BIOCHEMICAL STUDIES OF PIRICULARIA ORYZAE

Part IV. Production of Amylase by P. oryzae and its some properties*

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It is evident that the biochemical behaviors of P. oryzae are fundamentally attributed to its enzyme systems. Up to the present, however, there have been not so many researches about enzymes of this fungus. Presence of several kinds of enzyme has been known, such as proteinase[1,2], dipeptidase[2], polypeptidase[2], xylanase[3], amylase[3,4] L-amino acid oxidase[5] and transaminase[6]. With amylase among these enzymes, YOSHII[7] studied the effects of cephalothecine to the excretion of amylase by this fungus and TANAKA et al[4] reported the influence of inorganic nitrogen sources such as nitrate and ammonium salt to the activity of exo-and endo-amylase. In the study of the change of amylase activity during the cultivation of this fungus in which sucrose was used as carbon source, it was demonstrated that the activity was parallel to the weight of mycelium and the sudden decrease of activity in later stage seemed to be due to the decomposition of this polyase by proteinase[3]. Moreover, from the comparative study of two carbon sources, glucose and xylose, in the successive preculture, TANAKA and TSUJI[3] regarded this amylase as the constitutive enzyme of this fungus. On the other hand, the metabolic adaptability of this fungus to nutritive circumstances and, in relation to this fact, the adaptive increase of L-amino acid oxidase activity were recognized by the authors[5,8].

In the biochemical study of blast disease of rice plant, it is most important to know if this fungus possesses the ability to show the adaptive increase of amylase activity as in the case of L-amino acid oxidase. In present paper the change of amylase activity in the

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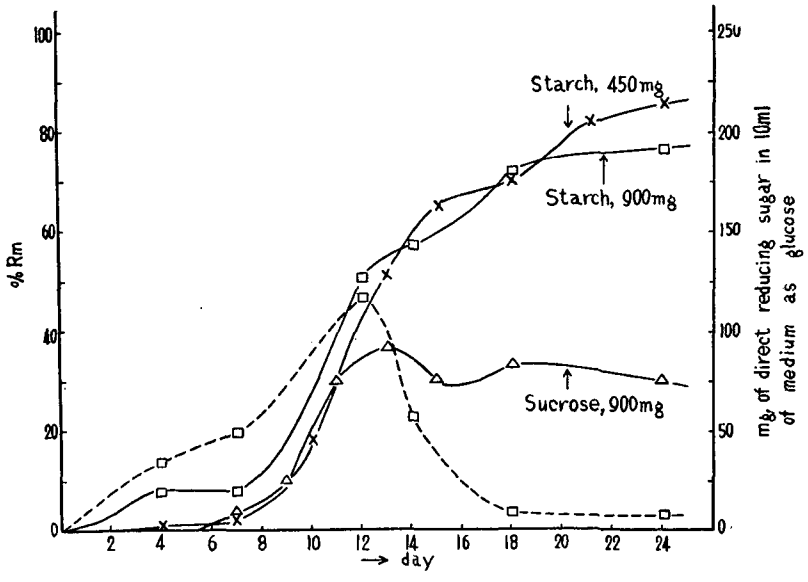
presence or absence of starch as substrate, the influences of amino acids to the production of this enzyme, some properties of partially purified enzyme preparation and moreover the crystallization of this amylase are recorded.

Experimental and Results.

1. Amylase activity in the presence or absence of starch. P. oryzae was grown at 27–8°C in 30 ml of medium containing 900 mg of sucrose, inorganic salts and growth factors[9]. At appropriate intervals, the contents of two culture flasks were filtered together and the clear filtrate was subjected to determination of exo-amylase activity and direct reducing sugar. In second experiment sucrose was replaced by 450 mg or 900 mg of soluble starch and the same determinations were



Full line indicates amylase activity and dotted, direct reducing sugar. Reaction mixture consisted of 15 ml of filtrate, 10 ml of 1% soluble starch soln., M/15 phosphate buffer soln. (pH. 6 9) and 5 ml of water; temperature, 38–9°. After 120 mins' incubation, increase of reducing value as maltose was determined by Fehling-Lehrman-Schoorl's or micro-Bertrand's method. In every determination, control value was subtracted.

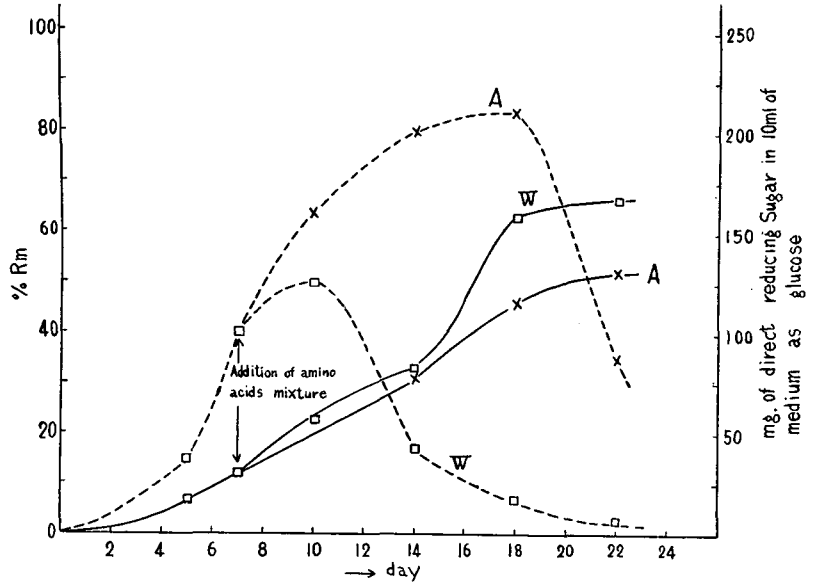
Fig. 1. Changes of amylase activity and direct reducing sugars amount in culture medium containing starch and sucrose.

TABLE 1. Changes of pH and iodine color test of culture medium containing soluble starch.

Day		4	7	10	12	13	14	15	18	21	24
Medium contg. 900 mg of starch	pH	5.3	5.5		6.2		6.4		7.4		7.4
	iodine color	blue	blue		bluish purple		purple		slightly yellow		color-less
Medium contg. 450 mg of starch	pH	4.7	6.0	6.8		6.9		7.1	7.3	7.4	7.4
	iodine color	blue	blue purple			reddish purple		color-less			color-less

made, including the observation of iodine color test. These results are presented in Fig. 1 and Tab. 1. In the determination of amylase activity in the filtrate, especially in that containing soluble starch as inductive substrate, some notice must be paid to the increase of reducing value observed during 120 minutes' incubation without the new addition of starch as substrate. This increase seems to be due to the attack of amylase present to the partially degraded starch remaining in the filtrate. In practical analysis in such a case, it is a most difficult to distinguish the increase of reducing value due to starch newly added from that derived from the remaining degraded starch. This blank increase during the incubation time, however, reduced to almost negligible value at least thereafter, when the iodine color test indicated purple~red. Besides this, it was found that amylase possessed much greater affinity towards native starch than degraded starch, when the two kinds of polysaccharide just described were incubated together[10]. Accordingly the amylase activity detected in relatively earlier stage of cultivation, which was calculated from the difference between the value obtained in the presence of starch as substrate and that in the absence of starch is not so far from the true activity.

2. Effects of amino acids on amylase activity.— This fungus was grown in each of twenty culture flasks, containing as much the same amount of soluble starch and other salts as mentioned above. On the seventh day of cultivation 20 ml of the aqueous solution of twenty amino acids was added into the each of ten flasks up to the total volume of 50 ml. The concentration of single amino acid was M/1000 and the total concentration of amino acids M/50. The amino acid mixture consisted of DL-valine*, L-leucine*, DL-isoleucine*, DL-threonine*, DL-phenylalanine*, DL-methionine*, L-tryptophane*, L-arginine



Full line indicates amylase activity as % of hydrolysis and dotted line shows direct reducing sugar. Capital A shows the experiment in which amino acids mixture was used and W, the experiment in which water was used in place of amino acids. Determinations of activity and reducing value were made by the same methods as in Fig. 1.

Fig. 2. Changes of amylase activity and direct reducing sugar amount in the presence or absence of the mixture of twenty amino acids.

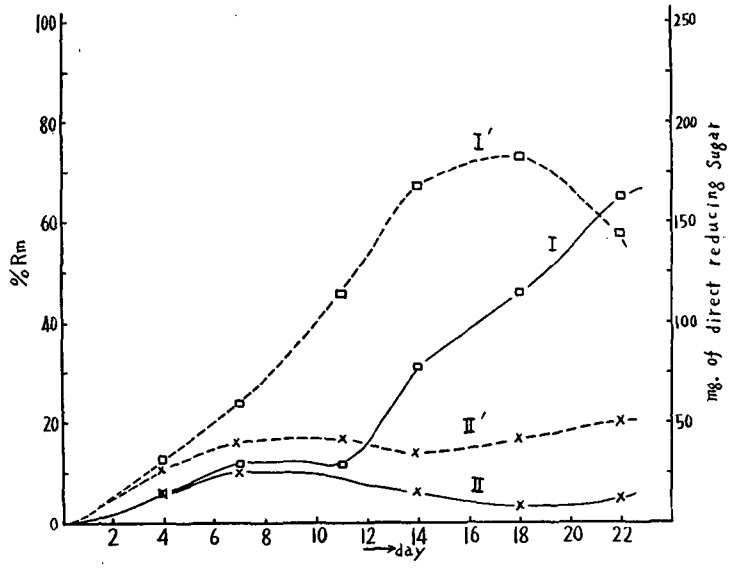
TABLE 2. Changes of pH and iodine color test in the experiments represented in Fig. 2.

Day	5	7	Addition of amino acids	10	14	18	22
pH	5.3	5.8	W	6.1	6.4	7.2	7.6
			A	5.1	4.4	4.7	5.0
Iodine color	blue	bluish purple	W	reddish purple	yellow	colorless	colorless
			A	brown	yellow	colorless	colorless

hydrochloride*, L-lysine hydrochloride*, L-histidine hydrochloride*, DL-alanine, DL-serine, L-aspartic acid, L-glutamic acid, L-hydroxyproline, L-tyrosine, L-cysteine hydrochloride, L-taurine, DL-α-amino-n-butyric acid and glycine. In place of amino acids mixture, 20 ml of water

was added into the other ten flasks. The results obtained are presented in Fig. 2 and Tab. 2.

3. Influences of "essential" and "nonessential" amino acids. These twenty amino acids are divided into two groups, that is, the ten "essential" amino acids*) and the other ten "nonessential" for the nutrition of rat[12]. The effect of each group to the adaptive production of amylase was examined respectively. In total volume of 30 ml, soluble



Determinations and expressions are the same as shown in Fig. 1. Full line I expresses the amylase activity of medium contg. "essential" amino acids and II, that of medium contg. "nonessential; dotted line I' shows the direct reducing sugar amount in the medium contg. "essential" amino acids and II', that of medium contg. "nonessential".

Fig. 3. Changes of amylase activity and direct reducing sugar amount of the culture solutions containing "essential" and "nonessential" amino acids.

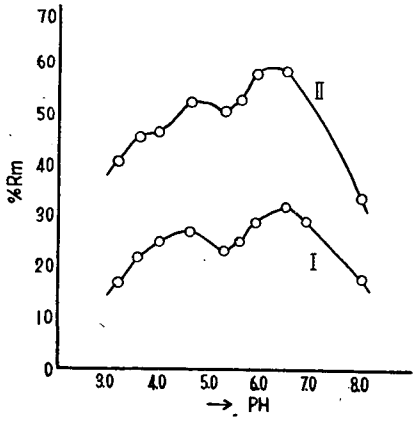
starch (900 mg), other salts and "essential" or "nonessential" amino acids were contained. The concentration of each amino acid was M/600. At appropriate intervals, enzymic activity and weight of mycelium were measured as described above (Fig. 3 and Tab. 3).

4. Partial purification of amylase and its some properties. Partial purification of amylase was made almost according to NISHIDA[13]. This

TABLE 3. pH and iodine color reaction of medium contg. "essential" and "nonessential" amino acids and the weight of mycelium in each medium.

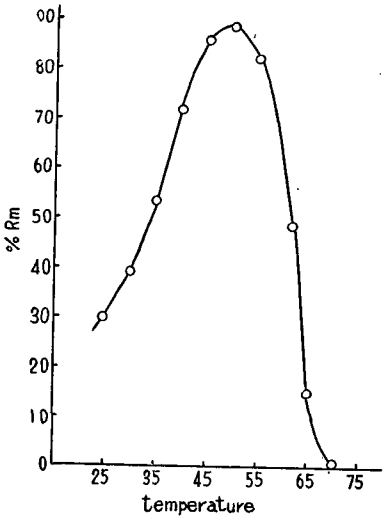
Day		4	7	11	14	18	22
pH	essential amino acids added	4.7	4.7	4.4	4.6	5.0	5.2
	nonessential amino acids added	3.4	3.3	3.3	—	3.8	4.4
Iodine color	essential	blue	blue	reddish purple	yellow	color-less	color-less
	nonessential	blue	blue	blue	blue	blue	blue
Weight of mycelium (mg)	essential	—	21.3	—	52.8	106.2	113.8
	nonessential	—	9.2	19.4	36.4	70.8	80.2

Mycelium was filtered from the medium, washed with water and dried at 100°; values express the average of two mycelium.



Reaction mixture: 0.5/25 ml enz. soln.+buffer soln., 5 ml+1% sol. starch soln, 5 ml+water, 4.5 ml; tempt., 38-9°. I shows % Rm after 60 mins' incubation and II, % Rm after 120 mins'.

Fig. 4. Effect of pH on the activity of partially purified amylase.



Experimental conditions was the same as in Fig. 4, except that pH was held at 5.9; time, 120 mins.

Fig. 5. Effect of temperature upon partially purified amylase activity.

blast disease fungus was cultured for twenty days in the medium containing soluble starch and other salts as mentioned above and to 250 ml of clear filtrate whose % Rm was 76.0, N-acetic acid was added till the pH-value reached 6.4–6.5 and about 125 ml of 3% tannin solution was added under vigorous stirring. This mixture was kept at 0°C for twenty hours. Cloudy precipitate was separated by centrifugation, washed with cold acetone in the centrifuge until the supernatant acetone became colorless, dissolved in 10 ml of distilled water and filtered. 25 ml of cold acetone was added to this filtrate under stirring and the mixture was kept at 0°C for half an hour. The precipitate separated was washed twice with cold acetone and kept in vacuo. Yield, 5.8 mg of grayish white powder. The enzyme preparation obtained by this procedure was stable in this state for more than three weeks. This preparation was redissolved in 25 ml of distilled water and the solution was employed for the determination of optimum temperature and pH. The results are shown in Fig. 4 and Fig. 5.

5. Determination of amylase-type.— In this experiment it was examined whether this amylase belonged to α-type or β-type. The dextrine formation power was measured at pH 5.9 according to WOHLGEMUTH[14] (expressed as X) and its modified method[15] (denoted as D). The value of X of the original filtrate whose % Rm was 76.0 after twenty days cultivation was 1.1–1.3 and $D_{120'}^{38°}$ was 1.4–1.7. Moreover, $D_{120'}^{40°}$ of the aqueous solution of partially purified amylase described in experiment 4 was 2.8. The stability of this partially purified amylase preparation towards acid-and heat-treatment was also examined. The amylase preparation used in this test was obtained by the same procedure with that presented in experiment 4 from 225 ml of the filtrate whose % Rm was 77.3. This enzyme preparation was dissolved in 25 ml of distilled water. To 2 ml of this aqueous solution, 8 ml of water was added and the mixture was kept at 70°C for just fifteen minutes. Immediately after that time, it was cooled with running water to room temperature and subjected to determination of amylase activity at pH 5.9 and 38°C. During 120 minutes incubation, almost no increase of reducing value was recognized and accordingly the iodine color remained unchanged. In next experiment, 2 ml of the cold aqueous solution just mentioned was brought to pH 3.4 with ice-cold N-acetic acid and kept at 0°C for fifteen minutes. Then 0.1 N solution of sodium hydroxide was added up to pH 6.4, the original pH of amylase solution. The activity of this amylase solution was measured at

pH 5.9 and 38°C in total volume of 30 ml. 73.5 per cent of the activity of untreated amylase solution was recovered in this test and iodine color remained blue. These data show that the exo-amylase produced by P. oryzae is mainly of β-type.

6. Crystallization of amylase—The general principle of procedure was that according to FUKUMOTO[16]. This fungus was grown for 21 days in the medium containing 900 mg of soluble starch per flask. 725 ml of clear filtrate was concentrated in vacuo below 40°C to a small volume and filtered. This filtrate, 24 ml, was saturated with solid ammonium sulfate (saturation degree, 0.8), allowed to stand overnight and centrifuged. The precipitate collected was dissolved in 5 ml of distilled water, filtered from insoluble materials, and washed with water. The amount of filtrate and washing water was 18 ml. This fractionation procedure was repeated further twice. About 20 ml of enzyme solution obtained in the third procedure was dialyzed in cellophan bag against running water for seven hours and successively distilled water for sixteen hours. The content of the bag was concentrated to 10 ml as described above. To this aqueous solution absolute ethanol was added drop by drop under vigorous stirring to the concentration of 60 per cent (v/v). Grayish turbidity was observed. After the mixture was allowed to stand at 0°C for one hour, the precipitate was collected by centrifugation. Furthermore, small amount of enzymatically active precipitate separated out from the supernatant when it was kept at 0°C overnight. The aqueous solution of first and second precipitate was subjected once more to alcohol precipitation procedure just mentioned. In this case, however, the concentration was raised up to 70 percent. 13 ml of the aqueous solution of the precipitate obtained was fractionated with ammonium sulfate at the saturation degree, 0.80 and the solution of precipitate obtained was again dialyzed against running water and distilled water for fifteen hours and thirty five hours respectively. 8 ml of cold acetone was added drop-wisely to 12 ml of cold dialyzed solution, which was found to be strongly active towards soluble starch, under continuous stirring and the mixture was allowed to stand at 0°C. On the next day silky stream of very fine particle was observed on stirring and fine rod-shape-like particles were microscopically found out. This particle, however, lost its shape into liquid when it was allowed to lie on the slide glass for some minutes. After seven days' incubation at 0°C, these particles showed microscopically sharp parallelogram-like shape and the shape changed no longer

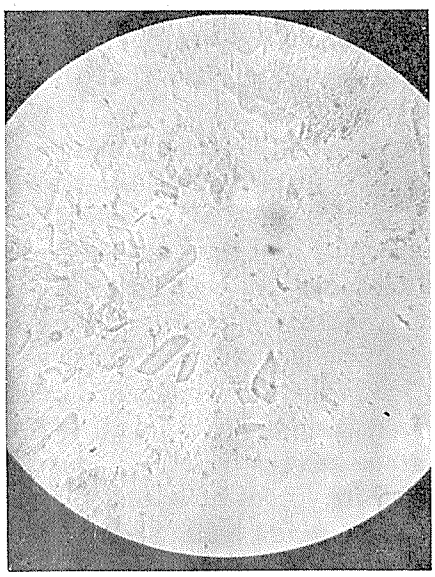


Fig. 6. Crystalline amylase (β-type) of P. oryzae.

on being allowed to stand for several hours (Fig. 6). This crystal produced qualititatively remarkable amount of reducing sugar from soluble starch at pH 5.9 and 40°C and yet the iodine color of the reaction mixture remained original blue color. But the yield was a most unsatisfactory and so further investigation of this crystalline amylase preparation was not performed reluctantly.

Discussion

The study of the biochemical behaviors of p. oryzae towards the nutritive circumstances is a most important in order to protect rice plant from the blast disease. It was already observed by the authors that, when this fungus was nourished with amino acid as carbon source, activity of L-amino acid oxidase was increased[5]. This finding enables us to expect the biochemical adaptability of this fungus to starch, one of the most common constituents of host plant. It was reported that soluble starch was not so good carbon source for P. Oryzae[11] and also amylase of this fungus seemed to be constitutive enzyme[3]. But the production of amylase by this fungus in the presence of soluble starch was over again examined by the authors. The change of amylase ac-

tivity in the presence of soluble starch as carbon source was compared with that in the presence of sucrose (Fig. 1) and it was found that the activity in soluble starch medium was much higher than that in sucrose medium. In latter medium, maximum % Rm 37.2 was attained on thirteenth day of cultivation, followed by almost constant activity thereafter (% Rm, 30-32). On the other hand, in the former medium containing soluble starch, % Rm 50.8 was measured on twelveth day and continuous increase of activity was found until the maximum % Rm 72.1 was attained on eighteenth day. Thereafter almost no change of activity was observed. These facts indicate that in the presence of soluble starch the production of amylase was raised nearly twice as much, compared to the production of amylase in sucrose medium. Moreover, good growth was observed macroscopically in this case and the formation of spore was also observed on eleventh day. These findings represent the biochemical adaptability of this fungus: this blast fungus seems to possess the ability to maintain its growth obstinately, corresponding smoothly to the nutritive conditions of host plant.

The increase of the amount of the direct reducing sugar in the starch medium, calculated as glucose, was approximately correspondent to that of amylase activity as far as twelveth day. Thereafter, sudden decrease of reducing value was measured, in spite of the further steady increase of amylase activity. In later stage of incubation, when the nearly constant amount of reducing sugar, i. e., 8–10 mg per 10 ml of medium, was measured, the change of amylase activity was no more observed. The same tendency was also observed during the incubation with sucrose: the amount of reducing sugar reached the maximum value, i. e., 242 mg per 10 ml of medium on nineth day, thereafter followed by the steep decrease of reducing sugar, i. e., 5.8 mg on eighteenth day and the amylase activity showed continuous increase as far as thirteenth day, thereafter followed by nearly constant activity level, though it was much lower than in starch medium. These findings suggest the presence of a certain coupling between the production of amylase and the energy metabolism[17], not to speak of the adaptive formation to α-1.4-glucosidic linkage of starch[18),19)]. Even when the value of % Rm showed 72–77 during 120 mins incubation in the determination of activity, the iodine color of the reaction mixture was purple—purplish red. This fact may indicate that a greater part of amylase secreted in the medium is of β-type. The difference in the

amount of soluble starch added, i. e., 450 mg and 900 mg respectively, showed not so remarkable influences upon amylase activity, change of pH, iodine color and the amount of residual reducing sugar.

Hokin reported the effectiveness of amino acids, especially "essential" amino acids, upon amylase synthesis and secretion by pigeon pancreas slices[20]. The effect of twenty amino acids mixture was examined and it was found that this mixture had not direct effect stimulating the production of amylase and, on the contrary, the activity of amylase was slightly reduced in this experiment, compared with the case of no addition of amino acids. These results are in good accord with those obtained by NAKAMURA and SUGAWARA[18]. It is noteworthy that the remarkable increase of residual reducing sugars was detected in this case and the time showing the maximum amount of residual sugars was far prolonged, in comparison with the case of no amino acids. This may be ascribed to the relative increase of residual sugars which is due to the rapid utilization by P. oryzae of α-keto acids formed from amino acids added[8,9].

The addition of amino acid mixture disturbed the further development of pH of culture medium as observed in the case of no amino acids and the difference of the pH observed in two cases seems not to have direct influences upon the production of amylase, at least in the present experimental conditions (Fig. 2).

The amino acids mixture just mentioned involved two nutritional groups for the rat, i. e., "essential" amino acids and "nonessential". The effect of each group on the amylase production was examined (Fig. 3). In the case of "essential" amino acids added, the processes of the changes of amylase activity and residual sugars were almost the same as those in Fig. 2. On the other hand, in the case of "nonessential" acids, both the activity of amylase produced and the amount of residual sugars were found in much lower level, especially in the later stage of cultivation. It is evident from the results indicated in Fig. 2 that pH of medium are not so closely related to the amylase production. Accordingly, the much smaller amylase activity in this case may be due to the direct inhibitory effect of "nonessential" amino acids on the amylase production. If this is the case, some inhibition should be recognized in the preceeding experiment in which the mixture of twenty amino acids was used. But the fact was found to be the reverse. One possible explanation of this fact is that the inhibitory effect of "nonessential" amino acids may be covered by

"essential" amino acids. Then it may be possible to think about the indirect stimulating effect of "essential" amino acids. At any rate, it was found that amino acids did not take positive part in the production of amylase and it might mainly depend upon the inorganic nitrogen source[17].

The optimum pH-range of partially purified amylase preparation was 5.9–6.5. In the more acidic pH-range this preparation was relatively stable and 76 per cent of the maximum activity wss recovered even at pH 3.2. In the more basic range, however, more rapid inactivation was observed. It was already shown that the relatively broad optimum pH-range was characteristic of the saccharogenic amylase of Asp. Oryzae[21]. When this preparation showed % Rm of 60 and 89, the iodine color of reaction mixture remained purple and reddish purple respectively. Optimum temperature was 45–50°C and higher temperature resulted in sudden decrease of activity. During the incubation at 50°C for 120 minutes, % Rm reached 89.1 and yet the iodine color test gave reddish purple color. The very small dextrine formation power of this preparation was concluded from the results in experiment 5. When this preparation was treated at pH 3.4, 73.5 per cent of activity of untreated preparation was recovered, though its whole activity was lost by the heat-treatment at 70°C. These findings seem to indicate that this amylase preparation is of so-called β-type or saccharogenic amylase type. In addition, the supernatant separated by centrifugation from the first precipitate which was caused by addition of tannin was concentrated below 40°C and three times as much volume of cold acetone was added to this concentrated solution. The precipitate obtained was found to be nearly enzymatically inactive. Thus it is most probable that the principal part of exo-amylase of P. oryzae is of β-type. The determination of true type of this amylase, for instance, β-type, saccharogenic amylase type and gluc-amylase type[22], will be attained by further research.

In order to obtain pure crystal of this amylase, purification procedure was repeated several times and only once parallelogram-like crystal was obtained. Although the very unsatisfactory yield prevented us from making detailed research of this crystal, qualititative investigation indicated that this crystalline amylase was of so-called β-type.

Summary

The adaptive production of amylase by P. oryzae was recognized and the amylase activity in the presence of soluble starch was raised nearly twice as much, compared to that in the presence of sucrose as carbon source. The mixture of twenty amino acids which "essential" and "nonessental" amino acids were involved in did not show positive effect upon the production of amylase and it seemed that "nonessential" amino acids inhibited the production of amylase and this inhibitory effect was covered by "essential" amino acids.

Some properties of partially purified amylase preparation were examined and it was concluded that this preparation was of β-type.

Parallelogram-like crystalline amylase was obtained, though its yield was a most unsatisfactory. This crystal appeared to be qualititatively of β-type.

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