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Purification and properties of α-amylase from *Aspergillus oryzae* MIBA316

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An alpha-amylase was purified from a culture broth of *Aspergillus oryzae* MIBA316. The enzyme hydrolyzed effectively amylopectin, amylose and starch, and cleaved maltopentaose to form a mixture of maltotriose and maltose. Glucose was not produced from maltose. The N-terminal sequence of the first 10 residues and other molecular properties were identical with those of Taka-amylase A.

Keywords: *Aspergillus oryzae*, α-amylase, glucoamylase, maltopentaose

Aspergillus species have attracted attention for their role in fermentation of oriental food products or industrial application of hydrolytic enzymes. Within the range of carbohydrolases produced by this species, the majority has been considered as α-amylase and glucoamylase[1-3)]. Strain MIBA316 has been utilized for specific fermentation of Japanese sake at Akita Konno Co.

In the present study, α-amylase was purified from the culture broth of strain MIBA316 and its enzymatic and molecular properties, especially the cleavage pattern of maltooligosaccharides, were characterized.

Materials and methods

Cultivation of A. oryzae MIBA316

MIBA316A was cultivated at 30℃ for 2 days on medium I agar (3%) plate (pH 5.5), which contained 0.5% KH_2PO4, 0.1% $NaNO_3$, 0.5% soluble starch, 1.0% peptone, and 0.1% $MgSO_4{\cdot}7H_2O$. Cells grown were collected, suspended in 30 ml of 0.85% NaCl. The 1-ml aliquots of cell suspensions obtained were inoculated to 500 ml-flasks, containing 50 ml of medium I and shaken at 30℃ and at 100 rpm for 6 days. The cultures were filtered through a glass filter (pore size, G1) and concentrated to 100 ml in vacuum at room temperature.

Purification of enzyme

Step 1. The culture concentrate was dialyzed against 10 mM potassium phosphate buffer, pH 6.8 (named as PPB) and centrifuged at 10,000 rpm for 30 min at 4℃ to remove insoluble materials.

Step 2. The clear supernatant solution was applied to DEAE-Sephacel column (1.5 x 36.2 cm), which was equilibrated with PPB. After washing thoroughly with PPB, the column was eluted with a linear gradient (700 ml) conducted between PPB in a mixing chamber and PPB/1.0 M NaCl in a reservoir (each 6 ml /tube). The active enzyme fractions were pooled, concentrated on Amicon PM-10 membrane filter, and subjected to centrifugation as described above.

Step 3. The concentrate (2.5ml) was applied to a Sephacryl S-200 column (2.5 x 93.4 cm),which was equilibrated with PPB/0.5 M NaCl/0.02% sodium azide (each 3 ml/tube). The active fractions eluted from the column were collected and concentrated.

Step 4. The concentrate was rechromatographed on Sephacryl S-200 column, as described in step 3.

The active fractions eluted from the column were concentrated, dialyzed against PPB and pooled as the final preparation.

Enzyme assay

α-Amylase activity was assayed as follows: The reaction mixture (2.0 ml) contained 80 mM maleate -NaOH buffer (pH6.8), 10 mM amylopectin, and the diluted enzyme solution. After incubation for 2-10 min at 37℃, the reaction was terminated by boiling for 5min. Reducing power was determined by using 3,5-dinitrosalycilic acid[4]. When one of the oligo-saccharides was employed as substrate, glucose formed was determined by using glucose oxidase kit[5]. One unit (U) of the enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol substrate for 1 min at 37℃ and at pH 6.8. Protein was assessed by the method, as described before[6].

Molecular weight determination

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was run according to the method, as previously reported[7,8]. The molecular weights of proteins were determined by using standard molecular weight markers (29,900 – 112,000).

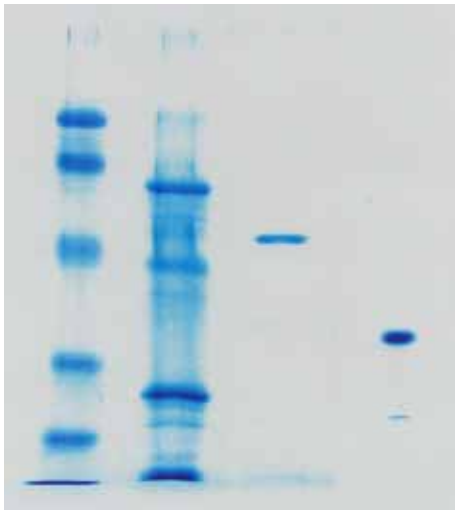
Results and discussion

The final preparation of enzyme was purified 12.6-fold over culture broth, the yield being 25.3 % (Table 1). This preparation gave a single protein band in both native PAGE and in SDS-PAGE (Fig. 1). The molecular weight of the enzyme was estimated to be 50,000. The N-terminal amino acid sequence of the first 10 was determined to be Ala-Thr-Pro-Ala-Asp-Trp-Arg-Ser-Gln-Ser-. The activity was maximal at 55℃, and the enzyme was most active at pH4.5. These properties were identical to those for Taka-amylase A[9].

The enzyme effectively hydrolyzed amylopectin and amylose (Table 2). On the other hand, glycogen, pullulan and panose were not effective substrates. Neither glucose liberation nor an increase in reducing power was confirmed when one of the following saccharides was employed as the substrate; isomaltose, isomaltotriose, and isomaltotetraose.

Table 1. Purification of α-amylase from *A.oryzae* MIBA316

Step	Total Protein (mg)	Total Activity (Unit)	Specific activity (U/mg Protein)	yield (%)	purity (fold)
1	817.2	4437	5.1	100.0	1.0
2	250.0	4878	19.5	110.0	3.6
3	73.3	2276	31.1	51.3	5.8
4	17.4	1124	64.5	25.3	12.6



1 2 3 4

Fig.1 Native and SDS-PAGE of *A. oryzae* MIBA316 α-amylase

Lanes1 and 2, molecular weight markers (112,000, 81,000, 49,900, 36,200, 21,000) and (68,000, 45,000, 29,900 and 14,100): lanes 3 and 4, purified α-amylase (2.5 μg each) in SDS-gel and in native gel,respectively.

Hydrolysis of amylose and amylopectin, respectively by this enzyme was followed by incubating 20-μl each at 0.5, 1, 2, 4 and 24 h intervals. Maltose increased throughout the reaction, whereas maltotriose decreased continuously, and glucose appeared after 1 h incubation. Such hydrolysis pattern indicates the linkage to be split in maltosaccharides is the non-reducing terminal one.

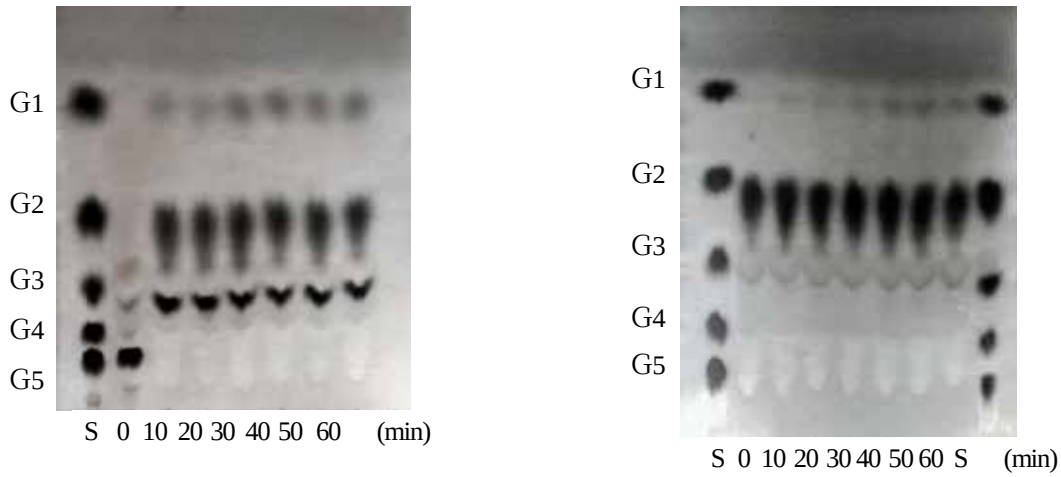


Fig. 2. A paperchromatogram of the product from maltopentaose (*in left panel*) and maltose (*in right panel*) at time intervals indicated of *A.oryzae* MIBA316 α-amylase. S,.standard maltooligosaccharides. G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, malttopentaose. Twenty μl aliquots of the respective reaction mixtures were applied on Advantec Toyo NO.51A filter paper. N-butanol/ pyridine/deionized water (6:4:3, v/v) as solvent, and spots detected using silver nitrate, as described by Walker and Campbell (*J. Bacteriol*. 86:681-688, 1963).

Table 2 Hydrolysis of various Substrates by *A. oryzae* MIBA316 α-amylase

Sugars	Relative activity (%)	
	I	II
0.5% amylopectine	**100**	-
0.25% amylose	**99**	-
0.25% soluble starch	**37.5**	-
0.25% glycogen	2.10	-
0.25% pullulan	0	-
10mM panose	0.00	-
10mM maltose	-	1.07
10mM maltotriose	-	100
10mM maltotetraose	-	68.80
10mM isomaltose	-	0.00
10mM isomaltotrise	-	0.00
10mM isomaltotetrase	-	0.19

I, assayed by using dinitrosalycilic acid
II, determined by using glucose oxidase.

Maltopentaose was degraded to maltotriose and maltose. However, maltose could not be degraded to glucose (Fig.2). It was also observed maltotetraosee was converted to a mixture of maltotriose and maltose, and that maltotriose was slowly decomposed to maltose and glucose. Maltose was not effective substrate of this enzyme to yield glucose (Table2, Fig.2).

Amylose (0.5 %) was incubated for 30 and 60 min with 0.2 U enzymes, and subjected to the polarimetric analysis. Comparable experiments were conducted with same units of glucoamylase from *Rhizopus* sp. and α-amylase from *Bacillus subtilis*, respectively. After 60 min treatment, the optical rotation was 0.4 degree with glucoamylase. But the degree was 1.5 and 1.8 for MIBA enzyme and α-amylase, respectively. The anomeric configuration of products revealed that this enzyme was an α-amylase.

MIBA enzyme had the same N-terminal amino acid sequence (10 residues) as Taka-amylase, and the other molecular properties were not discriminated. From these findings along with the results reported in the references, MIBA enzyme is assigned to α-amylase.

Today the strain has been extensively employed as useful host for cloning of carbohydrolase derived from the *Aspergillus* species and the gene encoding for α-glucosidase which might be classified into the same

amylase family was isolated from *A. oryzae*[10].

The genes for *A. oryzae* MIBA316 glucoamylase were cloned in specific strain of *A. oryzae* that has been employed as safety host-vector system[12]. A similar type of *A. oryzae* enzyme was successfully constructed in *A.nidulans* strain and well characterized[11].

It should be noted that strain MIBA316 could produce α-amylase and glucoamylase. Recently, these enzymes secreted by *A.niger* are reported to be products formed by proteolytically processing of a precursor[12]. These activities have been effectively used fermentation of rice and other starches.

More detailed study on the enzyme structure of MIBA316 strain deduced from its nucleotide sequences is under progress[13].

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