그리고 탄소원을 0.5%, 질소원을 0.5% 수준으로 사용했을 때 효소 생성을 극대화하였다. 그리고 배지의 초기 pH를 6.5, 배양온도를 35℃로 유지했을 때 효소생성이 높았다. Sepharose CL-6B 젤 여과 및 DEAE-Sephacel 이온교환수지를 사용하여 부분정제한 효소활성의 최적조건은 pH6.5, 온도 70℃였다. 효소는 pH8.0에서 가장 안정하였으며, 60℃ 이상에서 불활성화 하였다. Fe^{++}, Hg^{++} 및 Ag^{+}는 세균으로부터 효소 생성을 억제하였으며, 아울러 효소활성도 완전히 저해하였다. 효소의 기질에 대한 K_m값은, 1.7%(corn starch), 1.4%(wheat starch), 2.5%(rice starch) 및 1.0%(potato starch)였다.

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