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A Newly Isolated *Rhizopus microsporus* var. *chinensis* Capable of Secreting Amyloytic Enzymes with Raw-Starch-Digesting Activity

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A newly isolated active producer of raw-starch-digesting amyloytic enzymes, *Rhizopus microsporus* var. *chinensis* CICIM-CU F0088, was screened and identified by morphological characteristics and molecular phylogenetic analyses. This fungus was isolated from the soil of Chinese glue pudding mill, and produced high levels of amylolytic activity under solid-state fermentation with supplementation of starch and wheat bran. Results of thin-layer chromatography showed there are two kinds of amyloytic enzymes formed by this strain, including one α-amylase and two glucoamylases. It was found in the electron microscope experiments that the two glucoamylases can digest raw corn starch and have an optimal temperature of 70°C. These results signified that amyloytic enzymes secreted by strain *Rhizopus microsporus* var. *chinensis* CICIM-CU F0088 were types of thermostable amyloytic enzymes and able to digest raw corn starch.

Keywords: *Rhizopus microsporus* var. *chinensis*, raw starch digesting, amyloytic enzymes, screening and identifying

Among the amyloytic enzymes applied in starch-related industries, α-amylase and glucoamylase are the two principal ones. The α-amylase is an endoenzyme that catalyzes at the interior sugar bonds of the starch molecule, producing maltodextrins as end products, whereas the glucoamylase is an exoenzyme that acts on the starch molecule from the nonreducing end, releasing glucose as the final product [19]. Amyloytic enzymes with a wide range of specificities are available, but researchers continue to search for new amyloytic enzymes that could create new applications.

The traditional course of enzymatic starch saccharification is a high-energy-consumption procedure, which significantly enhances the cost of starch-related products. Therefore, to reduce the cost of starch processing, the importance of enzymatic saccharification of raw starch without cooking has become well recognized recently [24, 34]. This has generated a worldwide interest in the discovery of several raw-starch-digesting amyloytic enzymes that do not require gelatinization and can directly hydrolyze the raw starch in a single step [26, 34].

The microorganisms reported to be active producers of amyloytic enzymes capable of digesting raw starch have mostly been fungi, such as *Aspergillus* sp., *Rhizopus* sp., *Penicillium* sp., *Thermomyces* sp., and *Saccharomyces* sp. [4, 8, 9, 15, 20, 21, 28, 29]. The α-amylase secreted by fungi are normally heat-labile, with temperature stability between 40°C and 60°C and easily loses activity at up to 60°C. The glucoamylases secreted by fungi tend to exhibit multiplicity, which are thought to be several mechanisms: mRNA modifications, limited proteolysis, variation in carbohydrate content, or the presence of several structural genes [16].

However, there are few reports about filamentous fungi that could produce both glucoamylase and α-amylase concurrently [27]. Therefore, the ability of excreting synchronously both glucoamylases and α-amylase has stimulated much interest worldwide, because both enzymes might be used together in different stages of starch saccharification. In order to raise the utilization ratio of starch and save the cost of saccharification, much effort had been made on co-displaying on yeast [26, 32], mixed fermentation between filamentous fungi and *Bacillus* [31], and so on.

In the present study, we isolated a *Rhizopus microsporus* var. *chinensis* CICIM-CU F0088 capable of producing amyloytic enzymes with raw-starch-digesting ability. Results

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of purification and characterization of the three amyloytic enzymes showed that one α-amylase and two glucoamylases are produced by the fungus concurrently.

Materials and Methods

Microorganism Selection

A three-step strategy was carried out for microorganism selection: the first step iwas to search for fungi grown on potato dextrose broth (PDA; DIFCO 254920) with additional 1% corn starch, called medium A. Two-gram samples collected from raw-starch-rich areas were homogenized in sterile physiological saline of pH 5.0, incubated at 30°C for 1 h, and then streaked (a loop of such homogenized culture) on the surface of medium A. After incubation at 30°C for 24 to 72 h, all morphologically contrasting colonies were purified. Pure cultures were picked up and stored on PDA slants at 4°C.

The second step was to verify whether raw-starch-digesting enzymes could be secreted by those fungi. A selection medium B called trypan blue–raw starch, in a test tube including 1% raw corn starch, 1% trypan blue, 0.14% $(NH_4)_2SO_4$, 0.20% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 2% agar, was used. Selected fungi were inoculated onto the test tube in turn. After incubation for 3 days, strains with raw-starch-digesting ability would show a ring-shaped transparent band at the top of medium B. The length of transparent band was proportional to the raw-starch-digesting ability to a certain extent.

The third step was to ensure the composition of amyloytic enzymes secreted by fungi. Selected fungi were inoculated into medium C, including wheat bran 14 g in salt-solution (20 ml) containing starch 0.3 g, $(NH_4)_2SO_4$ 0.2 g, K_2HPO_4 0.06 g, and $MgSO_4 \cdot 7H_2O$ 0.02 g in every 500-ml Erlenmeyer flask. Fermentation process and crude enzymes preparation were carried out as described below. Thin-layer chromatography (TLC) was carried out to determine the hydrolyzed products of raw starch at different incubation times, as depicted in a laer section below.

Identification of the Microorganism

Morphological characteristic. The established taxonomic system of *Rhizopus* [25] was followed for identification of strains. The strains were cultured on potato dextrose broth (DIFCO 254920) at 30°C. Slide mounts were prepared and examined under a light microscope (Axiostar plus, Zeiss, Oberkochen, Germany). Response to temperature was determined by measuring the diameters of colonies on agar plates incubated at temperatures of 30°C, 35°C, 40°C, and 45°C.

Sporangiospore morphology was evaluated by scanning electron microscopy (Quanta-200; FEI, Holland). The samples with visible sporulations were prefixed by soaking the whole apparatus in 3% glutaraldehyde for 1 h and postfixed in 1% osmium tetroxide for 5 min with three washings in 0.1 M phosphate buffer after each fixation. After dehydration in a series of ethanol, the samples were dried in a critical point drier (CPD-030; BAL-TEC, Switzerland), coated with platinum (SCD-005; BAL-TEC, Switzerland), and observed under the SEM (Quanta-200; FEI, Holland).

Molecular phylogenetic analysis by 5.8S gene and flanking ITS. Genomic DNA was extracted from fresh cultures using a modified protocol [5]. For the sequence analysis, the ITS1–5.8S–ITS2 rDNA region of the fungi was amplified by PCR using primer set pITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and pITS4 (5'-TCCTCCGCT TATTGATATGC-3'). The PCR mixture (50 μl) contained 37.5 μl of deionized water, 5 μl of PCR buffer, 4 μl of dNTP, 1 μl of each primer (10 pmol/μl), 1 μl of template DNA, and 0.5 μl of *Ex Taq* DNA polymerase (Takara, Kyoto, Japan). The PCR amplification was performed in an automated thermal cycler (PTC-200; BIO-RAD, U.S.A.) with the following conditions: predenaturing at 95°C for 5 min; 30 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 1 min, and prolonging at 72°C for 1 min; before the extension at 72°C for 10 min, final cooling to 4°C. Detailed operating steps followed Li *et al.* [11].

DNA was sequenced on both strands with an Applied Biosystems Model (3130) automatic DNA sequencer, and a BigDye Terminator v3.1 kit (Applied Biosystems, U.S.A.). Sequence analysis was performed using Sequence Scanner v1.0 software, and then compared with sequences in the GenBank databases using the BLAST program.

Determination of the Number of Amylolytic Enzymes by Active Staining on Native-PAGE

The number of amylolytic enzymes was determined by active staining on native-PAGE (7%) [36]. The gel of electrophoresis was washed by super-purified water, submerged into 1% soluble starch–acetic acid solution (pH 6.0), maintained at 60°C for 10 min, and then stained with KI/I_2 solution.

Enzymes Purification

Preparation of crude enzymes. After cultivation at 30°C for 72 h in medium C, 100 ml of 100 mM sodium phosphate buffer (pH 6.0) was dispensed into each flask, and each flask shaken at 200 rpm for 1 h. The extraction was repeated twice, and the pooled enzyme extract was centrifuged at 10,000 ×*g* for 15 min at 4°C, and the clear supernatant was used as the source of amyloytic enzymes.

Ammonium sulfate precipitation. The crude enzyme extract was precipitated by slowly adding solid ammonium sulfate to saturation between 45% and 80%. The pellet obtained after centrifugation (12,000 ×*g*, 15 min) was dissolved in a minimal amount of 30 mM acetate buffer (pH 6.0), and desalted on HW-40F (TOSOH) to remove redundant ammonium sulfate. The active fractions were pooled and concentrated by lyophilization.

Bio-Rad Model 491 Prep Cell. Redissolution fractions of protein concentration were loaded onto a Bio-Rad Model 491 Prep Cell, with an electrophoresis Ornstein–Davis buffer system (pH 8.8), and washing system (sodium acetate–acetic acid, pH 6.0). The active fractions were concentrated and desalted by Ultrafree-MC Centrifugal Filter Units.

Enzyme Activity and Protein Concentration Assays

The α-amylase activity was determined by microplate-based starch – iodine assay [36]. In a typical run, 40 μl of starch (Sigma) solution (1.0 g/l) and 40 μl of enzymes in 0.1 M phosphate buffer at pH 6.0 were added into 96-well microplates (Eppendorf). After 30 min of incubation at 50°C in a microplate reader (SpectraMax Plus384; Molecular Devices company, U.S.A.), where the assayed enzymes were most active, 20 μl of 1 M HCl was added to stop the enzymatic reaction, followed by the addition of 100 μl of iodine reagent (5 mM I_2 and 5 mM KI). Following color development, the absorbance at 580 nm (A_{580}) was measured using the microplate reader. One unit of α-amylase activity was defined as the disappearance of an average of 1 mg of iodine-binding starch material per minute in the assay reaction.

Glucoamylase activity was determined in 0.1 M phosphate buffer, pH 5.0, with 1% maltose. Twenty-five μl of enzyme was added to

100 μl of the buffer. The samples were incubated for 15 min at 60°C and for 5 min at 100°C to stop the reaction. The glucose concentration was determined by an immobilized enzyme biosensor and flow injection analysis (SBA-40C, Research Institute of Shandong Academy of Science, Key Laboratory of Biosensor, China) based on the GOD-POD method. One unit of glucoamylase activity was defined as the activity that forms 1 μmol glucose in 1 min under standard conditions.

Protein concentration was determined according to the dye binding method of Lowry *et al.* [12] using bovine serum albumin as the standard.

Purity Verification and Determination of the Molecular Mass

Polyacrylamide gel electrophoresis (PAGE) under native conditions was carried out to confirm the numbers of amylolytic enzymes [33]. The gel after electrophoresis was marked in gelatinized and cooled (R.T.) starch (1%) prepared in sodium acetate buffer (50 mM, pH 5.0) and incubated at 60°C for 10 min. Subsequently, the gel was stained with iodine reagent (3%). The amylolytic enzymes bands were visualized as transparent bands on a dark-blue background [1].

The purified amylolytic enzymes obtained from Prep Cell were ascertained for their purity on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), using the proteins of a prestained ladder (SM0661; MBI Fermentas, Hanover, MD, U.S.A.) as reference proteins. Their apparent molecular masses were determined on a Protein KW-802.5 Shodex Gel Filtration column (300 mm×8 mm I.D.).

Identification of Starch Hydrolysis Products by TLC

A 100-μl aliquot of 1% (w/v) raw starch or raw corn starch and 100 μl of enzyme (0.3 U) were incubated at 60°C for 12 h under a drop of toluene to prevent microbial growth. The hydrolyzed products were identified qualitatively by TLC using silica gel plate, 20×20 cm, (Merck), and glucose and maltose were used as standards. The spots were developed by the diphenyl amine method [2].

Raw Corn Starch Hydrolysis

A 200-μl reaction mixture containing 100 μl of 1% (w/v) raw corn starch solution (0.1 M sodium acetate buffer pH 6.0) and 100 μl of enzyme solution with the enzyme activity of 4 U/ml was incubated at 60°C for 72 h, centrifuged at 3,000 ×*g* for 10 min, and the supernatant separated with gentle sedimentation. The sedimentation was gently washed three times with pure water, and then photographed by electron microscopy in order to prove the raw corn starch hydrolysis ability of amyloytic enzymes. Boiled enzyme was taken as a control.

Effects of pH and Temperature on the Activity and Stability of the Purified Enzymes

The pH optimum of the purified enzyme was determined by assaying activity at various pHs (2.5 to 8.0) at 50°C for α-amylase, and 60°C for glucoamylase. Stability against different pHs was determined by incubating the enzyme solution for 60 min at the specified pHs and assaying residual activity at various times using the methods described above.

The effect of temperature on amyloytic enzymes activity was determined over the range of 30 to 80°C in 0.1 M phosphate buffer, pH 6.0 for α-amylase, and pH 5.0 for glucoamylase. The effect of temperature on stability was tested by pre-incubating the purified enzyme preparation at varying temperatures, ranging from 30 to 80°C for 6 h, and determining the residual activity at regular intervals of 15 min.

RESULTS

Isolation of Microorganism

A total of 154 samples collected from raw-starch-rich areas were homogenized and streaked on the surface of PDA medium with additional 1% raw corn starch. Then, 384 pure cultures were subcultured on PDA slants at 4°C, including strains from *Aspergillus* sp., *Rhizopus* sp., *Mucor* sp., *Fusarium* sp., and *Penicillium* sp. The second step was to verify whether raw-starch-digesting enzymes could be secreted by those fungi. After incubation for 3 days on trypan blue–raw starch medium, six fungi showed a ring-shaped transparent band at the top of the medium, including *Rhizopus* CICIM-CU F0088 (11 mm), *Rhizopus* CICIM-CU F0089 (9 mm), *Aspergillus* CICIM-CU F0090 (6 mm), *Penicillium* CICIM-CU F0091 (6 mm), *Mucor* CICIM-CU F0092 (4 mm), and *Fusarium* CICIM-CU F0093 (4 mm). The third step was to ensure the composition of amyloytic enzymes secreted by the fungi using TLC. Results showed only fungus *Rhizopus* CICIM-CU F0088 secreted at least two kinds of amyloytic enzymes; fungus *Rhizopus* CICIM-CU F0089, *Aspergillus* CICIM-CU F0090, and *Mucor* CICIM-CU F0092 produced only glucoamylase; and *Penicillium* CICIM-CU F0091 and *Fusarium* CICIM-CU F0093 excreted only α-amylase.

Because the overspreading fungal mycelia make it difficult to verify the ratio of clearing zone to growth zone, the traditional selecting medium in plate was unfavorable for accurately determining the raw-starch-digesting ability. Therefore, selection medium in test tube containing trypan blue–raw starch desirably solved the problem. Compared with traditional selecting medium in plate, such selection method needed much less medium, non-interfered with other fungi (one fungi in a single tube), and is easy to observe (trypan blue has the quality of combing with raw corn starch; in the condition of starch digested by enzymes, trypan blue will be released and the dark purple color turns to light purple), gives quasi-quantification (since the height of transparent band is proportional to the ability of raw starch digesting), and is especially suitable for batch operation.

Identification of Microorganism

Morphological characteristic. The criterion of *Rhizopus* taxonomy was monographed by Schipper, which divided the genus into three groups; the *R. stolonifer* group, the *R. oryzae* group, and the *R. microsporus* group, according to the characteristics of the sporangial apparatus and growth temperatures. Thereafter, the characteristics of morphology, growth temperature, and the data from mating experiments became the basic criteria for the species definition of *Rhizopus*.

Morphological characteristics examined under the light microscope showed a simple rhizoid, and erect morphology of sporangiophores. The length of sporangiophores was up

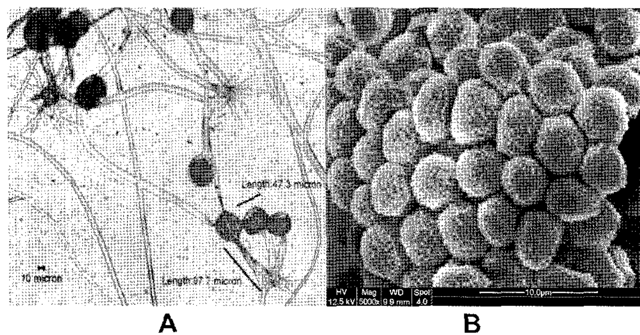

Fig. 1. Morphology of strain examined under light microscope (**A**) and sporangiospore morphology photographed by SEM (**B**).

to 0.5 mm, rarely up to 1 mm, and the maximal diameter of sporangia was 100 mm (Fig. 1A). Response to temperature was determined to be over 45°C. Spore morphology by SEM indicated that the sporangiospores of *Rhizopus* CICIM-CU F0088 were subglobose with minute spike-like projections on the surface (Fig. 1B), and the average diameter of sporangiospores was 5 μm.

All such morphological characteristics share very similar morphological features with *R. microsporus*. Thus, molecular phylogenetic analysis was carried out for further confirmation.

Molecular phylogenetic analysis using 5.8S gene and flanking ITS. The analysis of the ribosomal region *via* PCR has been widely employed for characterizing different fungal species [7]. The 5.8S gene and ITS regions were used in this study. Since the 5.8S gene is highly conserved, this region is used for the phylogenetic analysis of higher taxonomic levels, whereas the highly variable ITS regions are used for analysis of lower taxonomic levels.

A comparison of the ITS sequence of this strain with other related filamentous fungi showed that the ITS sequence of this strain had 98% similarity with *Rhizopus microsporus* var. *chinensis* (Fig. 2). Therefore, this strain was selected and identified; it was identified as a strain of *Rhizopus microsporus* var. *chinensis* and named *Rhizopus microsporus* var. *chinensis* CICIM-CU F0088.

Strain *Rhizopus microsporus* var. *chinensis* was first studied by Schipper and Stalpers [25]. According to their studies, strain *Rhizopus microsporus* var. *chinensis* belongs to thermotolerant fungi, with attributes of thermophilic possessing areas. Proteins secreted by thermotolerant fungi might be thermostable [19].

Determination of the Number of Amylolytic Enzymes by Active Staining on Native-PAGE

PAGE of the crude extract from strain CICIM-CU F0088 indicated the presence of three major amylolytic enzymes. Thermostable amylolytic activity containing activities of both α-amylase and glucoamylase was detected from *Rhizopus microsporus* var. *rhizopodiformis* [18, 19]. Among filamentous fungi, the ability for producing either α-amylase [23] or glucoamylase [10] alone is quite common. However, there are few reports about filamentous fungi that could produce glucoamylase and α-amylase concurrently [27].

Genetic diversity and post-translational proteolysis are the two principal reasons suggested for the multiple forms of amylolytic enzymes produced by filamentous fungi [22, 23].

Enzymes Purification

The overall scheme employed in the purification of amylolytic enzymes from CICIM-CU F0088 is summarized in Table 1.

The enzyme was purified by ammonium sulfate precipitation and desalted by HW-40F gel filtration chromatography. The active fractions were pooled and concentrated by

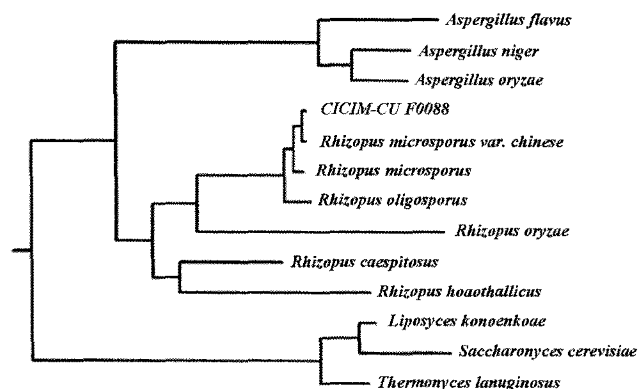

Fig. 2. Phylogenetic relationship of CICIM-CU F0088 and related filamentous fungus based on their ITS sequences.
The ITS sequence of the newly isolated strain has been deposited in the GenBank database (Accession No. EU304450).

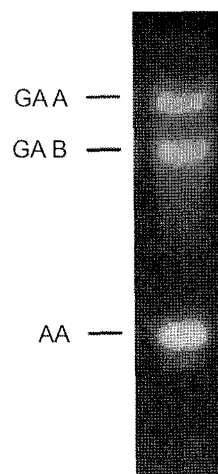

Fig. 3. Determination of the numbers of amylolytic enzymes by active staining on native-PAGE.

Table 1. Summary of the purification of amylolytic enzymes from CICIM-CU F0088.

Purification step		Total activity (U)		Total protein (mg)		Specific activity[c] (U/mg)		Purification (fold)		Yield (%)	
		GA[a]	AA[b]	GA	AA	GA	AA	GA	AA	GA	AA
Crude supernatant		1,500	1,103	600	380	2.5	2.9	1	1	100	100
Ammonium sulfate		705	474	64	32	11	15	4.4	5.2	47	43
Prep Cell	GA A	48		1.3		37		15		3.2	
	GA B	79		1.4		58		23		5.3	
	AA		32		0.6		53		18		2.9

[a]One unit is equivalent to 1 μmol of glucose released per minute.
[b]One unit is defined as the disappearance of an average of 1 μg of iodine-binding starch material per minute.
[c]Specific activity is expressed as 1 μmol of glucose released per minute in 1 mg of protein for GA, and the disappearance of an average of 1 μg of iodine-binding starch material per minute in 1 mg of protein for AA.

lyophilization. Proteins diluted to 1 mg/ml were loaded on Model 491 Prep Cell. In a typical run, samples are electrophoresed through a cylindrical gel. As molecules migrate through the gel matrix, they separate into ring-shaped bands. Individual bands migrate off the bottom of the gel where they pass directly into the patented elution chamber for collection. The α-amylase (AA) was eluted ahead of the two GAs, with a recovery of 2.9%; the AA was found to be a minor one compared with glucoamylase A (GA A) and glucoamylase B (GA B). GA B was eluted after AA, and GA A was the last one to be eluted. Such an elution sequence was consistent with their performances on native-PAGE.

The recovery was found to be 3.2% (GA A), 5.3% (GA B), and 2.9% (AA) with a fold purification of 15 (GA A), 23 (GA B), and 23 (AA).

Purity Verification and Determination of the Molecular Mass

Upon purification, three amylolytic enzymes were separated from one another and showed their apparent homogeneity with respect to protein (stained by Coomassie Brilliant Blue R-250 of purified amylolytic enzymes; Fig. 4, lanes d–f) and activity staining (activity staining of purified amylolytic enzymes shown in Fig. 4, lanes a–c).

The molecular masses of the purified enzymes were determined to be 55, 53, and 56 kDa by HPLC on a Protein KW-802.5 Shodex gel filtration column (data not shown). The relative molecular masses of the subunit estimated by SDS–PAGE were about 53, 52, and 54 kDa (Fig. 5), indicating that the three enzymes were all monomers.

For glucoamylase produced by *Rhizopus* sp., reports on molecular masses were normally between 49.3 kDa and 74 kDa, and they were all monomer enzymes [17]. With a closely evolutional relation with *Rhizopus oryzae*, GA A and GA B showed affinity to glucoamylase B (ABB22051.1), with a molecular mass of 53 kDa and 52 kDa, respectively.

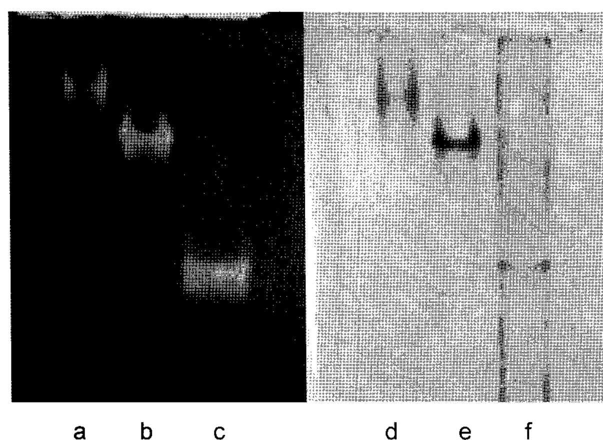

Fig. 4. Purity verification of the purified amylolytic enzymes from CICIM-CU F0088 by native-PAGE.
(a) GA A; (b) GA B; (c) AA-activity stained by KI/I_2, and (d) GA A; (e) GA B; (f) AA all stained by Coomassie Brilliant Blue R-250.

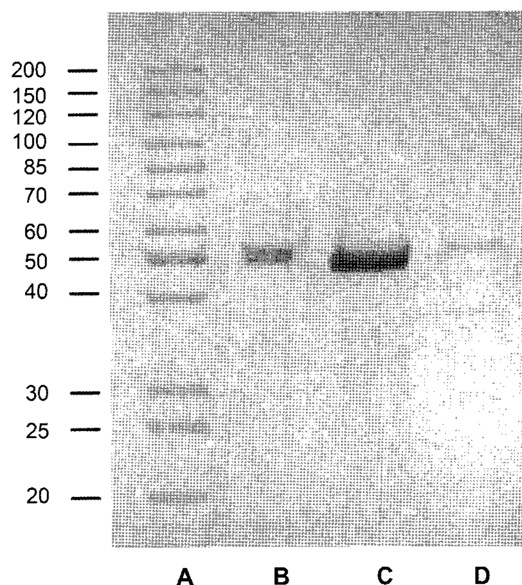

Fig. 5. Determination of the molecular masses of purified amylolytic enzymes from CICIM-CU F0088 by SDS–PAGE.
(**A**) Molecular size markers; (**B**) GA A; (**C**) GA B; (**D**) AA.

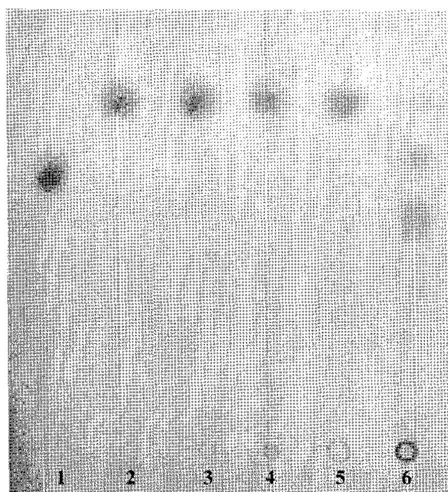

Fig. 6. Hydrolysis products released by the three enzymes, as analyzed by TLC.
1, Maltose; 2, glucose; 3, the crude enzyme acting on 1.0% (w/v) soluble starch for 1 h; 4, GA A; 5, GA B; 6, AA.

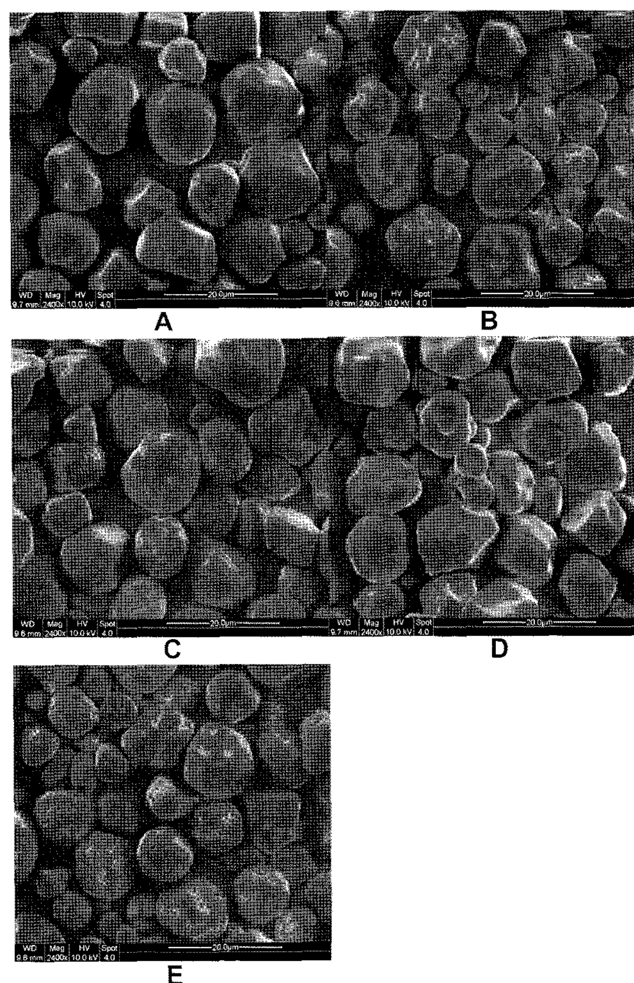

Fig. 7. Electron microscope photos of raw corn starch hydrolyzed by amylolytic enzymes.
(**A**) Raw corn starch hydrolyzed by boiled enzyme to serve as a control, (**B**) GA A, (**C**) GA B, (**D**) AA, (**E**) Three enzymes.

Identification of Starch Hydrolysis Products by TLC

Results of TLC showed that the final products of GA A, GA B, and AA were glucose, glucose, and maltose, which proved GA A, GA B, and AA to be glucoamylase, glucoamylase, and α-amylase, respectively. A similar ability of producing glucoamylase and α-amylase simultaneously by strain *Rhizopus microsporus* var. *rhizopodiformis* was previous reported by Peixoto *et al.* [18]. This was fascinating, since both enzymes might be used together in different stages of starch saccharification [35], which will reduce the production cost and avoid using heterologous systems for both enzymes secretion as described in a previous report [26].

Raw Corn Starch Hydrolysis

The raw corn starch hydrolytic ability of purified amyloytic enzymes could be observed clearly by electron microscopy. The unhydrolyzd native granules of raw corn starch were irregularly shaped, with smooth edges, and they varied in size between 5 and 15 μm (Fig. 7A). After digestion, it could be seen that the GA A excavated many pits or pinholes on the corn starch granules (Fig. 7B). Compared with GA A, GA B scooped relatively few pinholes on the corn starch (Fig. 7C). However, AA did not show the hydrolytic ability towards raw corn starch (Fig. 7D). With a mixed digestion by the three amylolytic enzymes, the interior portion of the granules became exposed and a hollow center was revealed (Fig. 7E), which signified that the granules were disrupted eventually.

A presumed hydrolyzing method of raw corn starch by the three enzymes might be as follows: at the early stage of the reaction, GA A acted as a major contributor of combination to raw corn starch, whereas GA B performed as a minor contributor, and formed starch molecules easy to be hydrolyzed by AA. Newly formed nonreducing ends of starch molecules by AA will be convenient to GA A and GA B, by splitting the original starch molecules. Along with the holes on starch being enlarged, the combination rate and hydrolysis efficiency increased dramatically [6].

The results of TLC and electron microscopy showed jointly that amylolytic enzymes from strain *Rhizopus microsporus* var. *chinensis* CICIM-CU F0088 had the ability of hydrolyzing raw corn starch, and its natural amylolytic enzymes system including both α-amylase and glucoamylase could be adequate for biotechnological applications.

Effects of pH and Temperature on the Activity and Stability of the Purified Enzymes

The summary of the properties of each enzyme is shown in Table 2. Enzymatic properties such as optimal pH, temperature,

Table 2. Summary of some enzymatic properties of amylolytic enzymes from CICIM-CU F0088.

	GA A	GA B	AA
Optimal pH	4.5	4.5	6.0
pH Stability	4.0–5.5	4.0–5.5	5.0–6.5
Optimal temperature (°C)	70	70	55
Thermal stability (°C)	70	70	60
Raw corn starch hydrolysis ability	Strong	Weak	N.D.

pH, and thermal stabilities of GA A and GA B were almost the same, but these values were quite different from those of AA.

In the case of GA A and GA B, optimum activity was found in the pH range of 4.0–5.5. Enzyme activity decreased drastically at pH below 4.0 or above 6.0. In the case of AA, the optimum range was from 5.0 to 6.5 with changes less than 10%. The enzyme activity of AA was unstable when pH was below 4.5 or above 7.0, and performed similarity with Taka-amylase [14].

For amyloytic enzymes from *Rhizopus* species, their optimum activities were in the pH range 4.0–5.5, such as the enzymes from Nahar *et al.* [13] and Ray [23]. For glucoamylases secreted by thermotolerent species, they were normally neutral pH optimum [9]. Compared with those strains, the optimum pH and pH stability found for *Rhizopus microsporus* var. *chinensis* CICIM-CU F0088 were comparatively similar with *Rhizopus* species.

Species of *Rhizopus microsporus* are an exception of the *Rhizopus* genus, mostly constituted by mesophilic organisms [18]. A strain of *Rhizopus microsporus* var. *rhizopodiformis* had an ability to grow up to 50°C and exhibited optimal amylase production at 45°C [19]. Similar to such report, the *Rhizopus microsporus* var. *chinensis* CICIM-CU F0088 in this study belonged to the species of *Rhizopus microsporus*, and also showed ability to grow at 40°C, and secreted amyloytic enzymes at a higher temperature than *Rhizopus oryzae*.

Because of the higher reaction rates when temperatures were higher than 50°C, the thermostable raw-starch-digesting amyloytic enzymes are of great importance for starch hydrolysis. In this point of view, the amyloytic enzyme from *Rhizopus microsporus* var. *chinensis* CICIM-CU F0088, which was optimally active at 70°C and displayed 98% of its peak activity at 75°C (data not shown), could be a good candidate for the efficient and quick hydrolysis of corn starch. The amylolytic enzymes produced by the thermotolerant fungus *Rhizopus microsporus* var. *chinensis* CICIM-CU F0088 are adequate for biotechnological applications.

The amyloytic enzymes of *Aspergillus oryzae*, *Aspergillus niger*, and *Rhizopus oryzae* [3, 30], which are the most common raw-starch-digesting strains, are heat-labile. Such an ability of being most active close to 60°C, and quick loss of activity at higher than 65°C, is suitable for yeast bread dough, while to reduce the cost of starch processing, a higher temperature will increase the speed of a reaction, reduce the time of hydrolysis, and finally result in more energy saving. Therefore, utilization of thermostable starch-digesting amyloytic enzymes is expected to show better hydrolysis of raw starch between 60 and 70°C without losing activity during prolonged incubation.

Thus, for industrial purposes, new thermostable amyloytic enzymes may make a significant contribution to the starch-related industry.

DISCUSSION

This is the first description of amyloytic enzymes, able to directly digest raw corn starch, produced by a strain of *Rhizopus microsporus* var. *chinensis*. New kinds of amyloytic enzymes that could create new applications have attracted global interest incessantly. In this article, two glucoamylases (GA A and GAB) and one α-amylase (AA) comprised the amyloytic enzymes system of the above strain. Prep Cell was carried out for enzymes purification for its unique character of separating proteins with similar properties; however, the processing capacity of Prep Cell was inefficient to acquire abundant pure enzymes, and barely enough enzymes were obtained for enzymatic properties research. Therefore, further study on larger-scale purification and more segmented characterizations produced by *Rhizopus microsporus* var. *chinensis* CICIM-CU F0088 are being carried out in our laboratory to interpret the mechanisms of thermostable ability and raw corn starch digestion.

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