Short Communication

N-Terminal Sequence of Amino Acids and Some Properties of an Acid-stable α-Amylase from Citric Acid-Koji (Aspergillus usamii var.)

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An acid-stable α-amylase (AA) was purified from an acidic extract of citric acid-koji (A. usamii var.). The N-terminal sequence of the first 20 amino acids of the enzyme was identical with that of AA from A. niger, but the two enzymes differed in molecular weight. HPLC analysis for identifying the anomers of products indicated that the AA hydrolyzed maltopentaose (G5) at the third glycosidic bond predominantly, which differed from Taka-amylase A and the neutral α-amylase (NA) from the citric acid-koji.

Key words: acid-stable α-amylase; N-terminal sequence; Aspergillus usamii; citric acid-koji; maltopentaose

In Kagoshima, citric acid was produced on a commercial basis by a koji of a variety of Aspergillus usamii,11 using waste pulp of sweet potatoes from starch production factories. The activity of AA besides glucoamylase (GA) was found in the acidic extract (pH 2.5) of the koji after 7-d culture.2 The Aspergilli are known to produce two types of α-amylase, i.e., AA in addition to the common NA.4 In this study, we purified AA from the acidic extract of the citric acid-koji, and studied the N-terminal sequence of amino acids and some enzymatic properties, especially the cleavage pattern of maltopentaose (G5), by comparison with the NA from the citric acid-koji.

Samples of citric acid-koji were kindly donated by Satsuma Kako Co. The AA was extracted with 5 volumes of chilled water from the koji after 50 h of solid culture, and the extract was adjusted to pH 4.0 and the CaCl2 content to 1 mM. After concentrating the solution by dialysis against saturated ammonium sulfate solution, the active fraction was passed through a Sephadex G-100 column previously equilibrated with 10 mM citrate buffer, pH 6.0. The pooled amylase fractions were chromatographed on a DEAE-Toyopearl 650M column with a gradient of 0.1–0.3 M NaCl in the same buffer. The acid-stable α-amylase was separated successfully from GA and NA on this chromatography.

The respective activities of AA, NA, and GA were assayed discriminately, based on the difference in the pH stability; a pre-incubation at pH 4.0 (37 C. 90 min) inactives NA alone without inactivation of other amylase activities. The enzyme reactions were done in 50 mM acetate buffer, pH 4.0, at 30 C, using 0.5% soluble starch as a substrate. The activities of the total amylase and GA were measured based on the amounts of reducing sugar and glucose produced57 by the Somogyi–Nelson method58 and glucose-oxidase method,7 respectively. Thus, individual activities of NA and AA were calculated from α-amylase activities with and without pre-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.59 N-Terminal analysis was done using a protein sequencer (Applied Biosystems, Procise Model 492). HPLC analysis was done to identify the anomeric configuration of products in the digests, using a YMC AQ-304 column60 and a monitor of differential refractometer. For this purpose, a short time of analysis is required to avoid mutarotation of the products; the reaction mixture passed through a molecular cut membrane (Millipore Molecut UF/10 TGC) was injected within 3 min of the reaction.

The 50-h citric acid-koji produced 4.5 units of amylase activity per gram. The relative activities of amylases in the extract were assayed to be 12.5% of AA, 9.5% of NA, and 78% of GA at pH 4.0. The preparation of AA with a specific activity of 19 units per mg had a single band on SDS-PAGE (Fig. 1), and was free from the common NA.

Fig. 1. SDS PAGE of the Purified Acid-stable α-Amylase (AA) from Citric Acid-koji.
The arrows indicate the migrations of standard proteins: 97000, phosphorylase b; 66000, bovine serum albumin; 45000, ovalbumin; 21000, trypsin inhibitor; 14400, lysozyme.

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The contamination with GA was estimated to be less than 2% from the amount of glucose produced, since z-amylase produces virtually no glucose with the digestion of soluble starch. The sugar content was 15% by the phenol-sulfuric acid method, using glucose as a standard. 110 The molecular weight of the enzyme was estimated to be 99,000 and 110,000 by SDS-PAGE (Fig. 1) and by gel filtration of HPLC (Shodex AF-102, data not shown), respectively, which indicates a monomeric enzyme.

The N-terminal sequence of the first 20 amino acids of the enzyme was identical with that of AA from A. niger111 (Table), but its molecular weight differed distinctly from that (Mw 54,000) of AA from A. niger. A high molecular weight of AA has been reported in the A. kawachii,121 but the sequence has not been reported. Based on the result of Boel et al.111 the whole peptide sequence of AA from A. niger was calculated to be about 65% the same as that of Taka-amylnase A, a typical NA from the Aspergillus, and contained the conserved peptide regions common to the amylase family.13,14 The AA from citric acid-koji had its optimum activity at a pH of 3.55 (at 37°C), and a temperature of 60-70°C (at pH 4.0). It is noted that the AA has a higher thermal stability than commercial Taka-amylnase A (Sigma Co., type X-A).

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<th>Table</th>
<th>The N-Terminal Sequence of Amino Acids and G5-Cleavage of AA and NA from Citric Acid-koji (A. usami var.)</th>
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Italic characters represent different amino acids between AA and NA. Bold character, G, denotes the glucose residue at the reducing end.

HPLC analysis showed that the AA hydrolyzed soluble starch to exclusively produce the z-anomers of maltodextrin (data not shown). The Km for soluble starch was 0.044% by the Somogyi-Nelson method. There have been to date few reports on the action pattern of AA. To investigate the cleavage pattern of maltodextrin, we tried to estimate the cleavage from the anomeric configuration of the products by the HPLC column. An z-amylase produces exclusively the z-anomer of product(s) corresponding to the cleavage. The column can separate anomers of products larger than maltose.20 Beta-anomer's peaks of maltodextrin (G3 to G8) lead to the corresponding z-anomer's peaks. The maltose peak can be detected on the chromatogram, but the anomer peaks cannot be separated. Figure 2 shows that the AA produced predominantly the z-anomer of maltotriose (G3). This indicates that the AA hydrolyzes G5 predominantly at the third glucose bond from the nonreducing end (Table).

The neutral z-amylase from citric acid-koji was also purified to be homogeneous on SDS-PAGE, from the extract of 27 h-koji by essentially the same method as above. The N-terminal sequence of the first 15 amino acids of the enzyme was found to be identical to that of Taka-amylase A130 and common NA's from other Aspergilli.14 180 The production of G3 in the equilibrated mixture of z- and â-anomers (Fig. 2) indicated that the enzyme hydrolyzed G5 at the second bond similarly to Taka-amylase A130 (Table). Thus, the cleavage pattern towards G5 could differentiate distinctly AA from NA.

This study has shown that AA from citric acid-koji (A. usami var.) has the same N-terminal sequence (20 residues) as AA from A. niger,111 but differing in molecular weight. The HPLC analysis for identifying the anomers of products was a useful tool for the cleavage study, and the distinct cleavage pattern of the NA towards maltopentaose suggests that AA has a different subsite structure79-211 from the ordinary NA. More detailed study on the action

Fig. 2. Anomeric Configuration Analysis of Products to Identify the Predominant Cleavage Points in G5 by the Enzymes, AA (A) and NA (B), from Citric Acid-koji.

The reactions were done in 20 mM acetate buffer, pH 4.8, for AA or pH 5.4 for NA, at 37°C. [1] 0.24 ml, [S] 2 mm. HPLC conditions: column, YM-C30-04, eluent, 0.04% Na2SO4, in distilled water; flow rate, 0.4 ml/min.
patterns and subsite structures of AA and NA from citric acid-koji will be reported elsewhere.

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References