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Purification and Properties of an Endoglucanase from *Aspergillus Oryzae* VTCC-F045

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Abstract: An extracellular endoglucanase (EG) from *Aspergillus oryzae* VTCC-F045 was purified 2.6-fold to homogeneity throughout Sephadex G100 gel filtration chromatography. Purified EG had a specific activity of 40.36 U/mg protein and a molecular mass of 36 kDa. Optimum temperature and pH were 55°C and 5.5, respectively. The enzyme was stable at up to 55°C and in pH range 5.0-6.0 with a residual activity of over 60% for 6 hours treatment. Metal ions, EDTA, organic solvents and detergents inhibited the enzyme.

Key words: *Aspergillus oryzae* VTCC-F045; characterization; endoglucanase; purification

INTRODUCTION

Cellulose is a major polysaccharide constituent of plant cell walls and one of the most abundant organic compounds in the biosphere (Hong *et al*. 2001). Biological degradation of cellulose involves the synergistic action of three enzymes: endoglucanase or carboxymethyl cellulase (CMCase) (endo β-1,4-glucanase, E.C. 3.2.1.4), exoglucanase or cellobiohydrolase (exo-β-1,4-glucanase, E.C. 3.2.1.91), and β-glucosidase (β-D-glucoside glucohydrolase, E.C. 3.2.1.21) (Bhat and Bhat 1997). Endo-β-1,4-glucanase randomly hydrolyzes internal β-1,4-D-glycosidic bonds in cellulose producing oligos and reducing polymer length, while exo-β-1,4-glucanase (cellobiohydrolase) cleave cellobiosyl residues from the non-reducing end of cellulose chain. Then, cellobiose is hydrolyzed by β-glucosidase to yield two glucose units.

Endoglucanase as well as other cellulases have a broad variety of applications in food, animal feed, brewing, paper pulp, and detergent industries, textile industry, fuel, chemical industries, waste management and pollution treatment (Bhat and Bhat 1997; Ole *et al*. 2002; Anish *et al*. 2006). Among theses enzymes, EGs have been well studied and are produced by various microbes (bacteria, yeast, and fungi), plants, and protozoans. Especially, the filamentous fungi *Aspergillus* spp. (*A. fumigatus*, *A. niger*, *A. oryzae*, *A. terreus*) are preeminent in endoglucanase production (Akiba *et al*. 1995; Chen *et al*. 2001; Gao *et al*. 2008; Elshafei *et al*. 2009). The present work described purificantion and characterization of an endoglucanase from *A. oryzae* VTCC-F045 deposited at the Vietnam Type Culture Collection.

MATERIAL AND METHODS

Chemicals:

Carboxymethyl cellulose (CMC) was from Biochemika. Sephadex G100 were supplied by Pharmacia Co. (Sweden); Tween 20 and Tween 80 from BioBasic Inc. (USA); SDS from Sigma (USA) and Triton X-100 from Merck (German). All other chemicals were of analytical grade unless otherwise stated.

Organism and Culture Conditions:

A. oryzae VTCC-F045 strain from the Vietnam Type Culture Collection (Institute of Microbiology and Biotechnology, Vietnam University Hanoi) was grown in 200 ml Erlenmeyer flasks containing 50 ml of nutrient medium with the following composition (w/v): 1% soybean, 1% CMC, 0.2% KH_2PO_4, 0.03% $CaCl_2.2H_2O$, 0.14% $(NH_4)_2SO_4$, 0.03% $MgSO_4.7H_2O$, 0.4% lactose, and supplemented with 1 ml of salt solution (18 mM $FeSO_4$, 6.6 mM $MnSO_4$, 4.8 mM $ZnSO_4.7H_2O$, 15 mM $CoCl_2$), pH 5.5. The strain was grown at 28°C for 5 days on a 200 rpm rotary shaking. The biomass was removed by centrifugation for 10 min at 8000x g and the culture supernatant was used for EG purification.

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Endoglucanase Assay:

Endoglucanase activity was determined by the method of reducing sugars formed by enzymatic hydrolysis of CMC (Nelson 1944). A reaction mixture of 500 µl of the crude or purified enzyme was incubated with 500 µl of 0.5% (w/v) CMC in 0.1 M sodium acetate buffer pH 5 for 5 min at 55°C. The reaction was stopped by the addition of 1 ml of combined copper reagent and heated for 20 min in a boiling water bath. The tubes were cooled down to room temperature and 1 ml of arsenomolybdate reagent was added to all the tubes. The amount of reducing sugar liberated in the reaction mixture was measured at 660 nm (Nelson 1944). Glucose was used as the standard reduced sugar for concentration estimation. One unit of active endoglucanase was defined as the amount of enzyme which liberated 1 µmol glucose per minute under the standard conditions.

Purification of Endoglucanase:

The culture was centrifuged for 10 min at 12500 rpm and ammonium sulfate (65% saturation) was slowly added to 40 ml crude enzyme solution with constant stirring. The mixture was left overnight at 4°C, and then centrifuged at 12500 rpm for 10 min at 4°C. The supernatant was carefully decanted and the precipitate was redissolved in 0.1 M acetate buffer pH 5.5 and dialyzed for desalination. The dialyzed fraction (15 ml) was applied to a Sephadex G-100 column (2.6 x 6 cm) pre-equilibrated with 50 mM potassium phosphate buffer pH 7.5 at a flow rate of 25 ml/h until OD_{280nm} was less than 0.01, then washed with the same buffer. The eluate was collected with 14 fractions; 2 ml per fraction. The fractions containing highly active endoglucanase were pooled and used as purified enzyme for characterization. All purification steps were carried out at 4°C, unless otherwise specified.

Gel Electrophoresis and Protein Concentration:

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (Laemmli 1970) with Biometra equipment. SDS-PAGE was usually performed on gel containing 12.5% (w/v) acrylamide according to the manufacture's recommendation. The gel was stained with Coomassie Brilliant Blue R-250 for protein. The protein concentration was determined using bovine serum albumin as standard (Bradford 1976).

Temperature and pH Optima Determination:

The pH and temperature optima of EG were determined by measuring the activity as described above using 100 mM potassium acetate buffer (pH 3.5-5.5) and potassium phosphate buffer (pH 6-7.5), and in the temperature range of 30-80°C, respectively.

Temperature and pH Stability:

For the determination of temperature and pH stability, purified enzyme (0.14 µg for each reaction) was preincubated at different temperatures 30-60°C and pH range (100 mM potassium acetate pH 4.5-5.5 and potassium phosphate pH 6-7.5) for 1-8 h, respectively. The residual activity was then determined as above mentioned.

Effect of Metal Ions, Organic Solvents, and Detergents:

Purified EG (0.14 µg for each reaction) was incubated in 4-12 mM of various metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Ag^{+}, and K^{+}) and EDTA, in 20% (v/v) of different solvents (methanol, ethanol, isopropanol, acetone, and n-butanol), and in 0.4-2% (w/v) of different detergents (Tween 80; Tween 20, SDS; and Triton X-100) at 37°C for 4 h. The residual activity was then determined.

RESULTS AND DISCUSSIONS

Purification of A. Oryzae Endoglucanase:

The EG production by *A. oryzae* VTCC-F045 in the culture medium was 3.31 U/ml (specific activity of 15.54 U/mg protein) after 5 days of cultivation. After precipitation, dialysis and application to the Sephadex G-100 column, total protein content was reduced by about 97% and the specific activity was increased to 40.36 U/mg (100%), indicating 2.59-fold purification with a yield of 6% (Table 1, Fig. 1A). The enzyme was relatively pure as confirmed by SDS-PAGE (Figure 1B, lane 1). The molecular weight of this protein was estimated to be 36 kDa.

Other EGs from *Aspergillus* strains were purified to homogeneity through a similar purification scheme involving ammonium sulfate precipitation, acetone fractionation, and various chromatography (gel filtration: Sephadex G-100, ion exchange: DEAE-Sephadex A-50, affinity: Sepharose-4B, exclusion). Purified EGs from

Aspergillus strains had different molecular weights from 24 to 80 kDa on SDS-PAGE: 24-39 kDa (*A. aculeatus* SM-L22, (Chen *et al*. 2001)), 25 kDa (*A. terreus* M11, (Gao *et al*. 2008)), 78-80 kDa (*A. terreus*, (Nazir *et al*. 2009)). EGs were purified 18-fold with 14% yield and a specific activity of 67 U/mg protein (*A. terreus* M11, (Gao *et al*. 2008)), 27-fold and 10.5% yield (*A. terreus* DSM 826, (Elshafei *et al*. 2009)), 40-fold and 1.32% yield (*A. terreus* AN_1, (Nazir *et al*. 2009)).

Table 1: Summary of purification of EG from *A.oryzae* VTCC-F045

Purification step	Total volume (ml)	Total activity (U/ml)	Protein (mg/ml)	Spec. activity U/mg of protein	Yield (%)	Purification fold
Crude enzyme	40	132.4	8,52	15.54	100	1.0
$(NH_4)_2SO_4$ precipitation	15	27.15	1.11	24.46	21	1.6
Sephadex G-100	7	7.91	0.196	40.36	6	2.6

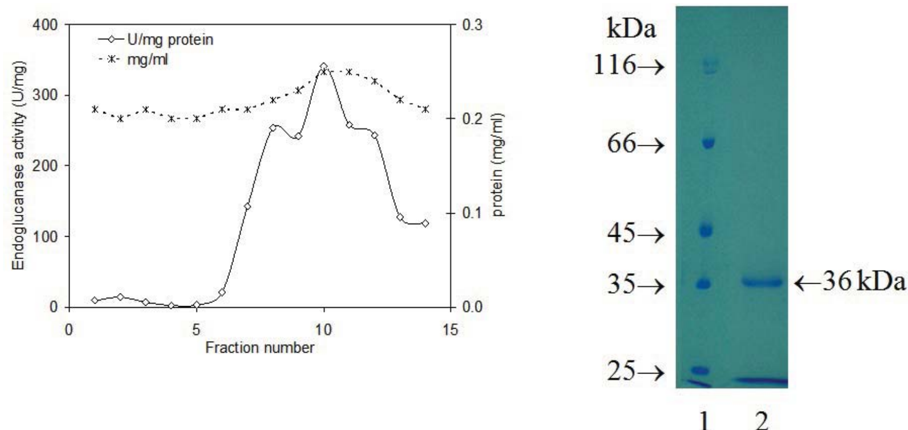


Fig. 1: Sephadex G-100 gel filtration chromatography of the endoglucanase from *A. oryzae* VTCC-F045 (A); SDS-PAGE of the purified endo-β-1,4-glucanase from *A. oryzae* VTCC-F045 through Sephadex G-100 (B); lane M: molecular weight marker; lane 1: the purified EG.

Temperature and pH Optima:

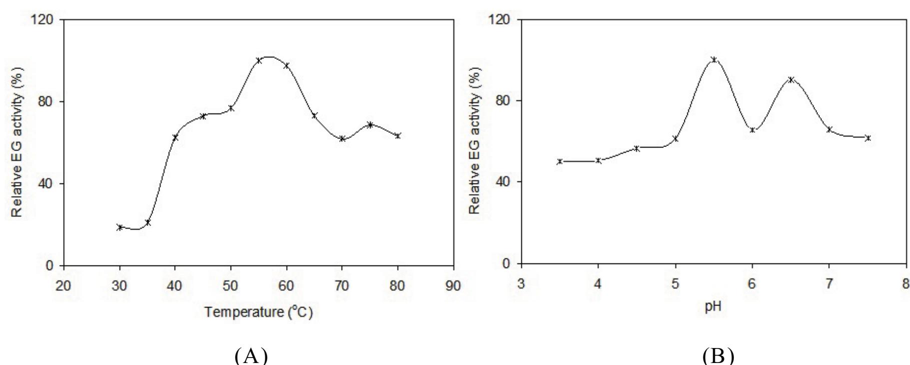
The *A. oryzae* EG activity increased gradually from 12.67 U/mg (19%) at 30°C to the maximum of 67.99 U/mg (100%) at 55°C (Fig. 2A) and then decreased gradually to 42.99 U/mg (63%) at 80°C. The EG activity gradually increased from 48.03 U/mg (50%) at pH 3 to the maximum of 95.88 U/mg (100%) at pH 5.5 and then decreased gradually to 59.02 U/mg (62%) at pH 7.5 (Fig. 2B).

Most EGs from *Aspergillus* strains showed a similar profile of optimum temperature (55-70°C) and optimum pH (3.5-5.0). The optimum temperature and pH for activity of the purified EG from *A. terreus* DSM 826 were found to be 50°C and pH 4.8, respectively (Elshafei *et al*. 2009), from *A. aculeatus* SM-L22: 55-70°C and pH 3.5-4.0 (Chen *et al*. 2001), from *A. terreus* M11: 60°C and pH 2 (Gao *et al*. 2008), from *A. terreus* strain AN_1: 60°C and pH 4 (Nazir *et al*. 2009), from *A. niger* IFO31125: 70°C and pH 6-7 (Akiba *et al*. 1995).

Temperature and pH Stability:

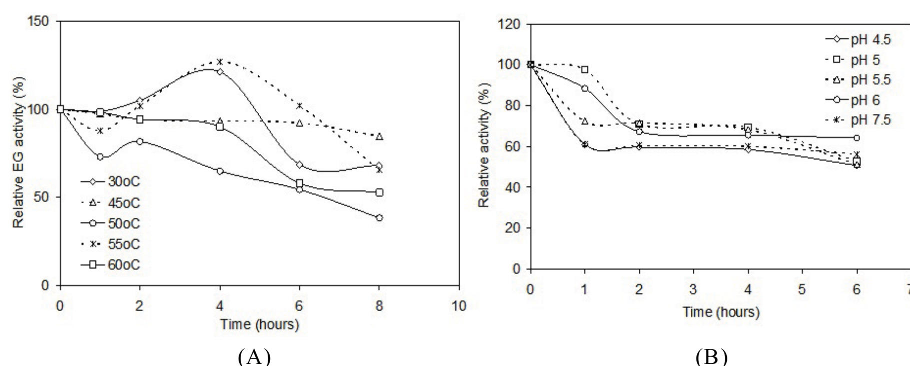
The *A. oryzae* EG was temperature stable up to 55°C and pH stable in pH range of 5-6, the residual activity increased by up to 21-27% after 4 hours treatment at 30-55°C (Fig. 3A) and over remained 72% after 1 hour treatment at pH 5-6 (Fig. 3B).

The purified endoglucanase from *A. terreus* DSM 826 could stand heating up to 50°C for 1 h without apparent loss of activity (Elshafei *et al*. 2009). However, the enzyme, incubated at 80°C for 5 min, showed about 56% loss of activity. The EG from *A. terreus* M11 was stable from pH 2 to 5 and retained more than 60% of its activity after heating at 70°C for 1 h (Gao *et al*. 2008). The EG from *A. terreus* strain AN_1 was stable over a broad range of pH (3-5) at 50°C (Nazir *et al*. 2009). The stable pH range for EG from *A. niger* IFO31125 was 5-10. The enzyme was very thermally stable and no loss of original activity was found on incubation at 60°C for 2 h (Akiba *et al*. 1995).



(A) (B)

Fig. 2: Temperature (A) and pH (B) optima curves of purified EG from *A. oryzae* VTCC-F045.



(A) (B)

Fig. 3: Temperature (A) and pH (B) stability curves of purified EG from *A. oryzae* VTCC-F045.

Effect of Metal Ions and EDTA:

The addition of metal ions and EDTA showed a slidely inhibitory effect on EG activity (Table 2). The addition of 4, 8 and 12 mM metal ions and EDTA decreased the EG activity by up to 16, 38 and 43%, respectively. Chen *et al*. (2001) also reported that the activities of all components from *A. aculeatus* SM-L22 were stimulated by Fe^{2+}. The addition of 25 mM of Co^{2+} and 50 mM of Zn^{2+} activated EG activity of *A. terreus* DSM 826 by about 83 and 25%, respectively. On the other hand, Hg^{2+} inhibited the activity of the EG by about 50 and 71% at a concentration of 25 and 50 mM, respectively (Elshafei *et al*. 2009). EG activity from *A. terreus* M11 was inhibited by 77% and 59% by 2 mM of Hg^{2+} and 2 mM of Cu^{2+}, respectively (Gao *et al*. 2008). EG from *A. niger* IFO31125 was inhibited by Hg^{2+} and Cu^{2+} but was not affected by other inhibitors of thiol enzymes such as p-chloromercuribenzoate and N-ethylmaleimide (Akiba *et al*. 1995).

Table 2: Effect of metal ions and EDTA on EG activity from *A. oryzae* VTCC-F045.

Metal ions (mM)	Residual activity (%)		
	4	8	12
Ag^{+}	84	99	73
Ca^{2+}	90	81	73
Co^{2+}	92	93	92
Cu^{2+}	96	92	87
EDTA	96	89	71
Fe^{2+}	87	64	58
K^{+}	103	86	83
Mn^{2+}	97	82	77
Ni^{2+}	95	92	70
Zn^{2+}	87	62	57

Effect of Organic Solvents and Detergents:

The addition of organic solvents tested (acetone, ethanol, isopropanol, methanol and n-butanol) at the final

concentration of 20% lead to 19-57% reduction in EG activity (Fig. 4A). n-Butanol and acetone strongly showed an decrease in EG activity by one half (Fig. 4A). All tested detergents at the concentration of 0.4-2% inhibited EG, Tween 20, Tween 80 and Triton X-100 decreased EG activity by up two third, especially, SDS at the concentration of 0.4-2% strongly reduced EG activity to 4-15% (Fig. 4B).

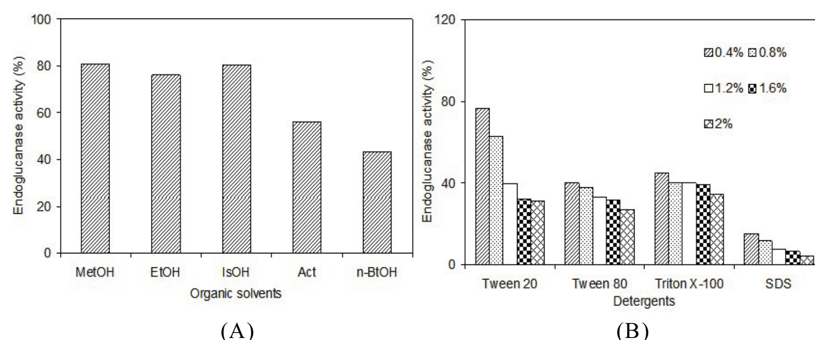


Fig. 4:. Effect of organic solvents (A) and detergents (B) on EG activity.

Conclusion:

An extracellular endoglucanase from *A. oryzae* VTCC-F045 was purified 2.6 folds through Sephadex G-100 gel filtration chromatography. The purified EG had a specific activity of 40.36 U/mg protein and was a monomeric protein with a molecular mass of 36 kDa. The optimal temperature and pH were at 55°C and 5.5, respectively. This enzyme was stable at pH 5-6 and at 30-55°C. EG was inhibited by tested metal ions, EDTA, organic solvents and detergents.

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