한국농화학회지 Vol. 21, No. 2, June 1978
J. Korean Agricultural Chemical Society

Studies on Acid-stable Alpha-amylase

Hack Joo Kim* and Si Myung Byun
Department of Biological Science and Technology
Korea Advanced Institute of Science (KAIS)
(Received April 25, 1978)

내산성 α-Amylase에 관한 연구

김 학 주* · 변 시 명
한국과학원 생물공학과
(1978. 4. 25)

SUMMARY

Acid-stable α-amylase was partially purified from *Paecilomyces subglobosum* by Sephadex G-150 gel filtration. About 7.7-fold purification was obtained and the partially purified preparation has 5.0 U of αamylase activity per mg of protein. Using this partially purified α-amylase, general properties were studied and it showed the maximal activities at the conditions of pH 4.0 and 38°C. High stability of the acid-stable α-amylase in acidic condition was observed, whereas thermal stability was similar to the conventional α-amylase.

INTRODUCTION

It has been known that mold produces two types of amylase. The one is α-amylase and the other is glucoamylase. α-amylase (EC 3.2.1.1) hydrolyzes randomly α-1,4-glycosidic linkage of starch and the iodine reaction of starch disappears rapidly. Glucoamylase (EC 3.2.1.3) hydrolyzes the α-1,4-linked glucose residue of starch successively from non-reducing ends of the chain to form glucose and the iodine reaction does not disappear until over 80% of starch is degraded.

Various kinds of microorganisms have been employed to produce those amylases and among them some strains had been extensively used in the industry, for example *Aspergillus*, *Rhizopus*, and *Bacillus* species. Generally some differences in enzyme properties have been found between the microbial amylase preparations and those amylases are less active either in the acidic pH or higher operating temperature.

From those different properties of amylase preparations, acid-active or acid-stable amylases are suitable for various uses under acidic conditions as digestive enzyme or in other fermentation industry using amylases.

Recent works(1,2,3) reported that *Aspergillus* and *Rhizopus* species produced the acid-active or acid-stable amylases and their properties were studied. Futhermore, new fungi, *Paecilomyces* species also produced the acid-active or acid-stable amylase (4,5).

In this work, we studied the preparation and

* 현주소 : 동아제약주식회사, 서울

characterization of acid-stable α-amylase of *Paecilomyces subglobosum.*

MATERIALS AND METHODS

Materials

Paecilomyces subglobosum obtained from the Department of Biochemistry, University of Minnesota was used in this experiment. Sephadex G-150 and silica thin layer chromatogram were purchased from Pharmacia Fine Chem. Co. Sweden and Eassman Kodak, U.S.A. respectively. Glycogen was obtained from Matheson Coleman and Bell Co. U.S.A. Other chemicals were analytical grades from Wako Pure Chemical Industries, Japan.

Enzyme Purification

Paecilomyces subglobosum was inoculated in the solid media of wheat bran: rice bran: water =5:3:4(W/W) and incubated at 30°C for 5 days. After 5 day-incubation, spores were formed and the surface turned to greenish-yellow. Hundred gram of this media was extracted with 2*l* of 0.01M citratebuffer, pH 4.0 by agitating for 30 min. The extract was centrifuged for 20 min at 9,000 rpm(Sorbal centrifuge, GS-3 rotor) and the clear supernatant was freeze-dried. Three g of the solid material was dissolved in 30ml of the buffer and dialyzed against 0.01M citrate buffer, pH 4.0 at 4°C overnight. The crude enzyme preparation was applied on the column of Sephadex G-150 (2.25cm×81cm) which was previously equilibrated with 0.01M citrate buffer, pH 4.0 at 4°C. The elution was performed with the starting buffer. The flow rate was adjusted to 4ml/20 min and 4ml fractions were collected. The protein peak which showed α-amylase activity was pooled and dialyzed overnight against water. The dialyzed enzyme preparation was freeze-dried and used for the characterization of the enzyme.

Enzyme assay

α-amylase was determined by the modification of the method of Street (6). The incubation mixture contained 0.2ml of 1% starch solution, dissolved in 0.1M citrate buffer,pH 4.0, 0.5ml of 0.1M citrate buffer, pH4.0, 0.2ml of 0.01N HCl. After this mixture was preincubated for 3 min at 37°C, 0.1ml of enzyme solution was added and the incubation was continued for 15 min. The reaction was stopped by adding 0.5ml of 0.4N trichloroacetic acid and 8.1ml of the distilled water and 0.4ml of $KI-I_2$ solution were added. Absorbancy at 578nm was measured and the amylase units are calculated from the number of glucosidic linkages which are hydrolyzed per min under the assay conditions as follows;

$$\text{Volume activity} = \frac{E_0 - E_1}{E_0} \times A \times \frac{1}{t} \times \frac{1}{v} \times 1000 \ (\text{U}/1) \text{ at } 37^\circ\text{C}$$

where E_0 : extinction of reference, corresponds $t_o=0$ min

E_1 : extinction of test with the enzyme solution after $t=15$min

A : 12.35μ mole amylose in the assay mixture

V : volume of sample in assay

t : incubation time

Reducing power was also measured by the method of Rick *et al.* (7) using 3,5-dinitrosalicylic acid. Glucoamylase activity was measured by determining the released reducing sugars by the method of Somogyi (5,8).

Thin Layer Chromatography of Enzymatic Products

Silica thin layer chromatography was carried out on Eastman Kodak silica thin layer chromatogram at room temperature by an ascending method. The developing solvent was n-propanol: water (85 : 15, V/V). Alkaline silver nitrate solution was used for detection of reducing sugars and $KI-I_2$ solution for detection of starch. Glucose and maltose were used as the authentic sugars.

Kinetic analysis

The partially purified enzyme in the previous Sephadex G-50 gel filtration step was used in this experiment. For the rate of hydrolysis of starch by the enzyme were determined under the incubation condition described in the enzyme assay, except varying the incubation time.

The pH dependence of the activity was determined under the standard assay condition except changing the pH of the incubation mixture. 0.1 M HCl-KCl buffers between pH2.0 to 3.0, 0.1 M sodium acetate buffers between pH3.5 to 5.5, 0.1M sodium phosphate buffers between pH6.0 to 7.5, and 0.1M Tris-HCl buffers between pH 8.0 to 9.0 were used. The effects of temperature on the enzyme activity were examined at 7 different temperatures in the range from 30°C to 65°C.

RESULTS AND DISCUSSION

Enzyme purification

When the mold grows in the solid medium previously described, either α-amylase or glucoamylase were not detected until the spores appeared. After 3 or 4 days when spores were formed, the surface of medium turned to greenish-yellow and high enzyme activities appeared rapidly. The highest activtty was obtained at 5 days incubation at 30°C. Same result was obtained when the mold was cultured in the liquid media of glucose (3%), peptone (2%), and other inorganic salts in the chemostat with 600 rpm agitation and aeration at 30°C. By extracting the enzyme from the solid media with 0.01 M citrate buffer, pH 4.0, the crude enzyme was prepared and freeze-dried since α-amylase was not precipitated with the ammonium sulfate fractionation. Fig. 1 showed the result of Sephadex G-150 gel filtration of the freeze-dried enzyme preparation.

First peak did not show any amylase activity and α-amylase activity appeared between tween two protein peaks. Fractions which showed α-amylase activity were pooled and freeze-dried (Enzyme preparption I). Enzyme preparation I showed 3 major bands with 2 or 3 more minor bands by 7% polyacrylamide gel electrophoresis of Gabriel's (9). The partially purified enzyme preparation (I) was not purified further by DEAE-Sephadex or Sepharose 4B-glycogen affinity chromatography (5). Second peak(Enzyme preparation II) showed glucoamylase activity

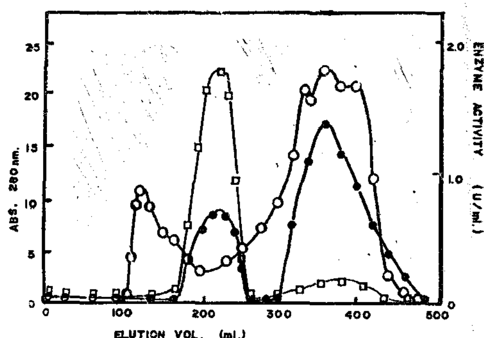

Fig. 1 Sepahadex G-150 filtration profile of amylases from *Paecilomyces subglobosum*. Column size: 2.25×81cm, flow rate: 4ml /20 min, fraction volume: 4ml, sample size :30ml (11.5mg of protein/ml), elution buffer :0.01M citrate buffer, pH 4.0

○···○···○ : Absorbancy at 280nm

●···●···● : Amylase activity by 3,5-dinitrosalicylic acid

□···□···□ : Amylase activity by starch-I_2

and purified as a single protein band on 7% polyacrylamide gel electrophoresis by Sepharose 4B-glycogen affinity chromatography (5).

To distinguish α-amylase and glucoamylase

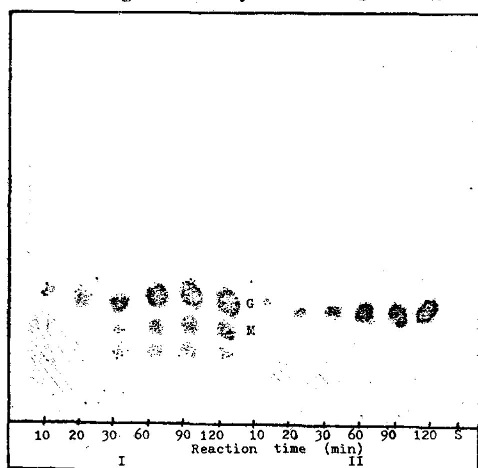

Fig. 2. Thin layer chromatogram of amylases-hydrolyzed products of starch at various incubation times

I : Time course of enzyme preparation I on starch

II : Time course of enzyme preparation II on starch

G: glucose, M: maltose, S: standard starch

Developing solvent: n-propanol: water=85:15 (V/V)

Coloring reagent: alkaline siver nitrate

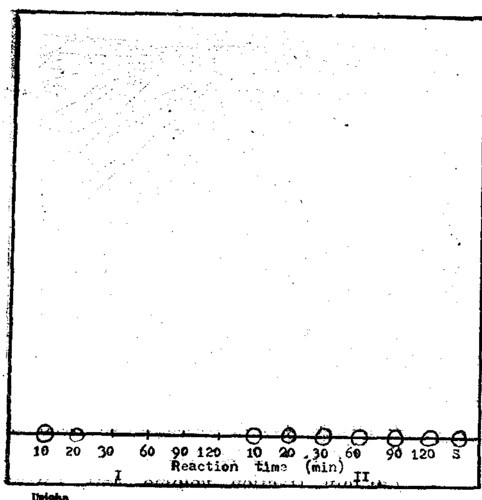

Fig. 3. Thin layer chromatogram of amylases-hydrolyzed products of starch at various incubation times

I : Time course of enzyme preparation I on starch

II : Time course of enzyme preparation II on starch

S: Standard starch

Developing solvent: n-propanol: water=85:15 (V/V)

Coloring reagent: KI-I_2 solution

activities, two enzyme preparations, I and II were subjected on 2% starch solution at various times and two microliters of reaction products were chromatographed on silica thin layer chromatogram using the solvent of n-propanol : water =85 : 15 (V/V). According to the results of Fig. 2 and 3, the enzyme preparation I showed α-amylase activity and II showed glucoamylase activity.

As shown in Fig. 2 and 3 enzyme preparation I hydrolyzed starch to form glucose, maltose, and lower polysaccharide of DP (degree of polymerization) as the reaction proceeded on time and the iodine-reaction disappeared at 20 minutes of the reaction time. Enzyme preparation II, however, produced only glucose and the iodine-reaction remained until the reaction proceeded for 2 hours. Iodine reaction on starch does not disapear until 80% of starch is degraded (1). According to the results presented we concluded that enzyme preparation I showed the α-amylase activity and the enzyme preparation II showed glucoamylase activity.

By the Sephadex G-150 gel filtration, the partially purified α-amylase had 5.0 U of the activity per mg of protein and showed 7.7-fold purification.

Enzyme properties

The partially purified preparation from Sephadex G 150 gel filtration was used in this experiment. As shown in Fig. 4 starch hydrolysis with acid-stable α-amylase from *P. subglobosum* was similar to those of Takaamylase (1). However mode of action was slightly different from them. At first higher dextrins were formed and the main product was glucose. This α-amylase also hydrolyze maltose which is different from acid-stable α-amylase of *Aspergillus*(1, 2). It was not clear, however, that this α-amylase possessed the ability of hydrolyzing maltose or maltase was contained as impurity since the enzyme preparation was not purified.

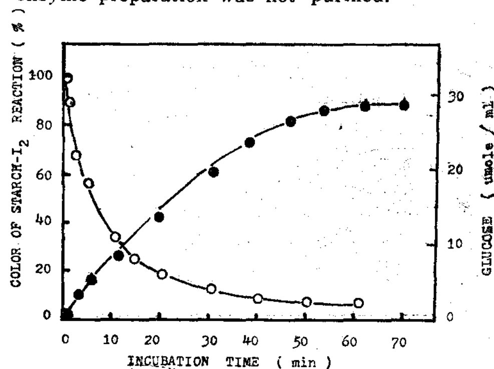

Fig. 4. Curves of starch hydrolysis with acid-stable α-amlyase of *Paecilomyces subglobosum*

●…●…● : Reducing sugar by 3, 5:dinitrosalicylic acid

○…○…○ : Color of starch-I_2 reaction

Fig. 5 shows the effects of pH for α-amylase activity. Maximal activity of α-amylase appeared at pH 4.0. Most of α-amylase had its optimal activity between pH 5 to 8 and almost no activity at pH 3.0, whereas the acid-stable α-amylase showed over 70% of the maximum activity.

To study pH stability of the enzyme α-amylase preparations were added to buffers of various pH

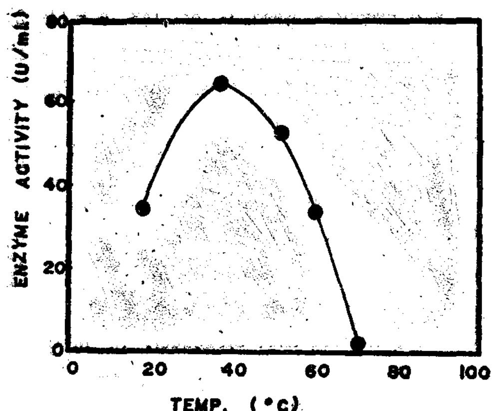

Fig. 5. Effect of pH on α-amylase activity

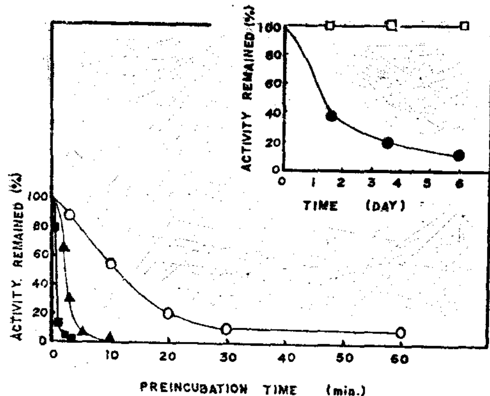

Fig. 6: pH stability of α-amylase activity
Enzyme was incubated in buffers of various pH at 37°C for 30 min and pH of the enzyme solution was adjusted to 4.0. Then the activities remained were assayed under the condition described in the text.

solution and incubated at 37°C for 30 min. Then the pH of the enzyme solutions was adjusted to pH 4.0 with diluted sodium hydroxide, and the activities remained were assayed. The results are shown in Fig. 6. As results the acid stable α-amylase from *Paecilomyces* retained most of its activity between pH 3.5 to 9.0. In general, pH stability of α-amylase was relatively low. As example, Taka-amylase A was inactivated completely at pH 2.2, maintaining only 4% of the original activity (1, 10). On the contrary the acid-stable α-amylase retained 58% of its original activity at pH 3.0 and more than 85% at pH 3.5. In akaline side this amylase seems stable.

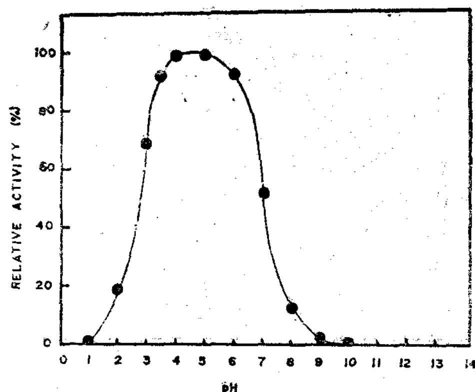

Fig. 7: Effect of temperature on α-amylase activity

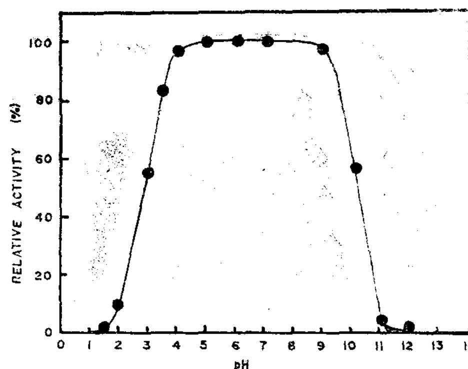

Fig. 8: Thermal stability of α-amylase activity
□···□···□ : 18°C　●···●···● : 37°C
○···○···○ : 50°C　△···△···△ ·· 60°C
■···■···■ : 70°C

An assessment of the effect of temperature revealed that the optimal temperature of this α-amylase was around 38°C as shown in Fig. 7. Heat stabilities of the acid-stable α-amylase are shown in Fig. 8. As results heat stability of the acid-stable α-amylase from *P. subglobosum* similar to the conventional α-amylase such as Taka-amylase (10).

In summary, the general properties of the acid-stable α-amalase from *P. subglobosum* are somewhat similar to those from *Aspergillus* except heat stability. Further purification of the enzyme is required to compare the characteristics of the enzyme with those of other acid-stable amylase.

요 약

*Paecilomyces subglobosum*이 생산하는 내산성 α-amylase를 Sephadex G-150으로 정제한 결과 순수정제가 되지 않았으나 glucoamylase와 분리되었다. 조효소를 사용하여 내산성 amylase의 일반성질을 조사한 결과 최적 pH는 4.0이었고 최적온도는 38°C이었다. 이 효소는 보통 α-amylase와 비교하여 pH에 대한 안정성은 매우 좋았으나 열 안정성은 비슷하였다. 전분에 대한 가수분해력이 좋았으며 생성물을 박층 크로마토그라피로 조사한 결과 말토스도 분해하는 것을 알았다.

REFERENCES

1. Minoda, Y. and K. Yamada, Agr. Biol. Chem., **27**, 806 (1963)
2. Okazaki, H., J. Agr. Chem. Soc. Japan, **24**, 88 (1950)
3. Wieland, H., Enzymes in Food Processing and Products, Reed, G. ed. 143, A.P.(1972)
4. Sawada, J.,T. Misaki, M. Okabe, H. Yasui, K. Haneda, and T. Okazaki, U.S. Patent, 3,652,400 (1972)
5. Kim, H.J. and S.M. Byun, Korean Biochem. J., **11**, (1978) in press
6. Street, H.V., in Methods of Enzymatic Analysis, Bergmeyer, H.U. ed. Vol. **2**, 898 (1971)
7. Rick, W. and H.P. Stegbauer, in Methods of Enzymatic Analysis, Bergmeyer, H.U. ed. Vol. **2**, (1971)
8. Somogyi, M.,J. Biol. Chem., **195**, 19 (1952)
9. Gabriel, O., in Methods in Enzymology, Jacoby, W.B. ed. Vol. **22**, p 505 A.P.(1971)
10. Takagi, T. and H. Toda, J. Biochem., **52**, 16 (1962)