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Studies on Enzyme of the Thermophilic Mold

Part. 3. Thermophilic mold amylase

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고온성 사상균의 효소에 관한 연구

(제 3 보) 고온성 사상균의 Amylase

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요 약

1. 내열성 사상균 *Humicola* 속의 Amylase를 정제하였다.
2. DEAE-Cellulose Column Chromatography로 2개의 활성구분으로 나누어 졌다.
3. 즉 pH 6.0, 0.05M와 0.5M의 인산염완충용액으로 용출시킬때 전자에서 당화형, 후자에서 호정화형 Amylase가 각각 분리되었다.
4. 이 당화형 Amylase의 최적 pH는 4.5~5.5이고, 안정 pH 범위는 4.0~9.0범위였다. 최적온도는 60~65°C로 다른 사상균의 그것보다 훨씬높고 80°C 이상에서는 불활성화 되었다.

The presence, in mold enzyme, of a saccharogenic amylase which seems to be similar to β-amylase of plant origin had been stressed by Tokuoka[1] and Corman et al[2]. Action on starch and purification of this enzyme had been performed by Kitahara et al,[3] Okazaki,[4] Phillips et al,[5] Kerr et al[6] and Weill et al,[7] especially, Phillips et al, suggested that saccharogenic amylase producting by the mold, *Rhizopus delemar*, splits glucose from the unreducing end group of starch molecule, and either passes by the anomalous 1,6-α-D-linkage in some manner of else hydrolyzes it very slowly, with difficulty. Recently, this amylase had been crystallized by Fukumoto et al.[8]

In the previous report,[9] the author et al found that the optimum temperature of a thermophilic mold cellulase was more higher than that of other cellulase.

In the present paper, the preparation of a thermophilic mold amylase and some enzymological properties of this enzyme was studied.

Experimental methods

1. Isolation of microorganism

Sample of manure was spread on agar plate as the following Table 1 and incubated at 50°C as long as to develop district colonies. Molds isolated were streaked on agar plate. Strains were incubated abundant at 50°C incubator, and the purest strain was used amylase forming tests as the following methods.

Table 1. Medium composition

Potato starch	3.0%
Ammonium citrate	1.0%
KH_2PO_4	0.3%
$MgSO_4 7H_2O$	0.1%
NaCl	0.05%
Distilled water	100ml

2. Amylase forming medium

Amylase forming medium was given in Table 1. One hundred m*l* of the basal medium was added to 500m*l* shaking flask and sterilized by autoclaving at 120°C for 30 minutes. The purest strains were cultured at a reciprocal shaker(130 strockes/minutes) for 40 hours at 40°C. After incubation medium was filtered and centrifuged (9,000 rpm) at 5.0°C for ten minutes and its supernatant was filtered by fiter paper. The filtrate was used for crude enzyme solution.

3. Enzyme production by jar fermentor

Out of some 200 strains of the thermophilic molds, amylase forming mold was isolated from screening method described above. *Humicola sp.* was found capable of producing amylase in the shaking culture medium. The culture medium contained the followings. Potato starch 3.0%, Ammonium citrate 1.0%, KH_2PO_4 0.3%, $MgSO_4 7H_2O$ 0.1%, NaCl 0.05%, tap water 100ml.

This *Humicola sp.* was cultured by using six liter capacity jar fermentor(Marubishi Laboratory Equipment Co., Ltd.) for 40 hours at 40°C, aeration 15L/minute, and agitation 250 rpm. The culture broth was filtered to remove mycelium and other solid materials. To the culture filtrate acid clay was added. Agitated for 30 minutes and filtered with Hyflo-Super-Cel to remove the acid clay. In this process maltose oligosaccharidase. pigments, clouding materials and other impurities were removed considerably.[10] The enzyme solution was concentrated at 30°C under reduced pressure to approximately one twentieth of the original volumn. The concentrated solution was dialyzed in cellulose tubing against running water for 48 hours and against 0.05% phosphate buffer solution of pH 6.0. The dialyzed crude enzyme solution was used for purification of amylase.

4. Determination of dextrinogenic and saccharogenic amylase

The reaction mixture, which concentrated five ml of one % dextrinized potato starch, one ml of McIlvaine buffer solution, one ml of distilled water and one ml of the enzyme solution was incubated at 60°C water bath, after incubation for ten minutes, the glucose produced in one ml of the reaction mixture was measured as saccharogenic amylase activity by the Micro-Bertland method.[11] The dextrinogenic amylase activity was assayed by the iodine reaction. One unit of amylase activity was defined as the amount of enzyme which forms 0.1mg of reducing sugar per one ml of enzyme solution under the above conditions.

5. Determination of Protein

The concentration of enzyme was determined by measuring, the absorbance at 280mμ using a Hitachi model EPU-2A photoelectric spectrophotometer.

6. DEAE-Cellulose column chromatography

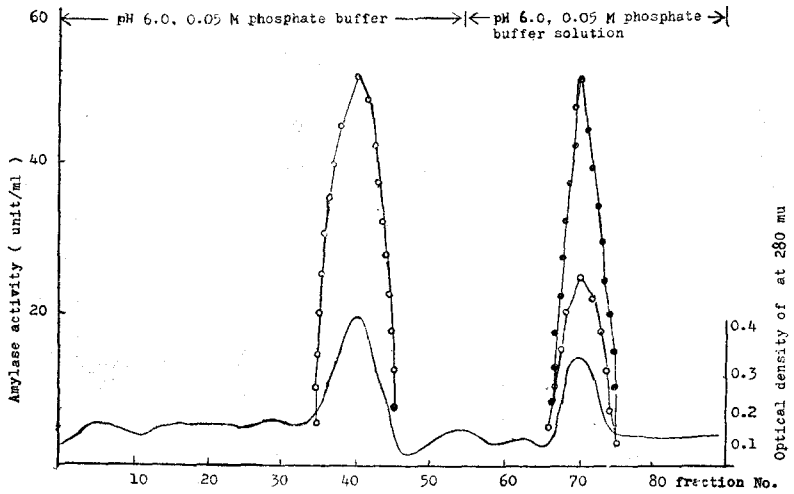
After dialysis against 0.05M phosphate buffer solution of pH 6.0 the enzyme solution was placed on a 1.6×35cm column of DEAE-Cellulose equilibrated with 0.05M phosphate buffer solution of pH 6.0. A step-wise elution was carried out with 0.05M and 0.5M phosphate buffer solution at the same pH respectively.

Results and Discussion

1. Fractionation of two types of amylase by DEAE-Cellulose column chromatography

A glass column(1.6×35cm) was packed with about four gram of DEAE-Cellulose and equilibrated thoroughly with 0.05M phosphate buffer solution of pH 6.0. The dialyzed enzyme solution was introduced on a column of DEAE-Cellulose described above. After absorption of the amylase was completed, the column was washed with 0.05M phosphate buffer solution of pH 6.0 and then eluted with 0.5M phosphate buffer solution of pH 6.0. The flow rate was 20ml per an hour and fractions of five ml was collected, optical

density at 280mμ, saccharogenic and dextrinogenic amlyase activity of these fractions were determined. As shown in Fig. 1, the amylase was separated into two fractions eluted with 0.05M and 0.5M phosphate buffer solution of pH 6.0. By DEAE-Cellulose column chromatograpy the amylase system was separated into two fractions. Saccharogenic amylase was present in fraction of 0.05M phosphate buffer solution of pH 6.0, while dextrinogenic amylase was present in fraction of 0.5M phosphate buffer of pH 6.0. In this step saccharogenic amylase was completely separated from dextrinogenic-amy lase. This result indicated that the amylase system produced by *Humicola sp.* was composed of saccharogenic amylase and dextrinogenic amylase activity.



—○— Saccharogenic amylase —●— Dextrinogenic amylase —— Protein at 280 mμ

Fig 1. Chromatography of amylase on a column of DEAE-Cellulose (1.5×30cm)

2. Optimum pH

After purified saccharogenic amylase fraction was adjusted to various pH values (pH 2.5—9.0) with McIlvaine buffer solution and 0.1 N hydrochloric aicd or 0.1 N sodium hydroxide solution respectively, incubated at 60°C for ten minutes, one m*l* of reaction mixtures was calculated by the residual activity (%).

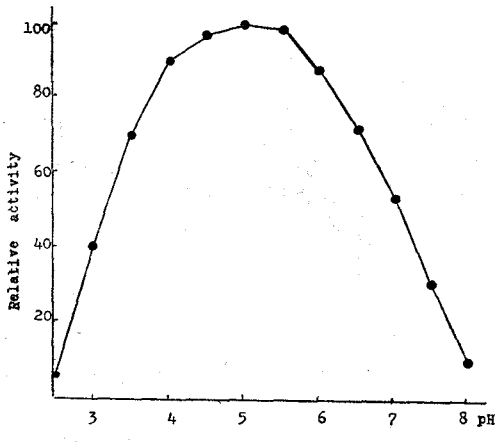


Fig 2. The pH dependence of saccharogenic amylase activity

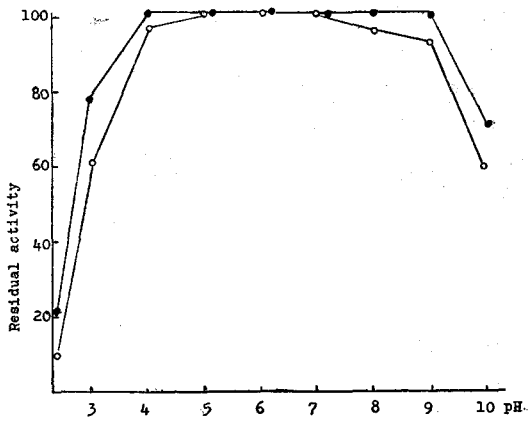
The result at various pH values are shown in Fig. 2.

As shown in Fig. 2, optimum pH of saccharogenic amylase activity was within 4.5 to 6.5, especially this saccharogenic amylase activity at acidic range was more stable then the neutral.

3. pH stability

Each fraction was adjusted to various pH values (from pH 2.5 to 9.0) with McIlvaine buffer solution and 0.1 N hydrrochloric acid or 0.1N sodium hydroxide solution, this mixture was incubated at 30°C for 12 hours, the residual activity was determined at pH 5.0 for dextrinogenic and saccharogenic amylase activity.

As shown in Fig. 3, the quite stable pH ranges



—○— Saccharogenic amylase activity
—●— Dextrinogenic amylase activity

Fig 3. The pH stability of saccharogenic and dextrinogenic amylase activity

were with 3.5-9.0, especially this fraction activity at alkaline range was more stable than acidic range, at the both outer sides of this range, conspicuous loss of activity was evident.

4. Optimum temperature

Effects of temperature on saccharogenic amylase activity was measured at various tempreature from 30 to 80°C, the relative activity was presented for 30 minutes and 60 minutes.

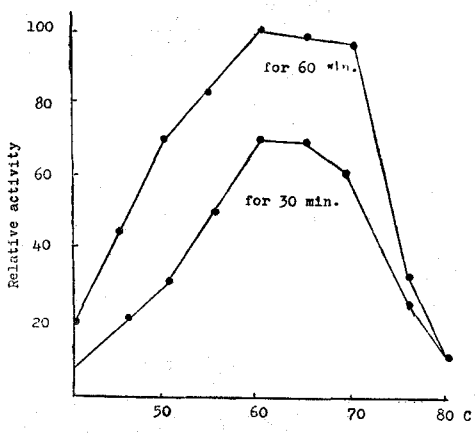


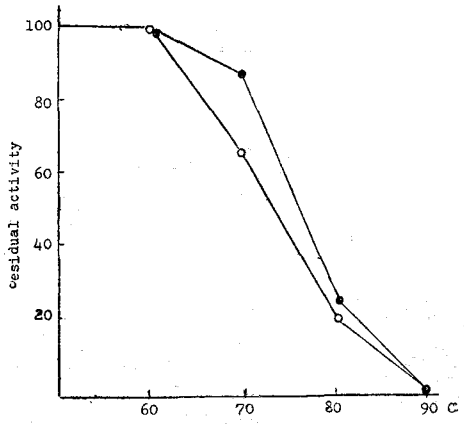
Fig 4. Temperature dependence of saccharogenic amylase activity

As shown in Fig. 4, the optimum temperature saccharogenic amylase was 65°C at pH 5.5. Above 70°C, the enzyme activity was rapidly inactivated.

The optimum temperature of fungal amylase was found in many experiments to be at 50-55°C with only very slight variation depending on strains, culture conditions and other experimental conditions.

5. Thermal stability

These enzyme fraction solutions were adjusted to pH 5.5 with various buffer solution, these mixtures were incubated at various temperature. After ten minutes incubation, these residual activities were determined.



—○— Saccharogenic amylase
—●— Dextrinogenic amylase

Fig 5. Thermal stability of saccharogenic and dextrinogenic amylase

As shown in Fig. 5, these amylases were stable at 60°C for ten minutes. The extent of the heat inactivation at 70°C was ten % for dextrinogenic amylase activity, 25% for saccharogenic amylase activity compared with 80% at 80°C for dextrinogenic and saccharogenic amylase activity. These amylase activities were completely inactivated at 90°C.

Summary

1. Purification of amylase system produced from *Humicola sp.* by a submerged culture was carried out.

2. By DEAE-Cellulose column chromatography amylase system was separated into two fractions eluted at 0.05M and 0.5M phosphate buffer solu-

tion of pH 6.0.

3. The saccharogenic amylase was mostly composed of. the fraction of 0.05M phosphate buffer solution of pH 6.0 while the dextrinogenic amylase was perseted in fraction of 0.5M phosphate buffer solution of pH 6.0

4. It was found that the optimum pH of this saccharogenic amylase was within the range of from 4.5 to 5.5, stable pH was within the range of from 4.0 to 9.0 and optimum temperature was 60-65°C. This amylase was stable at 70°C for ten minutes but completely inactivated 80°C above.

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