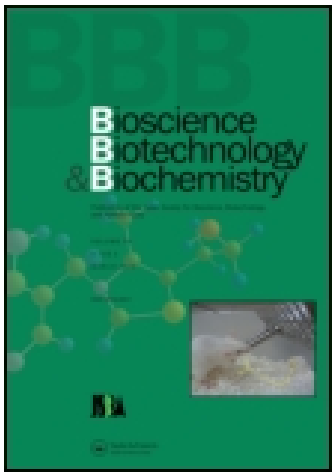

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Distinguishable Action between Acid-stable and Neutral α-Amylases from Shochu Koji (Aspergillus kawachii)

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Short Communication

Distinguishable Action between Acid-stable and Neutral α-Amylases from *Shochu Koji* (*Aspergillus kawachii*)

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Acid-stable (KAA) and neutral (KNA) α-amylases from *shochu koji* (*A. kawachii*) were purified and their actions towards maltooligosaccharides were studied. KAA could be distinguished from KNA by the following actions: with KAA, maltopentaose (G5) was preferentially hydrolyzed at the third glycoside bond, and the addition of potassium thiocyanate (KSCN) decreased the rate of CNP-release from 2-chloro-4-nitrophenyl-α-maltotrioside (CNP-G3).

Key words: acid-stable α-amylase; 2-chloro-4-nitrophenyl-α-maltotrioside; *Aspergillus kawachii*; cleavage pattern; maltooligosaccharide

In Kagoshima, sweet potato *shochu* (an alcoholic beverage) is produced by a *koji* (starter culture) of *Aspergillus kawachii*. The fermentation is under acidic conditions. Aside from glucoamylase (GA), the black *Aspergillus* group is known to produce two types of α-amylase, *i.e.*, the acid-stable (AA) and the common neutral (NA).[1,2] The three-dimensional structure and amino acid sequences of AA have been reported.[3,4] Few studies, however, have been done on its action towards maltooligosaccharides. In this study, two types of α-amylases, acid-stable (KAA) and neutral (KNA), from *Aspergillus kawachii* were purified and some of their properties were studied, especially, their action patterns towards maltooligosaccharides.

Seed-koji was purchased from Kawachi Gen-ichiro Shoten Co. KAA was extracted from wheat bran koji (90 g) of solid culture after 48 h at 35°C, with 10 volumes of 50 mM acetate buffer, pH 5.0, containing 1% NaCl. The enzyme solution, after saturation with ammonium sulfate to 25%, was then put on to a butyl-Toyopearl column. The column was washed with 25% saturated ammonium sulfate solution in 10 mM acetate buffer, pH 5.0, and then eluted by the same buffer without ammonium sulfate. Most of the GA could be removed by this chromatography. The pooled α-amylase fractions were dialyzed and chromatographed on a DEAE-Toyopearl 650M column with a gradient of 0–0.2 M NaCl in the same buffer. Two fractions of acid-stable α-amylase (KAA-1, KAA-2) and one fraction of neutral α-amylase (KNA) were obtained, each of which was further purified by a Superose 12 HR 10/30 packed column (Pharmacia Biotech). Samples of KAA-1 (39 U/mg), KAA-2 (37 U/mg), and KNA (147 U/mg) were obtained.

Activities of KAA, KNA, and GA were assayed discriminately by a method based on the difference in their pH stability[1]; preincubation at pH 3.5 (37°C, 3 h) inactivates KNA alone without inactivation of other amylase activities. The enzyme reactions were done in 50 mM acetate buffer, pH 5.0 at 30°C, using 0.5% soluble starch (Katayama Chemical) as substrate. The activities of total amylase and GA wee measured based on the amounts of reducing sugar and glucose produced,[5] by the Somogyi–Nelson method[6] and glucose-oxidase method,[7] respectively. Thus, individual activities of KNA and KAA were calculated from α-amylase activities with and without a first incubation under these acidic conditions. One amylase unit was defined as the amount of enzyme that can produce 1 μmol of product per min under the given conditions. Protein was measured colorimetrically, using bovine serum albumin as a standard.[8]

The 48-h koji produced about 200 units/g of amylase activity. The relative activities of amylases in the extract were 1 for KAA, 5.7 for KNA, and 4.4 for GA at pH 5.0. Each preparation of KAA-1, KAA-2, and KNA obtained had a single band on SDS–PAGE, and was free from α-glucosidase when PNP-α-glucoside was used as a substrate. The contamination of KAA preparations with GA was estimated to be less than 1% from the amount of glucose produced, using soluble starch as substrate, since α-amylase produces virtually no glucose upon the digestion of soluble starch.

The molecular weights (M_r) of KAA-1 and KAA-2 were estimated to be 70,000 and 97,000, respectively, by SDS–PAGE, and 67,000 and 110,000 by gel filtration of HPLC (Shodex AF-102). The results indicate KAA to be a monomeric enzyme. The M_r of KNA was 57,000 by SDS. Our result of two forms of AA are in fair agreement with that reported by S. Mikami *et al.*[9]

KAA-1 and KAA-2 from shochu-koji retained the full activity during incubation under acidic conditions at pH 3.5 for 3 h (at 37°C), while KNA was inactivated to less than 5% of the origianl activity. KAA-1 and KAA-2 demonstrated the optimum activity around pH 4.5 in 50 mM citrate buffer (at 37°C), while KNA had the activity at pH 5.0 (at 37°C). The optimum temperatures of KAA-1 and KAA-2 were around 65°C in 50 mM acetate buffer (at pH 4.0), while that of KNA around 50°C in the same buffer (at pH 5.4). The properties of KNA were similar to a commercial neutral type of Taka-amylase A (Sigma Co., type X-A).

To investigate the cleavage pattern of a maltooligosaccharide, the cleavage was estimated from the anomeric configuration of the products by HPLC,[10] using a YMC AQ-304 column (YMC, Kyoto). The column is known to separate anomers of products larger than maltose.[11] Figure

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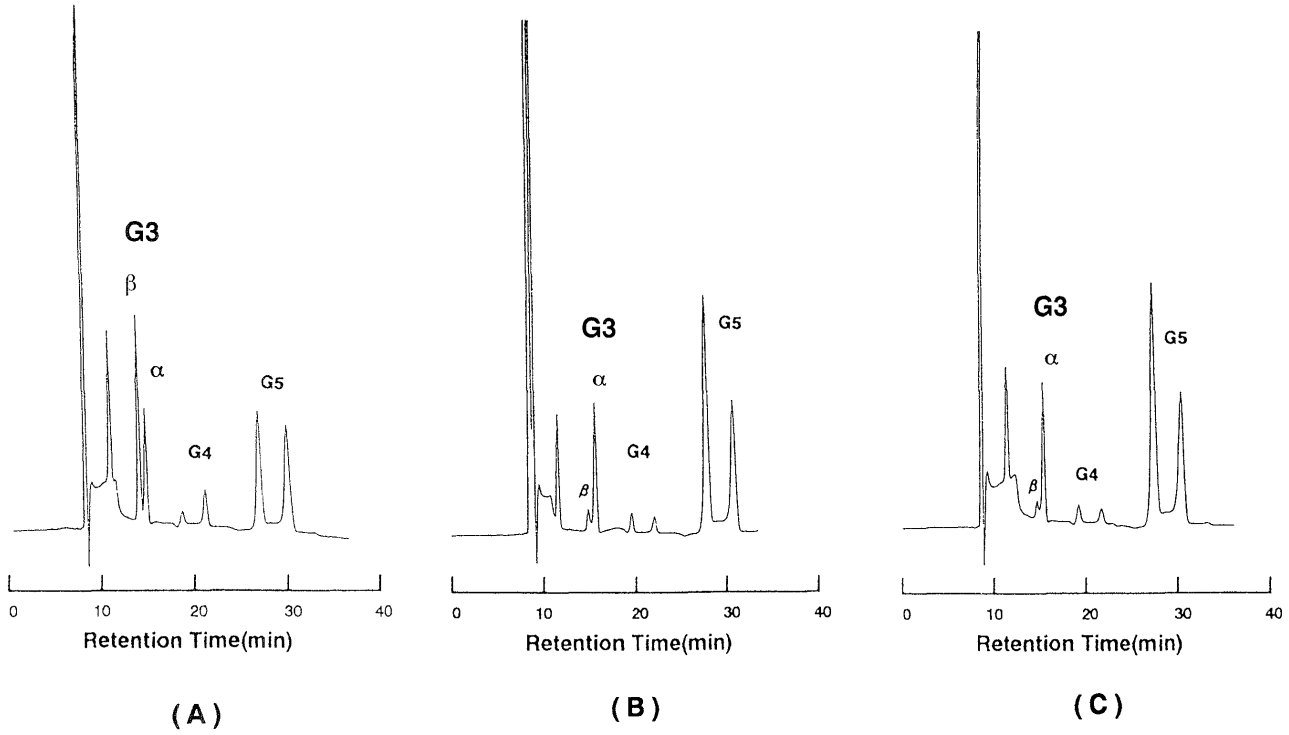

Fig. 1. Anomeric Configuration Analysis of Products to Identify the Predominant Cleavage Points in G5 by KNA (A), KAA-1 (B), and KAA-2 (C).
The reactions were done in 20 mM acetate buffer, pH 4.0, for KAA and pH 5.4 for KNA, at 37°C. [E], 0.3 U/ml; [S], 2 mM. HPLC conditions: column, YMC AQ-304; eluent, 0.04% NaN_3 in distilled water; flow rate, 0.4 ml/min; Detection, a RI detector.

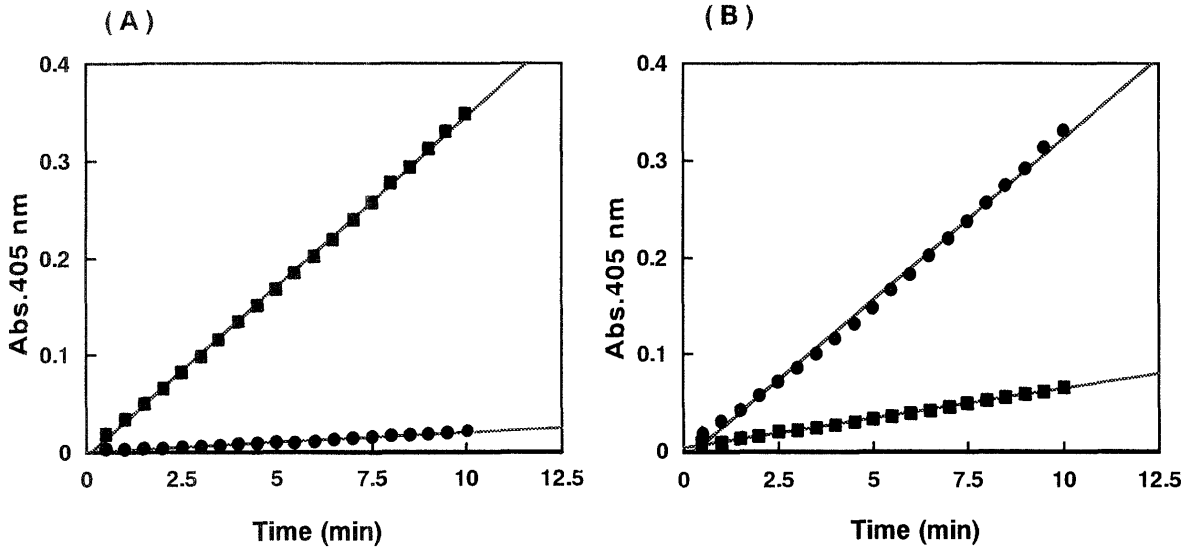

Fig. 2. Courses of the CNP-release from CNP-G3 by KNA (A) and KAA (B) in the Presence of 500 mM KSCN (—■—) and in the Absence (—●—).
The reactions of KNA (0.07 U/ml) and KAA-1 (0.04 U/ml) were done with 1 mM CNP-G3 in 20 mM acetate buffer, pH 5.4, at 37°C.

1 shows that KAA-1 and KAA-2 produced preferentially the α-anomer of maltoriose (G3) from maltopentaose (G5), while KNA produced an equilibrated mixture of α- and β-anomers of G3. This indicates that KAA hydrolyzes G5 preferentially at the third glycoside bond from the nonreducing end, while KNA splits the second glycoside bond. Thus, the cleavage pattern of KAA towards G5 was quite different from KNA, which was the same as those of AA and NA from citric acid-koji (*A. usamii* var.).[10] With maltohexaose (G6) and maltoheptaose (G7), the ratio of anomeric products were a little different between KAA and KNA, but less critical than with G5.

A new substrate CNP-G3 has been developed for use in the clinical assay of human α-amylase.[12] Its degradation pathway and the effect of KSCN addition of increasing on CNP-release have been investigated in the reaction of human salivary α-amylase.[13] Liberation of CNP from 1 mM CNP-G3 (Oriental Yeast) was continuously monitored at 405 nm with a UV recording spectrophotometer (Shimadzu UV265) under temperature-controlled conditions (37°C). Courses of CNP released from 1 mM CNP-G3 by both α-amylases, KAA and KNA, are shown in Fig. 2. The synthetic substrate was a good substrate for the KAA assay, since the rate of CNP-release was comparable to that of the degradation of soluble starch (about 70% with KAA). By the addition of 500 mM KSCN the rates with both forms of KAA were decreased to a fifth, while the rate with KNA was increased prominently (18-fold). Taka-amylase A was

affected by KSCN just like KNA. The two forms of KAA demonstrated the same action pattern, although they differ in molecular weight.

The results of this study have shown that KAA, aside from the difference in its stability at low pHs, could be distinguished from KNA by the following actions towards maltooligosaccharides: i) KAA cleaves preferentially the third glucosidic bond of G5 to give a formation of α-anomer of G3, while KNA cleaves the second bond to give an equilibrated mixture of α- and β-anomers of G3, ii) With KAA, KSCN addition depressed the rate of CNP-release from CNP-G3, while with KNA the addition increased the rate prominently. The latter action ii) has also been observed in the reactions of AA and NA from citric-acid koji (*A. usamii* var., unpublished results). It is possible to determine discriminately the activities of AA and NA in the crude extract based on the action. The preliminary result from this method was 1 and 5. 1 for the relative activities of KAA and KNA, respectively, in the koji extract, which was in good agreement with the result as described above. A moe detailed study on the action of AA and NA towards maltooligosaccharides will be reported elsewhere.

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