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Raw-Starch-Digesting Glucoamylase from *Amylomyces* sp. 4-2 Isolated from Loogpang Kaomag in Thailand

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Two types of glucoamylases I and II were produced by *Amylomyces* sp. 4-2 isolated from Loogpang Kaomag used in Thailand. Both enzymes, completely purified from solid wheat bran culture of the strain, hydrolyzed gelatinized glutinous rice starch completely but hydrolyzed gelatinized potato starch only to the extent of 60%. Glucoamylase I and glucoamylase II hydrolyzed glycogen completely and to the extent of only 50%, respectively. Both enzymes had the optimal activity on gelatinized potato starch between pH 3.5-5.0 at 55°C. Glucoamylase I (MW 68,000) had the ability to digest and be adsorbed onto raw starch whereas glucoamylase II (MW 50,000) lacked such activities. The optimal pH of glucoamylase I for raw starch digestion was between 3.5-4.5 and that for raw starch adsorption was 4.0.

INTRODUCTION

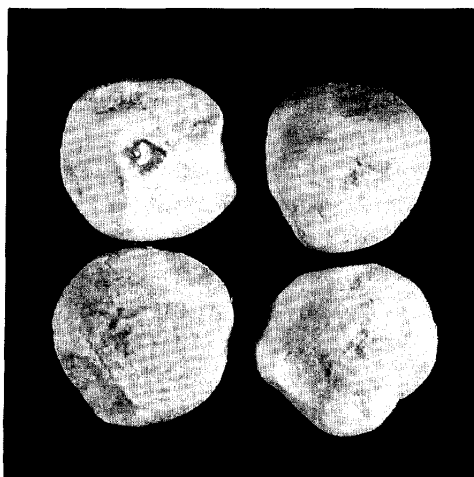
Glucoamylase that shows significant digestibility of raw starch is best known to be produced by some genera of molds such as *Aspergillus awamori* var. *kawachi* (Hayashida, 1975a), *Rhizopus* sp. and *Rhizomucor pusillus* (Kanlayakrit et al., 1987).

Amylomyces sp., previously classified as *Chlamydomucor* sp. (Ellis et al., 1969) is a monotypic fungus that has been found only in the inoculum preparations for rice and cassava fermentations in China and South East Asia, especially in Thailand. *Amylomyces* sp. generally found in Thai-seed-koji, called Loogpang Kaomag (Fig. 1), which is used as a starter for making sweet fermented rice (Kaomag) (Fig. 2), a Thai local dessert made from glutinous rice. This seed koji is also used for making a sweet sake called Grachae that can be done in any household. The growth of this unique fungus on starchy materials is nearly white with no cottony aerial growth. Consequently, it results in an attractive food product.

Wang et al. (1984) purified only one form of glucoamylase from *Amylomyces rouxii*. But, there is no report described about the multiplicity and raw starch digestibility of *Amylomyces* glucoamylases.

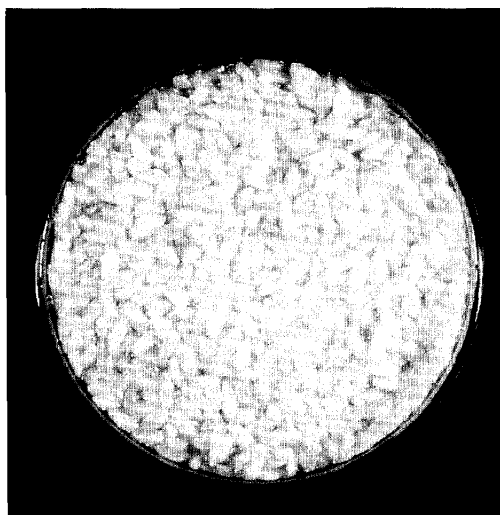
On the other hand, as previously reported (Hayashida, 1975a), *Aspergillus awamori* produced three types of glucoamylases; raw-starch-digesting glucoamylase I and raw-starch-nondigesting glucoamylase I' and II under the wheat bran solid culture or

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Loogpang Kaomog

Fig. 1. Picture of Loogpang Kaomag (diameter 1 cm).



Kaomag (sweet fermented rice)

Fig. 2. Kaomag (sweet fermented rice) Steamed glutinous rice was mixed with powder of Loopang Kaomag and incubated at 30°C for 3 days.

unregulated submerged culture. Based on the difference in hydrolysis curves on gelatinized potato starch and glycogen, *Aspergillus awamori* glucoamylase I which hydrolyzed glycogen completely was classified as type A. Glucoamylase I' and II which hydrolyzed glycogen of 80 and 40%, were classified as type B and C, respectively.

This paper describes the screening of *Amylomyces* strains from Loogpang Kaomag. Further, purification, characterization and classification of two types of raw-starch-digesting and raw-starch-nondigesting glucoamylase produced by *Amylomyces* sp. were also reported and compared with *Aspergillus awamori* glucoamylases.

MATERIALS AND METHODS

Isolation of *Amylomyces* strains

Amylomyces strains were isolated from eight samples of Thai-seed-koji (Loogpang Kaomag) obtained from the northeast provinces of Thailand by using the dilution plate method. Potato dextrose agar medium supplemented with 1% rice bran was used for culture isolation at 30°C, and stock culture.

Screening of glucoamylase-producing strains

The screening was carried out in a 500 ml-Erlenmeyer flask containing 50 g of wheat bran medium (wheat bran 25 g, rice hull 5 g, potato starch 0.5 g, tap water 25 ml : sterilized at 1.2 kg/cm² for 20 min). The inoculated culture of isolated strains were grown at 30°C for 4 days. Crude enzyme was extracted by mixing the culture of wheat bran medium with 150 ml of tap water and keeping for 5 hr at 4°C. After centrifugation at 9,000 rpm for 20 min, glucoamylase activity, raw-starch-digestibility and raw-starch-adsorbability of the crude enzyme were measured.

Amylase production

Enzyme production was carried out with wheat bran medium (wheat bran 75 g, rice hull 15 g, potato starch 1.5 g, tap water 75 ml ; sterilized at 1.2 kg/cm² for 20 min) in a 21-Erlenmeyer flask. The inoculated culture of *Amylomyces* was grown at 30°C for 4 days. Extraction of the enzyme was carried out by mixing the culture of wheat bran medium with 450 ml of tap water and keeping for 5 hr at 4°C. After filtration with 2 layer of gauze, the crude filtrate was used for enzyme purification.

Assay of amylase activities

In order to determine the amylase activity 1 ml of the enzyme solution was incubated with 5 ml of 1% potato starch solution, 1 ml of 0.1 M McIlvaine buffer, pH 3.8 and 1 ml of deionized water at 50°C. At a suitable time interval, 1 ml of the reaction mixture was taken and added into 0.01 N iodine solution. The change in color was observed, and the time required for the erythro (turning red with iodine) point was determined. One unit of α -amylase activity was defined as that which converted starch to the erythro point in 100 min under the above condition. To determine glucoamylase activity, 1 ml of the reaction mixture as above was taken after incubation for 10 min. and the reducing sugar formed were determined by the micro-Bertrand method (Klein, 1932) and the degree of hydrolysis was calculated as the percentage of theoretical

glucose for the hydrolysis curve. One unit of glucoamylase activity was defined as 1 mg of glucose produced in 1 ml of the above reaction mixture in 100 min under the above conditions.

Determination of raw starch digestion

A reaction mixture, which contained 0.3 g of raw corn starch, 36 ml of deionized water, 6 ml of 0.1 M citrate buffer of pH 3.6 and 6 ml of culture filtrate containing an equivalent of 15 unit of glucoamylase activity and 1 ml of toluene, was incubated at 30°C. At suitable intervals, reducing sugar formed in 1 ml of the reaction mixture was determined by the micro-Bertrand method (Klein, 1932) and the degree of hydrolysis was calculated.

Determination of raw starch adsorbability

A desired amount of the culture filtrate was added to 0.02 M citrate buffer, pH 3.6, to prepare an enzyme solution with an equivalent of 10 unit of glucoamylase activity. One gram of raw corn starch was added to 5 ml of the prepared enzyme solution and left to stand at 4°C for 15 min. After centrifugation, the glucoamylase activity of the supernatant fluid was assayed, and compared with that of the original amylase solution. The adsorption rate (AR) is defined according to the equation : $AR (\%) = ((B) - (A)) / (B) \times 100$. Where (A) indicates the residual glucoamylase activity after the adsorption test and (B) that of the original enzyme solution.

Molecular weight determination

The molecular weight of the enzyme protein was estimated with SDS-PAGE according to the method of Weber and Osborn (1969).

Protein determination

Protein content was determined by measuring its absorbance at 280 nm or by the method of Lowry (1951) with bovine serum albumin as a standard.

Isoelectric point determination

Isoelectric point was determined by chromatofocusing according to the method of Pharmacia Co., Ltd., (1983).

Purification of glucoamylase produced by *Amylomyces* sp. 4-2 The procedure for glucoamylase purification from crude enzyme was described as follows. All operations were carried at 4°C unless otherwise stated.

Step 1. Ammonium sulfate was added to the culture filtrate at a ratio of 60 g to 100 ml and it was kept overnight. The resulting precipitate was collected by centrifugation at 8,000 rpm for 20 min.

Step 2. Acid treatment The precipitate collected was dissolved in a minimal volume of water, and then adjusted to pH 2.6 with 1 N HCl and then kept at 4°C for 48 hr. After removal of the precipitate by centrifugation, the supernatant was adjusted to pH 5.0 with 1 N NaOH, and ammonium sulfate was added to the solution at a ratio of 20 g to 100 ml. The precipitate was discarded after being left for 24 hr at 4°C and an additional amount of ammonium sulfate was added at a ratio of another 25 g to the

original 100 ml. The solution was kept overnight and the precipitate was collected. This step was used to inactivate α -amylase.

Step 3. The precipitate was desalted with PVA Hollow Fiber Dialyzer (Kuraray Co., Ltd., Osaka, Japan) and lyophilized. The lyophilized sample was designated as crude enzyme.

Step 4. Gel filtration on Sephadex G-50 The crude enzyme was dissolved in a minimal amount of deionized water and applied to a column (5 x 90 cm) of Sephadex G-50 hydrated with deionized water. The column was eluted with the deionized water at a rate of 90 ml/hr. The fractions containing amylolytic activity were collected and lyophilized. This preparation was used for removing color impurities and cellulase.

Step 5. Column chromatography on DEAE-Sephadex A-50 The amylolytic fraction, containing both glucoamylase and α -amylase, was applied to a column (2.5 x 60 cm) of DEAE-Sephadex A-50 previously equilibrated with 0.05 M phosphate buffer, pH 5.5. The column was eluted with the same buffer at a rate of 70 ml/hr. The amylolytic-activity fractions were collected, dialyzed against deionized water, and lyophilized.

Step 6. Column chromatography on CM-Sephadex C-50 The sample from step 5 was dissolved in a minimal amount of 0.05 M phosphate buffer, pH 5.5 and applied to a column (2.5 x 60 cm) of CM-Sephadex C-50 previously equilibrated with the same buffer. The column was washed with a sufficient volume of the buffer at a rate of 40 ml/hr, followed by 400 ml of a linear gradient between 0 and 0.5 M NaCl in 0.05 M phosphate buffer, pH 5.5. The fractions containing glucoamylase and α -amylase activity were collected separately, dialyzed against deionized water overnight and lyophilized.

Step 7. Gel filtration on Bio-Gel P-ZOO The glucoamylase fraction from step 6 was applied to a column (1.8 x 150 cm) of Bio-Gel P-200 previously equilibrated with 0.05 M Tris-HCl buffer, pH 7.0 and eluted with the same buffer at a rate of 10 ml/hr. The fractions containing glucoamylase activity were collected, dialyzed against deionized water overnight, and lyophilized. The lyophilized sample obtained was designated as purified glucoamylase.

RESULTS AND DISCUSSION

Isolation and selection of glucoamylase-producing strains Thirty two fungi were isolated from eight samples of Loogpang Kaomag. Of these, eighteen isolates were identified as *Amylomyces* sp. according to Hesseltine and Ellis et al. (1965, 1966, 1969, 1973, 1976) and Ogasawara (1982). Among these, *Amylomyces* sp. 4-2 produced a little lower glucoamylase activity (32.5 units) than RA and SG strains (38 units) but its enzyme showed significantly higher raw-starch-digestibility (73.6%) and raw-starch-adsorbability (44.0%) onto raw starch than those of other strains (Table 1.). Thus strain 4-2 was selected for enzyme production and purification. This strain showed the following characteristics: colonies growing rapidly within 7 days at room temperature on potato dextrose agar medium with white mycelia: no rhizoid observed during growth; sporangiophores hyaline to light brown with yellowish content; sporangia abortive, globose, hyaline to brown, with enlarged apophysis; sporangiospores, globose, oval, ellipsoidal to irregular shaped; chlamydospores abundantly produced in

Table 1. Comparison of glucoamylase activity of culture filtrates from isolated strains of *Amylomyces* sp.^a.

Strain	Glucoamylase activity (units/ml)	Raw starch digestion (%)	Raw starch adsorption (%)
RA 1	40.0	13.7	10.2
RA 2	39.2	15.3	12.5
SR 1	32.5	9.9	14.7
SR 2	31.4	11.5	14.0
SG 1	37.5	12.2	31.9
SG 2	37.5	12.2	30.0
4 - 2	32.5	73.6	44.0
5 - 1	11.0	42.7	44.0
5 - 2	10.4	50.2	35.0

^a Isolated strains were cultivated in wheat bran medium at 30°C for 4 days.

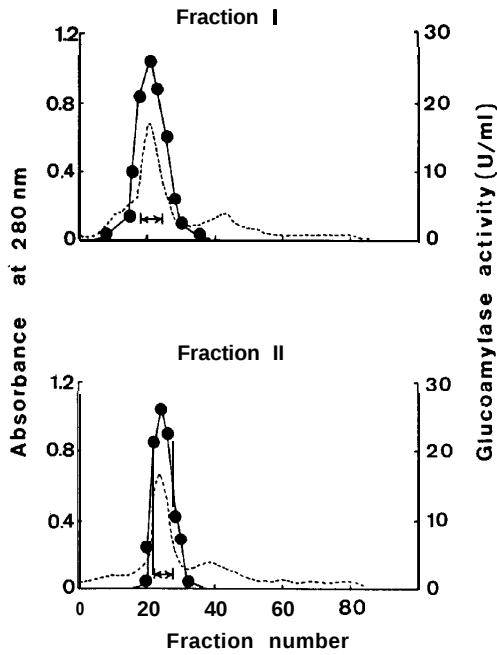


Fig. 3. Bio-Gel P-200 gel filtration of glucoamylases from peak I and peak II. Fractions of peaks I and II from CM-Sephadex C-50 column were applied onto a Bio-Gel P-200 column (1.8 x 150 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.0. Elution was carried out at a flow rate of 10 ml/hr with the same buffer. Three gram fractions were collected. Symbols : (.....), protein ; (●), activity ; (←→), collected fractions.

the aerial and substrate hyphae, hyaline to light brown, thick walled, globose, oval, ellipsoidal with granular contents, single and in series.

Purification of glucoamylase

When the sample from DEAE-Sephadex A-50 column was applied onto CM-Sephadex C-50 column chromatography, two peaks (peak I and peak II) of glucoamylases were obtained. Fractions of peaks I and II were separately collected and applied to Bio-Gel P-200 gel filtration and the result was shown in Fig. 3. In the peaks of the eluted fractions of both enzymes, there was a coincidence of protein concentration and glucoamylase activity. The purified enzyme from peak I (fraction.

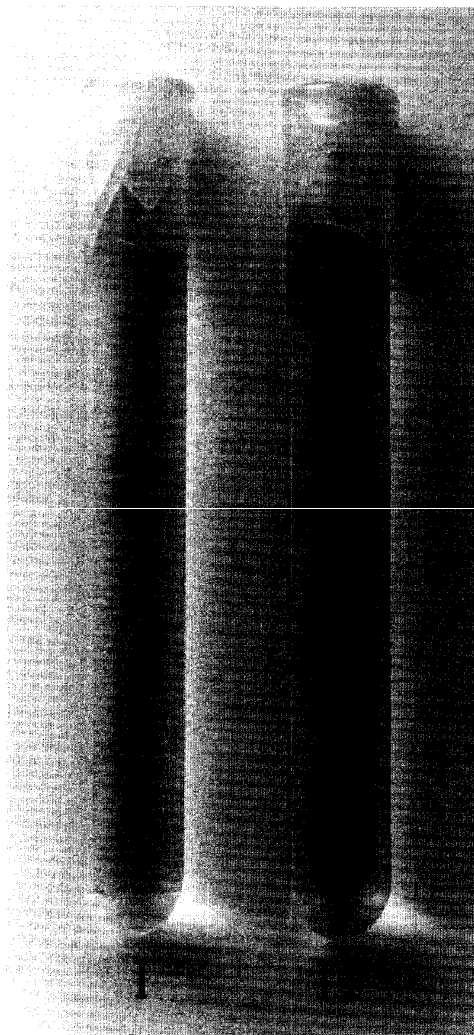


Fig. 4. Polyacrylamide disc gel electrophoresis patterns of the purified glucoamylases I and II. Electrophoresis in 7.5% polyacrylamide gel at pH 8.3 in Tris-glycine buffer was performed with a current flow of 4 mA per tube. Symbols : I, glucoamylase I ; II, glucoamylase II.

Table 2. Purification scheme for *Amylomyces* sp. 4-2 glucoamylase.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
Crude extract	62,800	189,000	3.0	100
60% (NH ₄) ₂ SO ₄ fractionation	20,700	128,000	6.2	68.1
Acid treatment	12,800	94,500	7.4	50.1
25-45% (NH ₄) ₂ SO ₄ fractionation	6,030	58,000	9.6	30.7
DEAE-Sephadex A-50	1,360	19,000	14.5	10.4
CM-Sephadex C-50				
glucoamylase I	241	5,470	22.7	2.9
glucoamylase II	289	7,920	27.4	4.2
Bio-Gel P-200				
glucoamylase I	147	3,770	25.6	2.0
glucoamylase II	215	6,600	30.7	3.5

No. 18-25) was designated as glucoamylase I, and that from peak II (fraction. No. 22-28) as glucoamylase II. Both enzymes displayed homogeneity in polyacrylamide disc gel electrophoresis indicating the presence of a single band protein as shown in Fig. 4.

The purification steps were summarized in Table 2. Final specific activities of glucoamylase I and II were about 25.6 and 30.7 unit/mg protein, respectively, with 5.5% total yield.

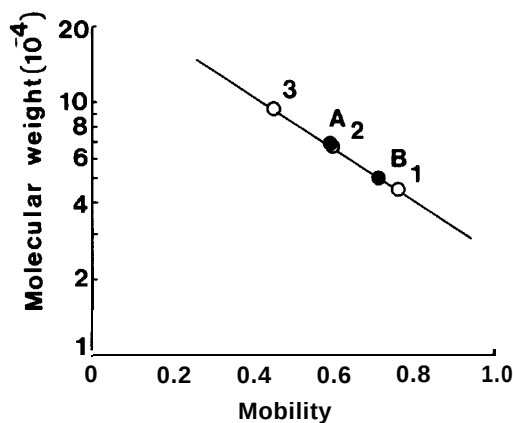


Fig. 5. Estimation of molecular weights of the glucoamylases by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Symbols (molecular weights of standard proteins) : 1, ovalbumin (45,000) ; 2, bovine serum albumin (68,000) ; 3, phosphorylase (94,000) ; A, glucoamylase I ; B, glucoamylase II. Experimental details are described in the text.

Properties of glucoamylase

The molecular weight of glucoamylases I and II was 68,000 and 50,000, respectively, as determined by SDS-PAGE (Fig. 5). The molecular weight of glucoamylase I was almost the same as that of *Rhizomucor pusillus* (MW 68,000) (Kanlayakrit et al., 1987) and *Rhizopus* sp. (MW 74,000) (Takahashi *et al.*, 1978) but smaller than that of glucoamylase I (MW 90,000) of *Aspergillus awamori* (Hayashida *et al.*, 1975a, 1975b, 1985). In the case of glucoamylase II, the molecular weight was smaller than that of *Rhizopus* sp. and *Aspergillus awamori*.

Both glucoamylases had optimal temperature and pH on gelatinized potato starch at 55°C and between pH 3.5-5.0, respectively. These enzymes were stable between pH 3.5-6.0 after keeping at 4°C for 24 hr, and up to 50°C in the case of 30 min-incubation. Glucoamylases I and II differed in their molecular weights and isoelectric point. The glucoamylase I, with molecular weight of 68,000, had an isoelectric point at 8.30 whereas the isoelectric point of glucoamylase II with molecular weight of 50,000 was at 8.85.

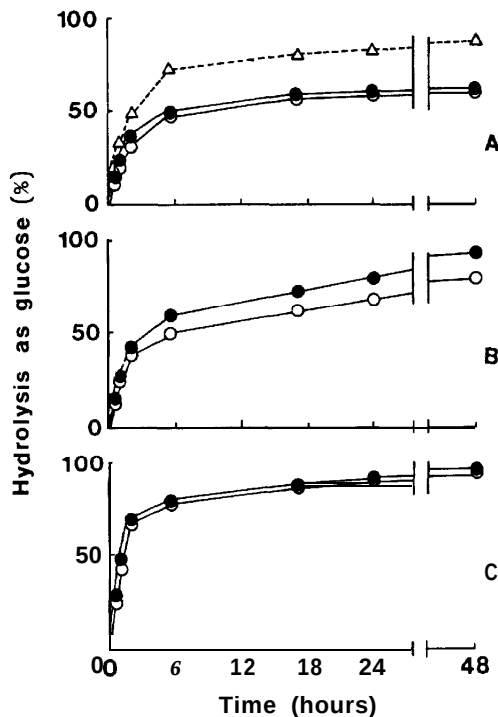


Fig. 6. Hydrolysis curves on gelatinized potato starch (A), glycogen (B) and glutinous rice starch (C) of *Amylomyces* glucoamylase I and II. One ml of each enzyme solution (15 units) was incubated with 100 mg cooked potato starch, glycogen or glutinous rice starch with 7 ml of 0.02 M acetate buffer, pH 4.2 at 40°C. At suitable intervals, reducing sugar formed was determined by the micro-Bertrand method and the hydrolysis degree was calculated. Symbols: (●), glucoamylase I; (○), glucoamylase II of *Amylomyces* sp. 4-2; (---△---), *Aspergillus awamori* glucoamylase I.

Hydrolysis curves of gelatinized substrates

The hydrolysis curves for 1% gelatinized potato starch, glutinous rice starch and glycogen was shown in Fig. 6, which indicated that both glucoamylases I and II hydrolyzed gelatinized glutinous rice starch completely but hydrolyzed gelatinized potato starch to the same extent i. e. at 60%. Glucoamylase I hydrolyzed glycogen completely and was classified as type A (Hayashida *et al.*, 1975a, 1975b, 1985) whereas glucoamylase II could hydrolyze up to 80% and classified as type B. During the incubation of gelatinized substrates, the erythro point was tested and it was found that there was no change in the iodine color, blue in potato and red in glutinous rice starch even after 48 hr of incubation. It indicated that there was no contamination of α -amylase. Increasing the unit of glucoamylase activity did not cause any significant change in the final hydrolysis of the substrates.

The hydrolysis curve of glucoamylase I on glycogen was the same as that of raw-starch-digesting glucoamylase I (type A) from *Aspergillus awamori* (Hayashida, 1975a). The hydrolysis pattern of glucoamylase II, was the same as that of raw-starch-nondigesting glucoamylase I' (type B) of *A. awamori* which hydrolyzed glycogen up to 80%. However, both *Amylomyces* glucoamylases I and II hydrolyzed gelatinized potato starch up to 60%, lower than those of glucoamylase I and I' of *A. awamori* with 90% hydrolysis, but almost the same as that of *A. awamori* glucoamylase II (type C). *Amylomyces* glucoamylase characteristically hydrolyzed gelatinized glutinous rice rather than gelatinized potato starch.

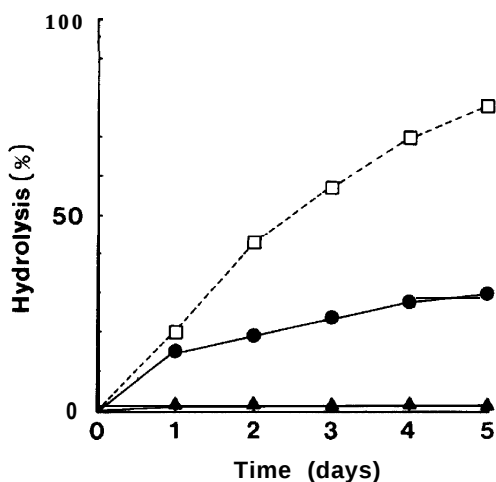


Fig. 7. Raw corn starch digestion by glucoamylase of *Amylomyces* sp. 4-2 compared with *Aspergillus awamori* glucoamylase I. The reaction mixture containing 0.3 g of raw corn starch, 6 ml of each enzyme solution (45 units/ml), 36 ml of deionized water with 1 ml of toluene, was incubated at 30°C. At suitable intervals, reducing sugar formed in 1 ml of the reaction mixture was determined by the micro-Bertrand method and the degree of hydrolysis was calculated. Symbols: (●), glucoamylase I; (▲), glucoamylase II of *Amylomyces* sp. 4-2; (---□---), *Aspergillus awamori* glucoamylase I.

Digestion of raw corn starch

Amylomyces glucoamylase I could digest raw corn starch whereas ***Amylomyces*** glucoamylase II could not (Fig. 7). The optimal pH for raw starch digestion was between 3.5-4.5. Glucoamylase I was adsorbed completely onto raw corn starch at pH 4.0 but glucoamylase II was not adsorbed at all. By using the same unit activity (270 units) of each enzyme, the ***Amylomyces*** glucoamylase I could digest raw corn starch at about 30% whereas ***A. awamori*** glucoamylase I digested it up to 80% for 5 days (Hayashida, 1975a). It was thus suggested that ***A. awamori*** glucoamylase I had about three times higher rate of digestion than that of ***Amylomyces*** glucoamylase I.

As mentioned above ***Amylomyces*** sp. 4-2 produced two types of glucoamylases ; glucoamylase I which digested and was adsorbed onto raw starch was thus designated as raw-starch-digesting glucoamylase I, glucoamylase II could neither digest nor be adsorbed onto raw starch was designated as raw-starch-nondigesting glucoamylase II. The result was different from that of ***A. rouxii*** as reported by Wang et al. (1984) which produced only one form of glucoamylase.

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