Degradation of Raw Starch Granules by α-Amylase Purified from Culture of Aspergillus awamori KT-11

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Received 23 September 2003, Accepted 1 November 2003

Raw-starch-digesting α-amylase (Amyl III) was purified to an electrophoretically pure state from the extract of a koji culture of Aspergillus awamori KT-11 using wheat bran in the medium. The purified Amyl III digested not only soluble starch but also raw corn starch. The major products from the raw starch using Amyl III were maltotriose and maltose, although a small amount of glucose was produced. Amyl III acted on all raw starch granules that it has been tested on. However, it was considered that the action mode of the Amyl III on starch granules was different from that of glucoamylase judging from the observation of granules under a scanning electron microscope before and after enzyme reaction, and also from the reaction products. Glucoamylase (GA I) was also isolated and it was purified to an electrophoretically pure state from the extract. It was found that the electron micrographic features of the granules after treatment with the enzymes were quite different. A synergistic effect of Amyl III and GA I was observed for the digestion of raw starch granules.

Keywords: Aspergillus awamori KT-11, α-amylase, Glucoamylase, Raw starch digestion, Scanning electron microscopy

Introduction

There are a large number of studies on the α-amylases (EC 3.2.1.1) of Aspergillus sp. Two kinds of acid stable α-amylase from Aspergillus kawachii have been reported (Mikami et al., 1987). There has also been reported an acid-stable and an unstable α-amylase from Aspergillus niger (Arai et al., 1968), crystals of Aspergillus oryzae enzyme (Fischer and Montmollin, 1951; Akabori et al., 1954), and some properties of A. awamori α-amylase (Bhella and Altosaar, 1984). A. awamori produces several potent amylolytic enzymes for bioindustry, and the mold also produces three forms of α-glucosidases (Yamazaki et al., 1977) and two types of glucoamylases. These are glucoamylase I that can be adsorbed onto raw starch, and glucoamylase II that can not be adsorbed (Ueda, 1981).

There are several microorganisms that have been found to produce raw starch digesting amylolytic enzymes. A. awamori (Ueda et al., 1974) and A. oryzae (Miah and Ueda, 1977) produced glucoamylases having activity for adsorption and digestion of raw starch. In addition, it has been reported that Rhizopus niveus glucoamylase was stronger than that of Aspergillus sp. (Yamamoto, 1995) for its raw starch degrading activity.

A strain named A. awamori KT-11 is a black mold that produces three kinds of amylolytic enzymes, and they have been identified as α-amylase (Anindyawati et al., 1998a), glucoamylase and α-glucosidase (Anindyawati et al., 1998b), in an extract from koji culture using wheat bran in the medium. We purified Amyl III (α-amylase), and GA I (glucoamylase) from a culture of this strain. Both purified enzymes, GA I and Amyl III, acted on raw starch. In addition, it was found that Amyl III was able to digest various raw starches.
The features of starch granules after treatment with enzymes and the susceptibility of raw starch to enzymes were quite different because of the action mode of each enzyme. In this paper, we describe the action of Amyl III on maltooligosaccharides and on various raw starches as compared with that of GA I.

Materials and Methods

Chemicals Soluble starch for the activity assay was purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). Raw starches for the activity assay and SEM were purchased from Ueda Chemical Co., Ltd. (Nara, Japan), and the starches were washed thoroughly with distilled water before use. Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Microorganism The black mold, which was isolated from the air in Indonesia, was identified as *Aspergillus awamori* from its morphological properties (Japan Food Research Laboratories, report No, OSS0100670, Osaka). The strain is referred to as *A. awamori* KT-11 in this study. The mold was cultivated for enzyme production, and the preparation of enzymes was carried out according to the method described previously (Anindyawati et al., 1998a).

Measurement of enzyme activities and protein concentration The soluble-starch-hydrolyzing activity (for α-amylase or glucoamylase) was carried out by incubating the mixture of enzyme (0.5 ml) and 0.25% of soluble starch (0.5 ml) in 50 mM acetate buffer (pH 4.8) at 37°C for 10 min. The amount of reducing sugar formed was determined by the Somogyi-Nelson method (Somogyi, 1952). One unit of the activity was defined as the amount of enzyme that released 1 µmol of reducing sugar; this was equivalent to glucose per minute under these conditions. α-glucosidase activity was carried out using 0.25% of maltotriitol as substrate under the same conditions as the soluble-starch-hydrolyzing activity. Raw-starch-hydrolyzing activity was assayed by using 1.25% of raw corn starch as a substrate under the same conditions as for the soluble-starch-hydrolyzing activity. The protein concentration was determined by measuring the absorbance at 280 nm on a spectrophotometer U-1100 (Hitachi, Ltd., Hitachi, Japan). Here we assume that the absorbance of a 1% enzyme solution was 10.0.

Preparation of Amyl III and GA I Amyl III was prepared according to the method described previously (Anindyawati et al., 1998a) with a slight modification. Approximate 10 g of a seed culture were inoculated onto a medium consisted of 500 g of wheat bran (Miyake Flour Milling Co., Ltd. Osaka, Japan) and 500 ml of tap water in a pan (32 x 21.5 x 8.5 cm in size, 3 cm in thickness). The pan had been sterilized twice for 20 min at 120°C. The medium and the culture was then incubated at 27°C for 5 days. The amylytic enzymes were extracted with 5 volumes of cold deionized water. The extract was filtered through a filter cloth and the filtrate was centrifuged at 5,000 rpm for 10 min to remove the contaminating spores. The resulting supernatant was further filtered through filter paper (Advantec Toyo, Tokyo, Japan). The filtrate was concentrated using hollow fibers (Amicon, Beverly, USA) as dense as possible and the concentrate was fractionated by precipitation with ammonium sulfate (zero to 0.3 saturation). The precipitate was dissolved in water and centrifuged at 5,000 rpm for 10 min to remove insoluble material. The supernatant was further precipitated with a 0.9 saturation solution of ammonium sulfate. The resulting precipitate was dissolved in deionized water and then concentrated, again using hollow fibers. This concentrate was subjected to chromatography using a DEAE Sephadex A-50, Sephadex G-100, Butyl Toyopearl, Bio-Gel A-0.5 and Superose 12HR10/30. Purified Amyl III showed as single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to the method of Laemmli, UK.

Thin layer chromatography (TLC) of enzymatic hydrolysis products The products from the reaction of soluble starch or raw starch with the enzymes were analyzed by TLC using a silica gel 60 plate (Merck, Darmstadt, Germany) in a solvent system of butanol-pyridine-water (6 : 3 : 1) and the two times development method at 37°C. The migrated products was visualized by heating the plate at 110°C in an oven, after it was dipped in ethanol-sulfuric acid (9 : 1).

Preparation of starch samples for the SEM Preparation of the starch samples before and after the enzymes treatment was as follows. Starch granules were washed with distilled water twice.

![Fig. 1. Hydrolyses of corn starch granules and cooked corn starch with Amyl III from A. awamori KT-11. Raw corn starch granules (A) and cooked corn starch (B) (20, 60 and 100 mg) were incubated with 7.5 mM of Amyl III in 50 mM acetate buffer (pH 4.8) at 37°C. ▲, ○ and □ indicate the substrate concentrations of 1%, 3% and 5%, respectively.](Image)
The washed starch granules were dehydrated by ethanol and then they were added to tert-butanol and freeze-dried. The samples (dry starch powder) were used for the observation under the SEM.  

**Scanning electron microscopy** Starch samples were mounted on SEM stubs with double-sided adhesive tape and the samples were coated with gold. Scanning electron micrographs were taken using

![Image](98x495 to 495x686)

*Fig. 2.* Incubation period dependent degradation of corn starch granules by hydrolyses with Amyl III from *A. awamori* KT-11. Raw corn starch granules (100 mg) were incubated with 7.5 mU of Amyl III in 50 mM acetate buffer (pH 4.8) at 37°C for (A) 2 h; (B) 8 h; (C) 24 h; (D) 48 h; and (E) 48 h without enzyme.

![Image](151x148 to 441x437)

*Fig. 3.* Identification of digests from various starches with Amyl III. Various starch granules (37.5 mg) from sago, waxy maize, sweet potato, tapioca, potato, rice and wheat were incubated with 5.6 mU of Amyl III in 50 mM acetate buffer (pH 4.8) for 2 h (lane 2), 8 h (lane 3), 24 h (lane 4), 48 h (lane 5) and without Amyl III for 24 h (lane 1). Each cooked starch (37.5 mg) was incubated with 5.6 mU of Amyl III in 50 mM acetate buffer (pH 4.8) for 2 h (lane 7), 8 h (lane 8), 24 h (lane 9), 48 h (lane 10) and without Amyl III for 24 h (lane 6). The details of each reaction conditions were described in the text. M, markers: G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose.
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Results and Discussion

Effect of the concentration of raw and cooked corn starch on Amyl III activity

The action profile of Amyl III with the substrate concentration was determined by incubating the enzyme solution with 1%, 3% and 5% raw and cooked corn starch in 50 mM acetate buffer (pH 4.8) at 37°C. Then, the amount of reducing sugar released was measured by the Somogyi-Nelson method. As shown in Fig. 1 (A), the reducing sugar released from the raw corn starch could not be detected until 24 h later. After this time, the amount of reducing sugar formed increased rapidly. The reducing sugar released increased with the amount of substrate. On the other hand, the reducing sugar released from cooked corn starch was observed immediately after the reaction has started, as shown in Fig. 1 (B). The high degradation rate of cooked corn starch was observed in the early stage of the enzyme reaction, although the rate decreased thereafter to constant rate. The products were maltotriose and maltose in both cases. But the products from raw corn starch contained a significant amount of glucose compared to the cooked corn starch. Maltotetraose was detected in the products from the cooked corn starch at the early stage of the reaction, but it was not detected in the hydrolysis reaction carried out with the corn starch.

The corn starch granules before and after digestion by Amyl III was observed using SEM as shown in Fig. 2. A number of holes was observed on the surface of granules acted by Amyl III, and the holes became deep and wide. These results indicated the existence of specific sites where Amyl III can adsorb and hydrolyze. Amyl III initially adsorbed onto the surface of granule and it degraded the
substrate for between 0 to 24 hour, then, the area of the surface of holes was increased. The case of the digestion of corn starch is explained by the fact that the amount of reducing sugar released from the granules was small until 24 hour, and then it increased rapidly after that. Amyl III molecules adsorbed onto the surface of holes and it degraded the inside of granules. When an excess of Amyl III was applied, the substrate degradation was exponentially increased and a prominent amount of the reducing sugar was released. Consequently, the concept of the usual substrate concentration was not applicable in the case of raw starch degradation, and it is thought that the surface area of a cave which was formed inside the starch granule is proportional to the substrate concentration.

Degradation of various starches by Amyl III

The degrading activity of Amyl III were assayed by incubating the mixture of enzyme and various raw or cooked starches in 50 mM acetate buffer (pH 4.8) at 37°C. Identification of the products was performed using TLC as shown in Fig. 3. The differences are remarkable in both the form and size of sugars with the different origins of starch. Amyl III acted on cooked corn starch to produce maltotriose, maltose and a small amount of glucose. The amount of oligosaccharides produced from potato starch was small when compared with the other different origins of starch. On the other hand, there were some differences in the production of oligosaccharides from raw starches. Maltose and maltotriose were detected as the main products from the waxy maize and wheat starches; however, the amount of products from sago and tapioca were very small and the products from sweet potato, potato and rice were almost nonexistent.

The digestion of various starch granules by Amyl III after 48 hour was observed using the SEM as shown in Fig. 4. The surfaces of waxy maize and wheat starch granules were changed after digestion with Amyl III. Some large holes appeared under observation with the SEM. With the starch granules of wheat and sago, the position where Amyl III acted was significantly changed. It was found that the structure around the granules surface is not uniform. Amyl III acted weakly on the surface of sweet potato, potato and rice starch granules, and so TLC did not detect the hydrolyzed products from these starches. These starches seemed to be degraded very slowly.

Although Amyl III acted on all types of tested starches, the features of these starches are widely different. The starch granules can be classified into two groups according to the digestion features seen with Amyl III. Sago, waxy maize, rice and corn starches constitute the group in which the holes were dug inside from a grain surface by Amyl III. Sweet potato, tapioca, and potato starches constitute the other group in which only a few hollows were observed, indicating that Amyl III acted mainly on the surface of starch granules. According to this classification, the former group represents starch from a seed origin, and the latter group represents starch from an edible root origin. Thus, we demonstrated that the action of Amyl III changes greatly with the origin of the starch, and it was presumed that the structure of the surface of starch was different from one origin to another.

Degradation of raw corn starch by Amyl III, GA I and Amyl III plus GA I

The degrading activity of Amyl III or GA I or both together were determined by incubating raw corn starch with enzyme in 50 mM acetate buffer (pH 4.8) at 37°C. Incubation period were 8, 24, 48, 72 h from left to right for each Amyl III, GA I, a mixture of Amyl III and GA I, and with out any enzyme. The experimental procedures were described in material and methods. M, markers: G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose.
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37°C, and then the amount of reducing sugar released was measured by the Somogyi-Nelson method as shown in Fig. 5. When Amyl III plus GA I was used for digestion of a starch granule, the amount of reducing sugar released increased remarkably, and the amount was 5 times higher than in the case of Amyl III alone, and 17 times higher than in the case of GA I alone. The products were identified by TLC as shown in Fig. 6. When Amyl III acted on raw starch, the products were the oligosaccharides G2 and G3. On the other hand, when GA I acted on raw starch, the reaction product was only G1. When Amyl III plus GA I acted on raw starch, reaction products were the comparatively bigger oligosaccharides and G2.

The corn starch granules after 72 h that were digested by Amyl III and GA I were observed under SEM, as shown in Fig. 7. Amyl III attacked the starch granules to form some big holes, while GA I formed some small holes on the surface of starch granules. It seems that GA I gives many holes on the surface area as compared with those of Amyl III. Glucoamylase acts on the $\alpha$-1, 4 glucosidic linkage to produce glucose. The surface of the starch granule is composed of non-reducing ends, and since there are few reducing ends inside the granule, it is considered that decomposition by GA I occurs only on the surface, without any decomposing inside. Amyl III, together with GA I, attacked by turns the granules in making some big holes and causing the breakdown of the granules after a long incubation. There is no important difference here in the decomposition manner as compared with the starch granule on which only Amyl III acted. However, when Amyl III and GA I were used together, it causes more holes per area, and the size of the hole is expanded further. The starch granule decomposed by GA I has many holes in the surface as compared with the starch granule decomposed by Amyl III. It is thought that GA I initially acted on the starch granule surface, and Amyl III

![Fig. 7. Microscopic observation of corn starch granules hydrolyzed with Amyl III, GA I and a mixture of both enzymes.](image-url)
adsorbed around the hole, and then the decomposition of starch granule extended further from the initial attachment points. G1, G2 and comparatively larger oligosaccharides were released as reaction products. The products differ from that released by GA I only.

Amyl III acts on the α-1, 4 glycosidic linkage of the inner granule and it released oligosaccharides. On the other hand, GA I recognizes a non-reducing end, and it released glucose. When Amyl III and GA I were used together for the digestion of the starch granule, it was thought that Amyl III plays the role of digestion to produce relatively larger oligosaccharide, and GA I acts for the release of rather smaller oligosaccharide. Maltose, released as a reaction product, is still produced in the later stage, even after a 72-hour incubation. The amount of reducing sugar is also increasing at a fixed rate. The release of comparatively large oligosaccharide was observed in TLC. These results indicated that the release of oligosaccharide is caused in several successive steps. Amyl III decomposed starch granules into G2 and G3, and GA I acted easily on G3 to decompose it into G1.

Conclusion

Amyl III (α-amylase) and GA I (glucoamylase) were purified to an electrophoretically pure state from the culture of Aspergillus awamori KT-11. Amyl III decomposed all starch granules that have been tested, and it generated mainly G1 and G2 as reaction products, and the action mechanisms of Amyl III depend on the type of starch granules. When Amyl III was used together with GA I for degradation of corn starch, the decomposition activity increased remarkably because of the synergistic effect of both enzymes.

References


