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PURIFICATION AND CHARACTERIZATION OF AN EXTRACELLULAR CHITINASE FROM *RHIZOPUS ORYZAE*

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Key words: *Rhizopus oryzae*, chitinase, purification.

ABSTRACT

A chitinase with about 50 kDa of molecular mass from *Rhizopus oryzae* was purified to electrophoretical homogeneity after DEAE Sepharose and Sephacryl S-200 chromatographs, with specific activity of 165.2 U/mg, 19.7% recovery and 4.3-fold of purification. It had optimal pH and temperature at 5.5-6.0 and 60°C, respectively, and was stable at pH 5.0-8.5 and below 50°C. Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+} and β-Me enhanced chitinase activity by 10-48% ,whereas Hg^{2+} and SDS strongly inhibited enzyme activity. This enzyme was identified that might be with both chitinolytic and amylolytic activity. According to the result of LC-MS/MS analysis, 4 identified amino acid sequences did not hit the same protein supposing that this enzyme is the first time reported.

I. INTRODUCTION

Chitin, composed of N-acetyl glucosamine by β-1-4 linkage, is one of the most abundant renewable polysaccharides in the world. It distributes in the cell walls of most fungi and exoskeleton of arthropod, including crab, shrimp, insect, spider, and etc. [19]. Some chitins and their derivatives with specific degree of polymerization (DP) were found to have antitumor, immunoadjuvant, hypolipidemic, hemostatic and antimicrobial abilities [10]. Marine waste is the most important sources of chitin. Over 80,000 tons of chitin is obtained from marine waste per year [23], which need to be more effectively utilized to prevent the impact on environment. Chitin is chemically and biochemically stable as well as biodegradable and non-allergenic [15]. Furthermore, many researches indicated that chitin and its derivative have multiple function including antimicrobial, acceleration of wound-healing, and growth of probiotics [1, 11].

Using chemical and enzymatic methods to degrade chitin into chito-oligosaccharides with specific DP would substantially increase its practicability. Chitinases are enzymes that can hydrolyze chitin into chito-oligosaccharides. They were, thus far, found in most of the organisms such as animals, plants and microorganisms even those without chitin struc ture [21]. Similar to classification of cellulolytic enzymes, chitinolytic enzymes can be crudely classified into β-N-acetylhexosaminidase (EC 3.2.1.52, known as exochitinases) and 1,4-β-poly-N- acetylglucosaminidase (EC 3.2.1.14, known as endochitinases) [19]. Exochitinases hydrolyze chitin at β-1,4-linkage of the terminal reducing sugar, while endochitinases cleave β-1,4-linkage randomly within chitin molecules. Many endochitinases from plants [21], fungi [13], and even human beings had been identified [4]. Chitinases, especially endochitinases, showed very high potential to the application of biocontrol. Most of plant chitinases are endochitinases [21] and able to degrade cell walls of fungi, which can consequently prevent themselves from pathogenic fungi [9]. However, most of microbial chitinases are exochitinases and with less effect on antifungal ability [21]. According to the previous studies, endochitinases had been found in several microbial species, such as *Bacillus* spp. [20, 22], *Pseudomonas fluorescens* [17, 18], *Clostridium thermocellum* [32] and *Vibrio proteolyticus* [8]. Most of them had antifungal activity, and could be applied on biocontrol against insects and pathogenic fungi in agriculture [19].

Rhizopus oryzae, a GRAS microorganism strain, is traditionally utilized on liquefaction and saccharification of cereal for brewing in the east. In this study, a novel chitinase from *R. oryzae* was purified and characterized.

II. MATERIALS AND METHODS

1. Materials

Rhizopus oryzae ATCC 200756 obtained from the American Type Culture Collection (VA, USA). The resins, DEAE sepharose and Sephacryl S-200, were products of Amersham

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Biosciences (GE Healthcare BioSciences Corp., MA, USA). All chemicals utilized in this report were purchased from Sigma Chemical Co. (MO, USA).

2. Cultivation of *R. oryzae* and Preparation of Crude Enzyme

R. oryzae was incubated on SIV agar plate (each liter medium contains 20 g soluble starch, 2 g urea, 5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 280 mg $CaCl_2 \cdot H_2O$, 20 mg citric acid, 15 mg $Fe(NO_3)_3 \cdot 9\ H_2O$, 10 mg $ZnSO_4 \cdot 7\ H_2O$, 1.8 mg $MnSO_4 \cdot H_2O$, 0.5 mg $CuSO_4 \cdot 5\ H_2O$, 0.5 mg $NaMoO_4 \cdot H_2O$, 15 g agar) for 5 days. The generated spores were collected and stored in 50% glycerol at -80°C. To prepare crude enzyme of chitinase, the spore of *R. oryzae* was inoculated to SIV medium to a final count of 10^6 CFU/mL. After 5-day incubation at 30°C and 120 rpm-rotation shaking, the inoculated SIV broth was harvested and filtrated through Advantec No. 1 qualitative filter paper (Toyo Roshi Kaisha, Ltd., Japan) and 0.22 μm membrane (Sartorius Stedim Biotech GmbH, Germany) to remove hyphae and spore of *R. oryzae*. The resulting filtrate was used as crude enzyme for the further purification and characterization.

3. Purification of Chitinase from Crude Enzyme

About 400 mL crude enzyme was concentrated by ultrafiltration (cutoff, 5 kDa) (Amicon Div., W. R. Grace and Co., Beverly, MA) and dialyzed against 25 mM Tris-acetate buffer pH 8.5 (TA buffer). The concentrated crude enzyme was chromatographed on a DEAE Sepharose Fast Flow column (2.6 × 5 cm), which was pre-equilibrated with TA buffer. After being washed with the same buffer, the chitinase was eluted by TA buffer containing 0 to 500 mM NaCl. The flow rate was 1 mL/min and 5 mL/tube of eluent were collected. The eluents with chitinolytic activity were collected and concentrated by Amicon ultrafiltration (cutoff, 5 kDa). The concentrated eluent was then chromatographed on Sephacry S-200 High Resolution column (2.6 × 90 cm), which was pre-equilibrated with TA buffer. The flow rate was 0.5 mL/min and 3 mL/tube of eluent were collected. Eluents with chitinolytic activity was collected for further analysis.

4. Determination of Protein Concentration

Protein concentration was determined by Bio-Rad Protein Assay. The reagent was purchased from Bio-Rad Laboratories, Inc. (CA, USA). The standard protein solution was prepared using bovine serum albumin.

5. Chitinolytic Activity Assay

Colloidal chitin was prepared by HCl swelling method [21] and applied as substrate in chitinolytic activity assay. To 0.5 mL of 1% colloidal chitin suspension, 0.5 mL enzyme solution was mixed and incubated at 50°C for 30 min with violent shaking under pH 6.0. To stop reaction, 1 mL of 2,4-dinitrosalicylic acid (DNS) reagent (containing 0.5 g/L DNS, 8 g/L NaOH and 150 g/L sodium, potassium tartarate) was added and incubated in a boiling water bath for 5 min to develop color [14]. Absorbance at 540 nm was measured to determine chitinolytic activity. One unit (U) of chitinolytic activity was defined as the amount of enzyme that can hydrolyze colloidal chitin and release 1 μg N-acetyl glucosemine equivalent within 1 min at 50°C.

6. Sodium Dodecylsulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The purity of enzyme samples during purification was determined by SDS-PAGE. Enzyme samples in dissociation buffer (62.5 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 5% β-Me and 0.01% bromophenol blue) was heated at 95°C for 3 min. The resulting samples were then analyzed by using 12.5% polyacrylamide gel [6] and stained by Coomassie Brilliant Blue R-250 [16]. Protein kit containing 14, 20, 29, 45, 66 and 97 kDa standard proteins was employed as markers.

7. LC-MS/MS Analysis

The purified chitinase was subjected to SDS-PAGE analysis. The specific chitinase band in the polyacrylamide gel was extracted and then digested by trypsin. The digested sample was further analyzed by LC-MS/MS using a QSTAR XL quadrupole time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA) serviced by Biotechnology Center, National Chung Hsing University.

8. Optimal pH and pH Stability

The optimal pH was determined by measuring the activity of chitinase at pH 3.5-9.0 (pH 3.5-5.5, 50 mM acetatee buffer; pH 5.5-7.5, 50 mM phosphate buffer; pH 7.5-9.0, 50 mM Tris-HCl buffer) [2]. To determine the pH stability, purified enzyme in various pH values of buffer (as shown above) was incubated at 50°C for 30 min. An equal volume of 0.2 M citrate buffer (pH 6.0) was added to maintain the pH at 6.0. The residual activity was measured by chitinolytic activity assay.

9. Optimal Temperature and Thermal Stability

The optimal temperature was determined by measuring the activity of chitinase at 30-50°C. To determine the thermal stability, the purified chitinase was incubated at 30-70°C for 30 min, and then chilled immediately in ice water for 5 min [2]. The residual activity was measured by chitinolytic activity assay.

10. Effect of Metal Ions and Chemicals

Purified chitinase in 50 mM citrate buffer (pH 6.0) with 5 mM Li^+, K^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Cr^{3+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+}, and with 5 mM β-mercaptoethanol (β-Me), urea, SDS and ethylenediaminetetraacetic acid (EDTA) were incubated at 50°C for 30 min [2]. The residual activity was measured according chitinolytic activity assay.

Table 1. Summary of purification of chitinase from cultural broth of *R. oryzae.*

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude	37.4	1423.5	38.0	100.0	1.0
DEAE Sepharose	4.2	418.9	99.7	29.4	2.6
Sephacryl S-200	1.7	280.8	165.2	19.7	4.3

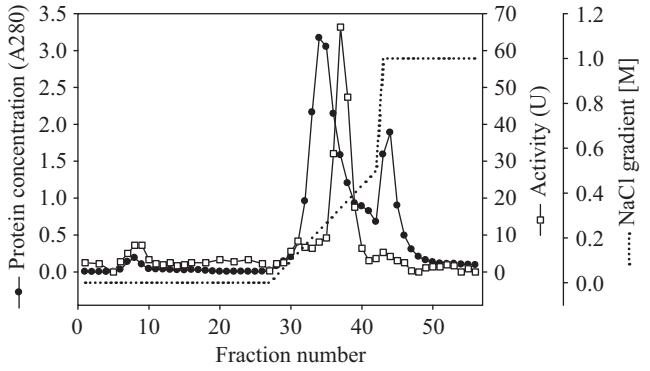

Fig. 1. DEAE Sepharose FF column chromatogram. The bed volume of the DEAE Sepharose FF was 26.5 cm^3 (2.6 × 5 cm). Flow rate was 1 mL/min and 5 mL per fraction was collected.

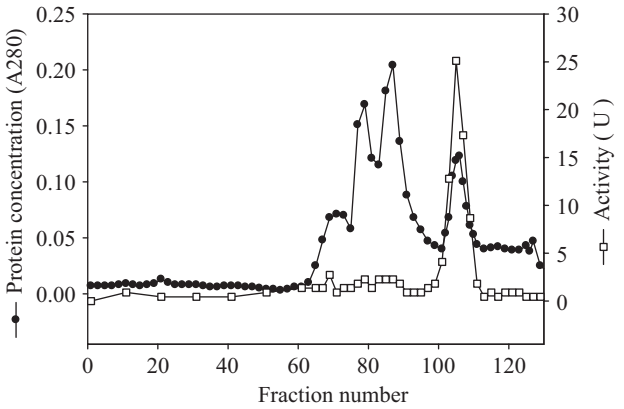

Fig. 2. Sephacryl S-200 HR column chromatogram. The bed volume of the Sephacryl S-200 HR was 478 cm^3 (2.6 × 90 cm). Flow rate was 0.5 mL/min and 3 mL per fraction was collected.

11. Substrate Specificity

For testing substrate specificity, various polysaccharides were applied on chitinolytic activity assay to replace colloidal chitin. Agarose, cellulose, carboxymethylcellulose sodium salt (CMC), soluble starch, Avicel®, chitosan, α-crystalline chitin, glycol chitosan and colloidal chitin in the concerntration of 0.5% were applied on chitinase assay. After 30 min of reaction, an equal volume of DNS reagent was added to stop the reaction and then incubated in boiling water bath for 5 min. Absorbance of 540 nm was measured to determine hydrolytic ability to the polysaccharides.

III. RESULTS AND DISCUSSION

1. Purification of Chitinase from Crude Enzyme

In DEAE Sepharose chromatography, fractions 37-39 were observed with chitinase chitinolytic activity (Fig. 1). The fractions of 37-39 were collected and concentrated by Amicon ultrafiltration (cutoff, 5 kDa). The concentrated eluent was further separated by Sephacryl S-200 column (2.6 × 90 cm) with 3 mL/tube and a flow rate of 0.5 mL/min. In Sephacryl S-200 column chromatogram, protein peak overlapped with chitinolytic activity peak, suggesting highly purified (Fig. 2). The enzyme was purified to electrophoretical homogeneity with a molecular mass of 50 kDa (Fig. 3(a)). At this stage, the sample was purified with specific activity of 165.2 U/mg, 19.7% recovery and 4.3-fold purification (Table 1).

To ensure the purified protein is target chitinase, crude enzyme was mixed with equal volume of colloidal chitin and iced for 1 hr. The mixture was then centrifuged. The protein

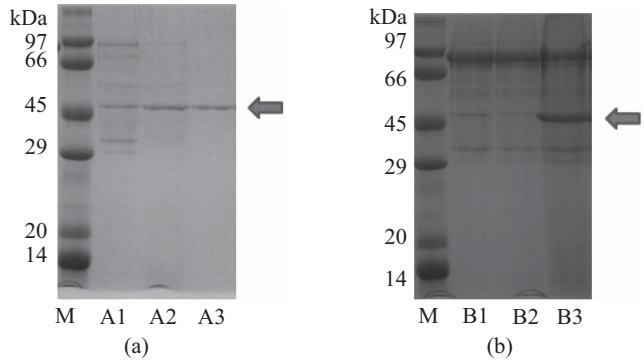

Fig. 3. (a) Profile of SDS-PAGE of chitinase: M, marker; lane A1, crude enzyme; lane A2, partial purified enzyme from DEAE sepharose FF chromatography; lane A3, purified enzyme from Sephacryl S-200 chromatography. (b) Adsorbing test of colloidal chitin with crude enzyme: M, marker; lane B1, crude enzyme; lane B2, protein un-accumulating in colloidal chitin; lane B3, protein accumulating in colloidal chitin pellet.

accumulating in colloidal chitin pellet and remaining in supernatant were analysed by SDS-PAGE individually. It was found that considerable quantity of chitin bonded protein with molecular weight of 50 kDa accumulated in the portion of precipitated colloidal chitin (Fig. 3(b), lane B3). The position of chitin bonded protein on the profile of SDS-PAGE was corresponding to the target protein that purified in this study

2. LC-MS/MS Analysis

By LC-MS/MS analysis and Mascot Search Results, 4 specific amino acid sequences (IVLGMPLYGR, QLFLLKQQNR,

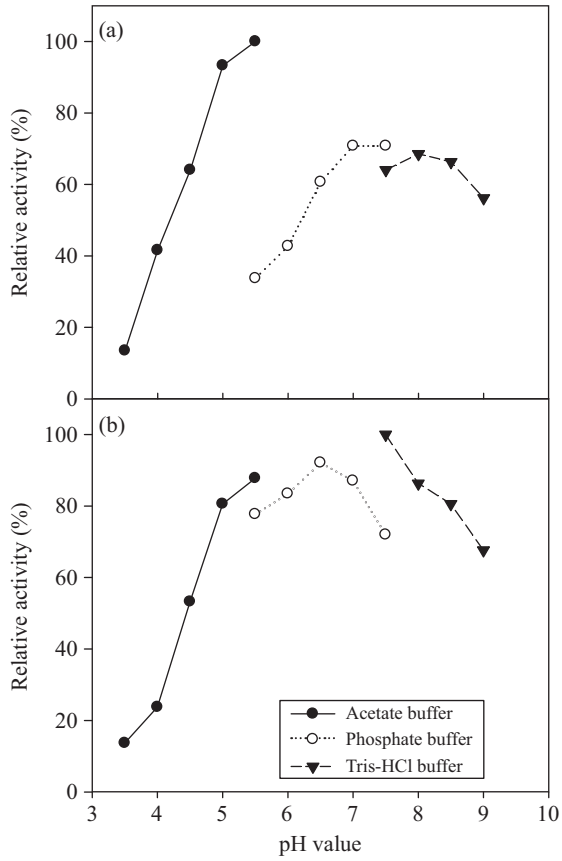

Fig. 4. pH effect on chitinase from *R. oryzae*. (a) optimal reaction pH and (b) pH stability.

HYPTDSWNDVGTNVYGCVK and NYNPQDIPADK) were identified, and can be hit to gi|115396398 (endochitinase 1 precursor, from *Aspergillus terreus* NIH2624), gi|358372245 (class V chitinase, from *Aspergillus kawachii* IFO 4308) and gi|119491681 (class V chitinase, putative, from *Neosartorya fischeri* NRRL 181). According to search result, 3 identified amino acid sequences hit to gi|115396398 (endochitinase 1 precursor, from *Aspergillus terreus* NIH2624); however the hit protein is with molecular mass of 44.7 kDa which does not match the molecular mass of the purified chitinase in this report. To our knowledge, there are still few chitinases from *Rhizopus oryzae* were reported. Yanai *et al*. [31] reported 2 chitinase encoded genes from *R. oligosporus*; however, the amino acid sequence does not match our results. Takaya *et al*. [24] reported intracellular chitinase gene encoded a 43.5 kDa protein in *Pseudomonas aeruginosa* that would smaller than that found in this study. Therefore, the extracellular chitinase of *R. oryzae* in this study is the first time reported.

3. Effect of pH and Temperature

The most efficient reaction pH and temperature for the chitinase were 5.5-6.0 and 60°C, respectively, through the breaking point of optimal pH was 8.0 (Fig. 4). The chitinase was stable at pH 5.0-8.5 and below 50°C (Figs. 4 and 5).

Table 2. Effect of metal ions and chemicals on chitinase from *R. oryzae*.

	Metals ions	Relative activity (%)	Metals ions	Relative activity (%)
(A)	None*	100.0	Co^{2+}	111.8
	Li^{+}	97.1	Ni^{2+}	89.1
	K^{+}	94.9	Cu^{2+}	56.3
	Mg^{+}	85.5	Zn^{2+}	69.8
	Ca^{2+}	124.6	Cd^{2+}	94.4
	Sr^{2+}	116.2	Hg^{2+}	0.2
	Ba^{2+}	118.6	Cr^{3+}	105.6
	Mn^{2+}	148.3		
(B)	Chemicals		Chemicals	
	β-Me	121.7	SDS	22.7
	Urea	103.6	EDTA	94.4

The purified chitinase was incubated with 5 mM metal ion or chemicals at 50°C for 30 min. The residual activity was then measured.
*The chitinase activity without additional metal ion or chemical added was defined as 100%.

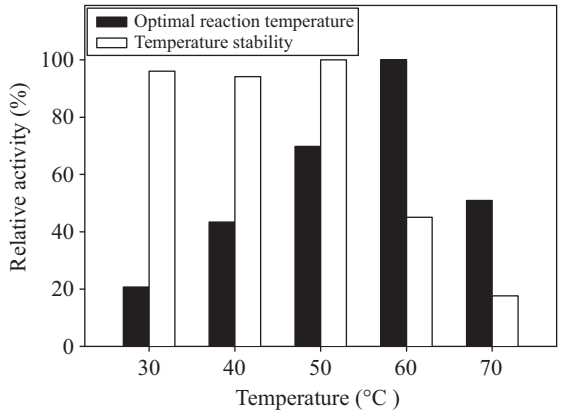

Fig. 5. Thermal effect on chitinase from *R. oryzae*.

Generally, chitinases from molds have lower optimal reaction pH and temperature, such as *Monascus purpureus* (pH4, 30°C) [27], *Penicillium* sp. (pH5, 40°C) [12], *Gladiolus gandavensis* (pH 5) [30]. However, the results of higher reaction pH and temperature were similar to chitinase from bacterial and plants, such as *Bacillus thuringiensis* (pH 6, 50°C) [3] and *Pseudomonas aeruginosa* K-187 (pH 8, 50°C) [25].

4. Effects of Metal ions and Chemicals

As indicated in Table 2, purified chitinase was moderately inhibited by Mg^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}, and highly inhibited by Hg^{2+} and SDS. Hg^{2+} can bind the thiol groups and interacts with tryptophan residues or the carboxyl group of amino acids [28]. SDS can interact with hydrophobic structure and cause destruction of protein. It would be the reason why Hg^{2+} and SDS strongly inhibited enzyme activity. Among the ions and chemicals, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+} and β-Me enhanced chitinase activity 10-48%. This result was similar to the chiti-

Table 3. Substrate specificity of purified chitinas.

Substrate	Relative activity (%)
Colloidal chitin*	100.0
Soluble starch	81.6
Cellulose	6.0
α-crystalline chitin	2.0
Chitosan	1.0
CMC	1.0
Agarose	0
Avicel	0
Glycol chitosan	0

* Utilization of colloidal chitin as substrate was defined as 100%.

nase from *Bacillus licheniformis* [7]. In this study, Mn^{2+} activated chitinase activity sharply, however, quite different result was described in *Pseudomonas aeruginosa* K-187 [26], *Aspergillus fumigatus* YJ-407 [29] and *Streptomyces viridificans* [5].

5. Substrate Specificity

Among the selected substrates, only colloidal chitin and soluble starch were dramatically hydrolyzed. Against colloidal chitin, soluble starch showed 81.6% activity units at absorbance at 540 nm. Supposing that the purified chitinase might have by-function of amylolysis. Cellulose, α-crystalline chitin, chitosan, CMC, Agarose, Avicel and glycol chitosan were not hydrolyzed by the purified chitinase from *R. oryzae* (Table 3).

IV. CONCLUSION

In summary, the purified chitinase from a chitinase-producing *R. oryzae*, a GRAS microorganism strain, in this study is the first time to be reported. Extracellular chitinolytic activity was observed and the chitinase was further purified. Through substrate specificity analysis, this enzyme might be a bi-functional enzyme that owns both chitinase and amylase activity and would be worth for further research.

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