Acid-stable α -Amylase of Black *Aspergilli*

Part III. Separation of Acid-stable α -Amylase and Acid-unstable α -Amylase from the Same Mold Amylase Preparation

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Received August 11, 1967

From the amylase preparation of black *Aspergillus* acid-stable α -amylase and acid-unstable α -amylase were separated by gel filtration on sephadex G-100 column. From the acid-unstable α -amylase fraction this enzyme was purified by fractionations with rivanol and acetone, and finally obtained as a homogeneous protein after gel filtration with sephadex G-50. Comparison of some general properties between the two α -amylases was carried out. Catalytic action was quite similar with both enzymes, but dextrinizing unit per mg enzyme protein of the acid-unstable α -amylase was about 5.6 times as large as that of the acid-stable α -amylase. The acid-unstable α -amylase was less heat-stable than the acid-stable α -amylase. Acid stability and pH-activity curve were compared with both α -amylases. High stability of the acid-stable α -amylase in acidic condition was observed, but, in alkaline range, it was more sensitive than the acid-unstable α -amylase.

As shown in previous papers,^{1,2)} the acid-stable α -amylase of black *Aspergillus* was obtained in the purified form and its catalytic action was quite similar to that of Taka-amylase A, although being different in its acid stability. In the subsequent papers, the physical and chemical properties of this enzyme protein will be compared with those of another acid-unstable α -amylase, for the purpose of elucidating the mechanism of this acid stability. As the acid-unstable α -amylase to compare with, Taka-amylase A is very favorable because its properties have been investigated in detail by many workers. But it may be better to compare with the acid-unstable α -amylase produced by the same mold strain simultaneously, namely by the same enzyme-forming system. Our mold amylase preparation of black *Aspergillus* contains, be-

sides the acid-stable α -amylase, a large amount of acid-unstable α -amylase and glucoamylase. So the separating method of these amylases was investigated, in which the acid-unstable α -amylase could be separated and purified from the enzyme preparation to a homogeneous protein.

In this paper, a separation and purification method of this acid-unstable α -amylase is reported and the general properties of this enzyme are shown comparing with those of the acid-stable α -amylase of the same enzyme preparation.

EXPERIMENTAL

Commercial amylase preparation of black *Aspergillus* "Sanactase" used as enzyme source in this experiments contains acid-stable α -amylase, acid-unstable α -amylase, glucoamylase and maltose-oligosaccharide transglucosidase. It also contains many sorts of other enzyme, such as protease, esterase and so on. As shown in the previous paper, to isolate the acid-stable α -amylase from this preparation, acid treatment, salting out with ammonium sulfate and precipitations

1) Y. Minoda and K. Yamada, *Agr. Biol. Chem.*, **27**, 806 (1963).

2) Y. Minoda, T. Koyano, M. Arai and K. Yamada, *ibid.*, **32**, 104 (1968).

with rivanol and acetone were carried out. Glucoamylase, transglucosidase and other enzymes could be eliminated by fractionations with ammonium sulfate, rivanol and acetone, but the acid-unstable α -amylase could not be separated from the acid-stable α -amylase by these procedures. So acid treatment was carried out to inactivate the acid-unstable α -amylase, and then the denaturated protein of this enzyme could be eliminated by fractionation. Now it is the present purpose to isolate the acid-unstable α -amylase in active form, and so acid treatment cannot be carried out and other separation method of the two α -amylase proteins must be devised. At first the chromatography on DEAE-cellulose was tested.

Chromatography on DEAE-Cellulose. Enzyme preparation was dissolved in phosphate buffer, pH 6.5 ($\mu=0.05$), dialyzed to the same buffer, and charged on DEAE-cellulose column bufferized with the same buffer. After washing, elution was carried out with the same buffer changing the ionic strength gradually from 0.05 to 0.4. Three active peaks were obtained. The one was glucoamylase peak, the second was considered to be maltose-oligosaccharide transglucosidase peak, and the third was α -amylase peak, but the two types of α -amylase could not be separated. This α -amylase fraction was subjected to rechromatography on DEAE-cellulose column by varied elution methods, but separation of the two

α -amylases could not be succeeded and most of the enzyme protein was inactivated and precipitated.

Gel Filtration on Sephadex G-100. Five grams of the enzyme preparation was dissolved in 100 ml of distilled water, 1 g of active carbon was added and the solution was centrifuged. To the decolorized solution ammonium sulfate was added, and the precipitate obtained from 0.4 saturation to 0.75 saturation was collected by centrifugation and dissolved in 25 ml of distilled water. The optical density at 280 $m\mu$ of this solution was 177. Fifty grams of sephadex G-100 was suspended in distilled water and poured into a large column (4×120 cm). After washing the column with M/50 calcium acetate, the enzyme solution was charged on the column and eluted with M/50 calcium acetate. The elution pattern is shown in Fig. 1. As shown in the figure complete separation of the acid-unstable α -amylase from the acid-stable α -amylase was not successful. But when the eluates from tube numbers 66 to 76 were collected, the acid-unstable α -amylase fraction without the acid-stable α -amylase could be obtained. So from this fraction the acid-unstable α -amylase was expected to be purified by the similar method as the acid-stable α -amylase.

Purification of Acid-Unstable α -Amylase after Gel Filtration.

The acid-unstable α -amylase fraction from the sephadex G-100 column was dialyzed successively, against running water for 2 days and against distilled water for a day. To the dialyzed enzyme solution, 1% rivanol solution was added to 1.6% of total volume and the mixture was centrifuged. To the supernatant 1% rivanol solution was added again to 14% of total volume and the precipitate was collected by centrifugation and dissolved in 2N acetate buffer, pH 5.5. To the yellow solution Fuller's earth was added in the ratio of 20 g to 100 ml solution and after 30 min, stir removed by filtration. The clarified enzyme solution was cooled to 2°C, added with acetone to make 48% of total volume and, after removing the precipitate by centrifugation, acetone was added again to the supernatant up to 57%. The precipitate was collected by centrifugation, dissolved in M/50 calcium acetate and subjected to fractional precipitation with acetone for several times to obtain purified enzyme preparation. OD specific activity of this preparation was 344.

Gel Filtration on Sephadex G-50. Similarly to the acid-stable α -amylase in the previous paper, an inactive protein peak appeared also from native

FIG. 1. Separation of α -Amylases on Sephadex G-100.

- : OD at 280 $m\mu$
- ×—: Acid-stable α -amylase
- △—: Acid-unstable α -amylase

enzyme protein of the acid-unstable α -amylase on fractional precipitation with acetone, so, after that precipitate was dissolved in M/50 calcium acetate and subjected to gel filtration on sephadex G-50, the active protein was collected and lyophilized to obtain purified enzyme protein. OD specific activity of this enzyme was 448. This preparation is a homogeneous protein not only on gel filtration but also on ultra-centrifugal pattern, on Tiselius electrophoretic pattern and on amino-terminal amino acid analysis as will be shown in subsequent papers.

Comparison of Some General Properties between Acid-Stable α -Amylase and Acid-Unstable α -Amylase. In Table I some general properties of the acid-unstable α -amylase are summarized, comparing with those of the acid-stable α -amylase of the same enzyme preparation. Assay methods were the same as those described in the previous papers. The modes of starch hydrolysis with both amylases were quite similar. The starch iodine reaction made standard red color at the time when 5.1% of starch was hydrolyzed, and the limit of starch hydrolysis was about 40% as glucose with both enzymes. Degradation products in the course of starch hydrolysis with the acid-unstable α -amylase were the same as those of the acid-stable α -amylase shown in the previous report on paper chromatography, and the main product from starch was α -maltose, having negative mutarotation. Maltase activity was absent.

Optical densities of one per cent (w/v) aqueous solutions at 280 m μ were not so different with both amylases. But the dextrinizing activity per mg

TABLE I. COMPARISON OF ACID-STABLE AND ACID-UNSTABLE α -AMYLASES OF *Aspergillus niger*

	Acid-stable α -amylase	Acid-unstable α -amylase
Dextrinizing activity	High	High
Saccharifying activity	Low	Low
Saccharifying degree at red point of starch iodine reaction	5.1%	5.1%
Limit of starch hydrolysis calculated as glucose	43%	39%
Main product from starch	α -Maltose	α -Maltose
Mutarotation of enzymatic product	—	—
Maltase activity	Absent	Absent
Dextrinizing unit per mg enzyme	160	900
OD specific activity	89	448
OD of 1% solution at 280 m μ	17.8	20.0

FIG. 2. Thermal Stability of Acid-Stable α -Amylase and Acid-Unstable α -Amylase.

Enzymes were incubated at each temperature for 15 min at pH 5.0 ($\mu=0.05$) and then the activities remained were assayed at 30°C.

—×—: Acid-stable α -amylase
—○—: Acid-unstable α -amylase

enzyme of the acid-unstable α -amylase was 5.6 times as large as that of the acid-stable α -amylase.

Thermal stability, pH stability and pH-activity curve of the two α -amylases were quite different. Fig. 2 shows thermal stability. Both α -amylases were dissolved in acetate buffer, pH 5.0 ($\mu=0.05$), and treated at each temperature for 15 min. After the treatment at 60°C, the acid-stable α -amylase retained about 90% of original activity and 28% even at 70°C. But the acid-unstable α -amylase was much sensitive to heat and lost its activity almost completely at 60°C for 15 min, being quite same as Taka-amylase A.

Effects of pH on both α -amylases are shown in Fig. 3. Enzymes were incubated at each pH at 37°C

FIG. 3. Effect of pH on Acid-Stable α -Amylase and Acid-Unstable α -Amylase.

Enzymes were incubated at each pH for 30 min at 37°C and then the activities remained were assayed at pH 4.8.

FIG. 4. pH-Activity Curves of Acid-Stable α -Amylase and Acid-Unstable α -Amylase.

—x—: Acid-stable α -amylase
—o—: Acid-unstable α -amylase

for 30 min. Buffers used were the same as described in the previous report. The acid-unstable α -amylase was inactivated completely at pH 3.0, but the acid-stable α -amylase retained about 90% of original activity at pH 2.2. On the other hand, in alkaline range, the acid-stable α -amylase was more sensitive than the acid-unstable α -amylase.

Fig. 4 shows pH-activity curves of the two α -amylases. Enzymes were dissolved in M/50 calcium acetate solution and assayed their activities at various pH values. The acid-unstable α -amylase of *Aspergillus niger* was proved to take similar behavior to Taka-amylase A. Although the acid-stable α -amylase held about 60% of its full activity at pH 2.5, the acid-unstable α -amylase showed no activity at this pH. On the contrary, at alkaline side, the acid-unstable α -amylase was more active than the acid-stable α -amylase.

DISCUSSION

From the data in this report, the acid-unstable α -amylase of black *Aspergillus* is quite similar to Taka-amylase A, α -amylase produced by *Aspergillus oryzae*, in its mode of action, thermal and pH stabilities, and seemed to be a popular type mold α -amylase. On the other hand, the acid-stable α -amylase is

the unique α -amylase which has never been found from animal or plant origins. In microbial field only black *Aspergilli* such as *Aspergillus niger*, *A. awamori*, *A. usamii* and *A. inuii* can produce this amylase so far as the authors have examined.³⁾ Although acid-stable α -amylase activities of these mold amylase preparations are variable on accounts of mold strain and culture conditions, they are considerably lower than acid-unstable α -amylase activities as shown in Fig. 1. But as the activity per mg protein of the acid-unstable α -amylase is about 5.6 times as large as that of the acid-stable α -amylase, the content of acid-stable α -amylase protein is not so small.

These two α -amylases could not be separated on DEAE-cellulose column. On the other hand, they could be separated by gel filtration on sephadex G-100. From the results the molecular size and shape of the acid-stable α -amylase seem to be different from those of the acid-unstable α -amylase.

The acid-stable α -amylase is more sensitive at alkaline side than the acid-unstable α -amylase, so the width of stable range of the two amylases is nearly the same. It was made obvious that the acid-stable α -amylase is more stable on heat. Have these facts some meaning in relation to the acid stability of these enzyme proteins or not?

The two α -amylases show quite the same mode of action each other, so their active sites are supposed to be very similar. Then the difference of the amino acid residues or other components around these sites and resulting conformational difference between the two enzymes may attribute to acid stability. It may hinder the active site and lower the activity of the acid-stable α -amylase.

3) Y. Minoda and K. Yamada, *J. Ferm. Association*, **20**, 254 (1962).