Title	泡盛麹菌に関する研究(第 2 報) : 泡盛醸造所から分離された泡盛麹菌のアミラーゼ及びプロテアーゼの 2,3 性質について(農芸化学科)
Author(s)	当山, 清善; 宮里, 興信
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Studies on *Aspergillus awamori*. II

On Some Properties of Amylase and Protease of *A. awamori* Isolated from *awamori* Breweries.

By

Seizen TOYAMA* and Koshin MIYAZATO*

I Introduction

In the previous paper[1], it has been indicated that the strains of *Aspergillus awamori* type isolated from *awamori*, "Tomodane-koji" or "Koji", employed as a starter for the production of *awamori*, a distilled rice wine made in the Ryukyus, were classified into four groups based upon their amylase and protease activity. As a result of comparison with the strains already isolated from the "Koji", it has been observed that the strains of one group isolated showed strong amylase activity. Because this group of *A. awamori* type was mainly isolated, it appeared of interest to investigate further some enzymatic properties of this group for the production of *awamori*. In view of the above reasons, a strain 29-2, which belongs to A group of *A. awamori* type in the previous paper, was selected for this investigation.

The work reported here deals with some enzymatic properties of amylase and protease using the selected strain, that is, the time course of the production of both enzymes during the cultivation on solid wheat-bran, the influence of temperature and pH on the β-amylase, and also influence of pH on the protease activity.

II Experimental methods

Organisms: Because it has been found that the strains of A group of *A. aaamori* type isolated from the *awamori* breweries gave strong activity, a strain 29-2 of this group was employed for the present investigation.

Preparation of enzyme solution: Enzyme solution was prepared according to the following procedures. Five grams of wheat-bran was mixed with 5 m*l* of water in a petri dish and sterillized at 120°C for 30 minutes. The sterilized bran was inoculated with conidia of the organism and incubated at 30°C. The solid culture was extracted with 50 m*l* of water, allowed to settle for an hour, was then filtered. This filtrate was employed as the enzyme solution for the assay of the activity of amylase and protease.

Activity assay of enzymes: β-amylase activity; The activity of β-amylase was determined as follows. A mixture of 1.0 m*l* of 1.0% soluble starch, 1.0 m*l* of enzyme solution, and 3.0 m*l* of buffer solution (pH 1.5~4.0: 0.1 M sodiumcitrate-HCl buffer, pH 4.0~5.5: 0.2 M sodium acetateacetic acid buffer, pH 5.5~7.5: 0.1 M McIlvain buffer, pH 7.5~

* Department of Agricultural Chemistry, College of Agriculture, University of the Ryukyus.

9.0: 0.2 M Tris-HCl buffer) was incubated for 15 minutes at 40°C, and the increase in reducing sugar was determined by Hanes method[2]. The activity was shown by the increase in the amount of glucose in the reaction mixture.

α-amylase activity ; The method of determination of α-amylase activity is the same procedures as described in the previous report[1]. The pH of the reaction mixture adjusted by using the same buffer solutions as described in the assay conditions of β-amylase. The activity was determined by a modification of Wohlgemuth method[3] and shown as Wohlgemuth value.

Protease activity ; The assay procedures for protease activity were carried out by the same method previously reported[1]. The reaction mixture contained 1.0 ml of 2% casein solution, 1.0 ml of enzyme solution, and 2.0 ml of buffer solution (the same buffer solutions as described in β-amylase). The reaction mixtures were incubated at 40°C for 20 minutes. The modification by Hagiwara of Anson's method was applied to determine the protease activity as was reported in the previous paper. The activity was represented as the increase of optical density (O.D.) at 660 μ by the use of a Coleman Spectrophotometer.

III Results

1. The production of amylase on wheat-bran culture

The strain 29-2 was grown on solid wheat-bran media at 30°C, and the activity of amylase was determined during the cultivation. The time course changes of β-amylase and α-amylase activity are shown in Fig. 1 and 2, respectively. As is evident from the figures, activities of both increased in proportion to the time of cultivation, and for both the maximal activities were reached within 60 hours after cultivation, and thereafter no change of their activities was observed.

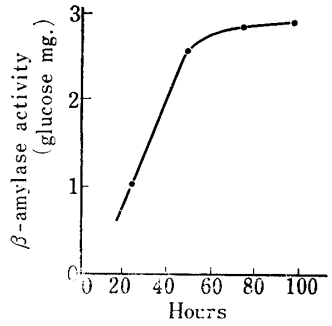

Fig. 1. The time course of production of β-amylase activity on wheat-bran culture by strain 29-2.

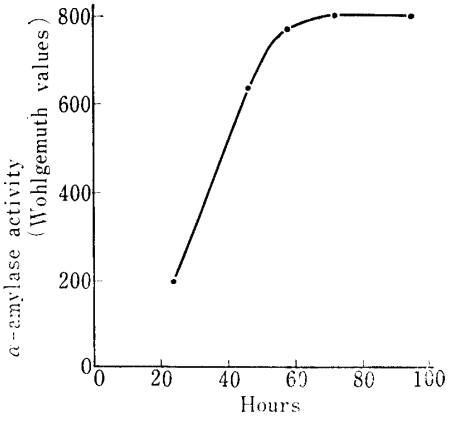

Fig. 2. The time course of the production of α-amylase on wheat-bran culture by strain 29-2.

2. The influence of pH on the amylase activity.

The influence of pH on the amylase activity of the strain 29-2 was investigated. The strain was grown on solid wheat-bran at 30°C for 70 hours, and the culture extract was employed for the estimation of α- and β-amylase activity. The activities of both were estimated at different pH values according to the assay conditions. The pH activity curves of both activities are in Fig. 3 and 4. The optima of the activity of α- and β-amylase

were over a wide range of pH values from 4.5 to 6.0 and from 3.5 to 7.0, respectively.

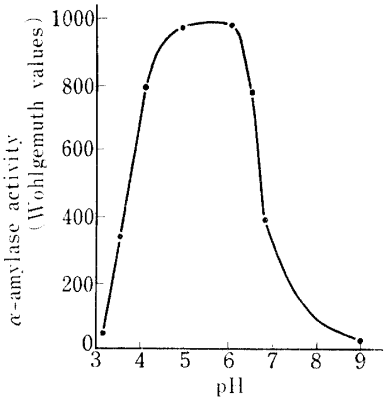

Fig. 3. The influence of pH on α-amylase activity of strain 29-2.

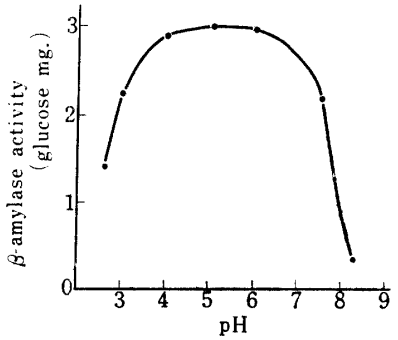

Fig. 4. The influence of pH on β-amylase activity of strain 29-2.

3. Influence of Temperature on β-amylase.

The influence of temperature on the activity of β-amylase was investigated for the enzme solution prepared from the culture extract of the strain 29-2. The enzyme solution was incubated at different temperatures at pH 5.5, and then the activity was determined by the method described in the assay conditions. As shown in Fig. 5, the maximum activity occurred at approximately 50°C, and gradually decreased above 55°C.

4. Thermal Inactivation of β-amylase.

In order to investigate the thermal stability of the β-amylase activity of the strain 29-2, the enzyme solution was heated at different temperatures at pH 5.5 for 30 minutes, after which its activity was determined. The data expressing the residual enzyme activity in percent of the initial activity is indicated in Fig. 6. The incubation of enzyme solution at 50°C reduced the residual activity to 25%, and at 55°C it was reduced to 70% of the unheated enzyme solution, and at 65°C its activity was almost destroyed.

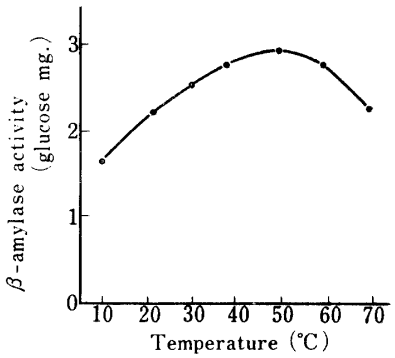

Fig. 5. The influence of temperature on β-amylase activity of strain 29-2.

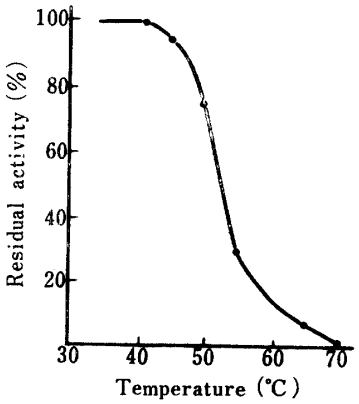

Fig. 6. Thermal inactivation of β-amylase activity of the strain 29-2.

5. pH Stability of β-amylase.

Because the fermentation for the production of *awamori* has been carried out in acid media, it is of interest to investigate the stability of β-amylase of the strain 29-2 at

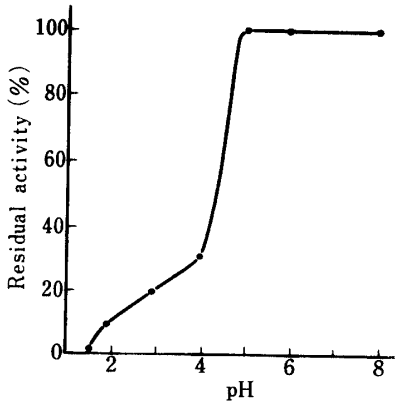

Fig. 7. pH stability of β-amylase activity of the strain 29-2.

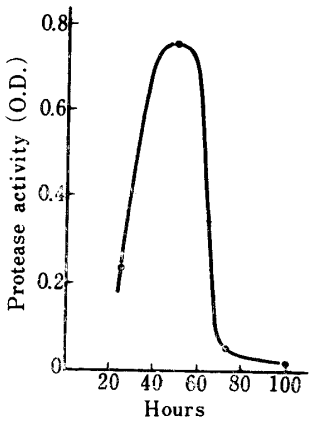

Fig. 8. The time course of the production of protease on wheat-bran culture by strain 29-2.

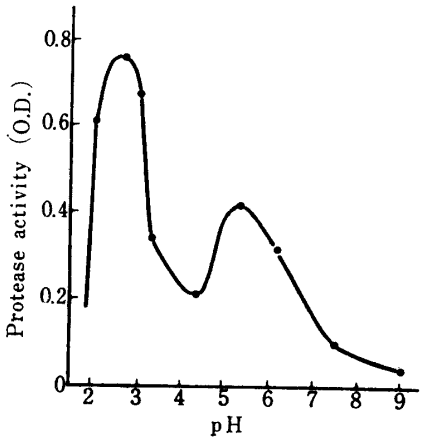

Fig. 9. The influence of pH on protease activity of strain 29-2.

different pH values. For this purpose, the enzyme solution was incubated at different pH values at 40°C for 30 minutes, and then its activity was estimated. The activity was represented as the data expressing the residual enzyme activity in percent of the initial activity. The results obtained are given in Fig. 7. The enzyme was found to be stable in the pH range 5.0 to 8.0, but a sharp drop in residual activity was observed below pH 5.0.

6. Production of protease on wheat-bran culture.

It is generally observed that members of the genus *Aspergillus* produce extracellular proteolytic enzymes[4,5]. In order to ascertain the protease production of the strain 29-2 during the cultivation on wheat-bran, the activity of protease on milk casein as a substrate was determined. Since the strain 29-2 has produced a great amount of protease in the acid range, as will be described later, the assay of the activity was carried out at pH 2.7. The time course change of protease production is shown in Fig. 8. The strain 29-2 bagan to produce protease remarkably in 20 hours after cultivation, and the maximum protease production was reached in 50 hours. Thereafter, the activity began to decrease rapidly, and was lost completely in 70 hours after cultivation.

7. Influence of pH on the Protease

It has been reported that the black *aspergilli*, *A. awamori*, *A. niger* and *A. saitoi*, produce acid proteases as major components of their protease systems[6]. In the previous report[1] it has been indicated that the strain 29-2 gave weak protease activity at pH 3.0 and pH 6.0. The influence of pH on protease activity of the strain at different pH values was investigated in this paper. The enzyme solution was prepared from the extract of a 50-hour-old-culture on wheat-bran, and the activity of protease on milk casein as a substrate was determined. The results are shown in Fig. 9. The strain 29-2 showed a high peak of protease activity at pH 2.7, and a secondary but lower peak at pH 5.5.

IV Discussion

In the previous paper[1], it has been shown that the strain os *A. awamori* type isolated from *awamori* "Tomodane-Koji" or "Koji" of *awamori* breweries were classified into four groups

according to their amylase and protease activity. That is, the strains which gave strong amylase and weak protease (at pH 3.0) activity, weak amylase and protease (at pH 3.0) activity, weak amylase and strong protease (at pH 3.0) activity and weak amylase and strong protease (at pH 3.0 and 6.0) activity were shown as A-, B-, C- and D-group, respectively. Because the strains of A-group have been mainly isolated, a strain 29-2 of this group was selected for the present investigation. In preliminaly experiments before preparation of this report, the time course of production of amylase of solid wheat-bran culture, and the influence of temperature and pH on the amylase activity of the each strain of above B- and C-group, and *A. awamori* Nakazawa IAM 2112 were investigated. As the results, it was observed that there were not any differences among these strains, except that the strain 29-2 of A-sroup gave highest amylase activity. The production of *awamori* has been carried out at pH below 4.0 during the fermentation to prevent the contamination of useless microorganisms. However, the amylase activity of the strain 29-2 was rapidly inactivated below pH 5.0 when the enzyme solution was kept at 40°C for 30 minutes (Fig. 7). Further work is required to clarify the relation between amylase activity and pH value during the fermentation in the breweries.

It has been previously shown that the strain of A-group had weak protease activity at pH 3.0 when milk casein was used as a substrate[1]. However, the strain 29-2 showed two peaks of protease activity (Fig. 9). One of them was observed at pH 2.7, and a secondary but lower peak at pH 5.5. As the results of preliminaly experiments on this report, it has been observed that the pH activity curves of the strains of B-group described above and *A. awamori* Nakazawa IAM 2112 were in agreement with that of the strain 29-2, whereas the strains of C-group showed one peak of protease activity at pH 2.7. From the pattern of the pH activity curve, it is considered that the strain 29-2 will produce acid-protease as major components of its protease system, and this result coincides with that of black *aspergilli* as has been reported by many investigators[6,7]. The maximum productin of protease of the strain 29-2 was observed in 50 hours after cultivation on solid wheat-bran. The strain of B-group and *A. awamori* Nakazawa IAM 2112 showed the same patterns of protease production as that of the strain 29-2, but the maximum protease production of the strains of C-group was present in 70 hours after cultivation.

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Summary

By using a strain 29-2 of *Aspergillus awamori* type isolated from *awamori* "Tomodane-Koji" or "Koji", some properties of amylase and protease were investigated. The results obtained were as follows :

1) The maximum production of α- and β-amylase were obtained within 50 hours when the strain was cultured on wheat-bran (Koji).

2) The optimum pH of α-amylase activity was in the range of pH values from 4.5 to 6.2, and that of β-amylase activity was from 3.5 to 7.0.

3) The optimum temperature for the activity of β-amylase was 50°C, and gradually decreased above 55°C.

4) The activity of β-amylase was stable above pH 5.0 when incubated at 40°C for 30 minutes, but it was rapidly inactivated below pH 5.0.

5) The highest activity of protease at pH 2.7 on milk casein as a substrate was found between 30 to 60 hours after cultivation on wheat-bran. The maximum peaks of the protease activity on milk casein were present at pH 2.7 and 5.5, and the peak of the former was higher than that of the latter.

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泡盛麹菌に関する研究 (第2報)

泡盛醸造所から分離された泡盛麹菌のアミラーゼ及びプロテアーゼの 2, 3 性質について (要約)

泡盛友種麹並びに麹中から分離された *Asp. awamori* Type 29-2 の麩麹抽出液を用いてアミラーゼ及びプロテアーゼの 2, 3 の性質を検討した結果次のことが明らかになった。

1) α 及び β-アミラーゼの生成は培養後 50 時間で最高に達した。

2) α 及び β-アミラーゼの作用至適 pH はそれぞれ 4.5～6.2, 3.5～7.0 にあった。

3) β-アミラーゼ活性の作用至適温度は 50°C にあり, 55°C 以上では暫時減少した。また酵素液を pH 5.0 以下に 40°C, 30 分間保つとその活性は急激に不活性化された。

4) プロテアーゼの生成は培養後 30～60 時間にあり, 70 時間以後では活性を殆んど示さなかった。プロテアーゼ活性の作用至適 pH を検討した結果 pH 2.7 に強い活性があり, pH 5.5 に弱い活性のピークがあった。