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Purification and Properties of a Novel Thermoactive Endoglucanase from *Aspergillus awamori* VTCC-F099

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Abstract: A novel extracellular endoglucanase from *Aspergillus awamori* VTCC-F099 was purified 3.08-fold to homogeneity throughout gel filtration and ion exchange chromatography. Purified EG had a specific activity of 65.3 U/mg protein and a molecular mass of 32 kDa. K_m and V_{max} were 5.83 mg CMC/ml and 333.33 U/mg protein, respectively. Optimum temperature and pH were 50°C and 5, respectively. The enzyme was stable at up to 40°C and in pH range 4.5-5.5 with a residual activity of over 80% for 24 hours treatment. Fe^{2+}, Cu^{2+} and EDTA activated EG activity whereas other metal ions, detergents and organic solvents inhibited the enzyme. These results suggested that EG from *A. awamori* VTCC-F099 could potentially be used as an additive in the feed for monogastric animals.

Key words: *Aspergillus awamori* VTCC-F099, novel endoglucanase, purification, characterization

INTRODUCTION

Cellulose is a major polysaccharide constituent of plant cell walls and one of the most abundant organic compounds in the biosphere (Hong, J., 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, M.K. and S. 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 (Anish, R., 2006; Bhat, M.K. and S. Bhat, 1997; Ole, K., 2002). 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, S., 1995; Chen, G., 2001; Elshafei, A.M. *et al*., 2009; Gao, J., 2008). In this study, we reported the purification and characterization of a novel thermoactive endoglucanase from *Aspergillus awamori* VTCC-F099 for first time.

MATERIALS AND METHODS

Chemicals:

Peptone, Tween 20 and Tween 80 was purchased from Bio Basic Inc., yeast extract from Difco, 3,5-dinitrosalicylic acid (DNS) from Fluka, carboxymethyl cellulose (CMC) from Prolabo, Triton X-100 from Merck. Sephadex G100 and DEAE-Sephadex A-50 were supplied by Phamarcia Co.; SDS from Sigma. All other chemicals were of analytical grade unless otherwise stated.

Organism and Culture Conditions:

The strain *Aspergillus awamori* VTCC-F099 purchased from Vietnam Type Culture Collection (Institute of Microbiology and Biotechnology, Vietnam University Hanoi) was grown on the liquid medium containing

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(g/l): KH_2PO_4 2, $CaCl_2$ 0.3, $MgSO_4.7H_2O$ 0.3, CMC 20, ammonium acetate 3, corncobs 30, Tween 80 1; Trace elements were also added, using 1% (v/v) solution of salts: 18 mM $FeSO_4.7H_2O$, 6.6 mM $MnSO_4$, 4.8 mM $ZnSO_4$, 15 mM $CoCl_2$, pH 6.5. The inoculated flasks were incubated for 4 days at 30°C on a rotary shaker at 200 rpm.

Endoglucanase Purification:

The culture was centrifuged for 10 min at 8000g. Ten ml of the crude enzyme (167 units) 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 then washed with the same buffer. The eluate was collected with 2 ml per fraction. A highly active endoglucanase pool of 20 ml through Sephadex G-100 column was further applied to DEAE-Sephadex A-50 ion exchange chromatography pre-equilibrated with 50 mM Tris HCl buffer pH 8 containing 50 mM NaCl (buffer A), then washed with the same buffer. The protein was eluted with 50 mM Tris HCl buffer pH 8 containing 1 M NaCl (buffer B) at a flow rate of 20 ml/h until $OD_{280nm} < 0.010$. The eluate was collected with 2 ml per fraction. The fractions containing highly active endoglucanase activity were pooled and used for characterization. All purification steps were carried out at 4°C, unless otherwise specified.

EG Activity Estimation:

Endoglucanase activity was determined by Mandels, M., (1976) with 0.5% (w/v) CMC in 100 mM potassium phosphate pH 6.5. The amount of reducing sugars released in the reagent solution at 50°C for 20 min were read at the wavelength of 540 nm (spectrophotometer UV-2500, Hewlett Package, USA). Glucose was used as the standard for the estimation of reducing sugars. One unit of the endoglucanase activity was defined as the amount of enzyme required to release 1 µmol glucose per minute under experiment conditions.

SDS-PAGE and Protein Concentration:

The homology and molecular mass of EG was determined by 12.5% SDS polyacrylamide gel electrophoresis with Biometra equipment (Laemmli, U.K., 1970). The gels were stained with Coomassie Brilliant Blue R-250 for protein. The protein concentration was determined using bovine serum albumin as standard (Bradford, M.M., 1976).

Kinetic Parameters:

The apparent kinetic parameters (V_{max} and K_m) were determined against CMC as substrate using Linewear-Burk plots.

Temperature and pH Optima:

The pH and temperature optima of EG were determined by measuring the activity as described above using 100 mM sodium acetate (pH 3.0-5.5) and potassium phosphate (pH 6-8) at 50°C, and in the temperature range of 30-70°C in 100 mM potassium phosphate pH 6.5, respectively.

Temperature and pH Stability:

For the determination of temperature and pH stability, purified enzyme (2 µg protein for each reaction) was preincubated at different temperatures 30-70°C for 6-72 h, and pH range 3.5-8 (100 mM sodium acetate pH 3.5-5.5 and 100 mM potassium phosphate pH 6-8) at 37°C for 2 h and pH 4-6 at 37°C for 6-72 h, respectively. The residual activity was then determined.

Effect of Metal Ions, Organic Solvents and Detergents:

Purified EG (2 µg protein) was incubated in 5-15 mM of various metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Ag^{+}, K^{+}) and EDTA, in 10-30% (v/v) of different solvents (acetone, methanol, ethanol, isopropanol, n-butanol), and in 0.5-2% (w/v) of different detergents (Tween 80, Tween 20, Triton X-100, and SDS) at 37°C for 2 h. The residual activity was then determined as described above.

RESULTS AND DISCUSSION

Purification of A. awamori Endoglucanase:

The EG production by *A. awamori* VTCC-F099 in the culture medium was 16.69 U/ml (specific activity of 21.19 U/mg protein) after 4 days of cultivation. This culture supernatant was applied to Sephadex G-100 gel filtration chromatography, then to DEAE-Sephadex A-50 ion exchange chromatography. The EG was purified 3.1 fold with 7.1% yield and a specific activity of 65.3 U/mg protein (Table 1). The molecular weight was estimated to be 32 kDa on SDS-PAGE (Fig. 1).

Table 1: Summary of purification of EG from *A. awamori* VTCC-F099.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude	7.88	166.86	21.19±0.038	100	1
Sephadex G-100	5.62	126.01	22.44±0.159	75.52	1.06
DEAE-Sephadex A-50	0.18	11.86	65.30±1.434	7.11	3.08

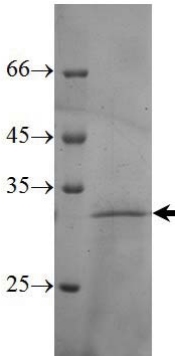


Fig. 1: SDS-PAGE of the purified EG from *A. awamori* VTCC-F099 through DEAE-Sephadex A-50 ion exchange chromatography; lane 1: the purified EG; lane M: molecular weight marker.

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, G., 2001)), 25 kDa (*A. terreus* M11, (Gao, J., 2008)), 78-80 kDa (*A. terreus*, (Nazir, A., 2009)). EGs were purified 18-fold with 14% yield and a specific activity of 67 U/mg protein (*A. terreus* M11, (Gao, J., 2008)), 27-fold and 10.5% yield (*A. terreus* DSM 826, (Elshafei, A.M. *et al*., 2009)), 40-fold and 1.32% yield (*A. terreus* AN_1, (Nazir, A., 2009)).

Kinetic Parameters:

The K_m and V_{max} values obtained for the purified EG from *A. awamori* VTCC-F099 were 5.83 mg CMC/ml and 333.33 U/mg protein, respectively. K_m is a measure of the apparent affinity of enzyme for its substrate. The K_m value (5.83 mg CMC/ml) was lower than that obtained for EGs from *A. niger* (52-80 mg/ml, (Hurst, P.L., 1977)) and from *S. sclerotorium* (8.7 mg/ml, (Waksman, G., 1991)), but higher than that (0.5 mg/ml) obtained for cellulase of *M. verrucaria* (Halliwell, G., 1965). The V_{max} (333.33 U/mg protein) obtained for the purified EG from *A. awamori* VTCC-F099 was higher than those obtained for the purified EGs from *A. terreus* AN_1 (Nazir, A., 2009) and *A. terreus* DSM 826 (Elshafei, A.M. *et al*., 2009) (200 and 4.35 U/mg protein, respectively) when CMC was used as substrate.

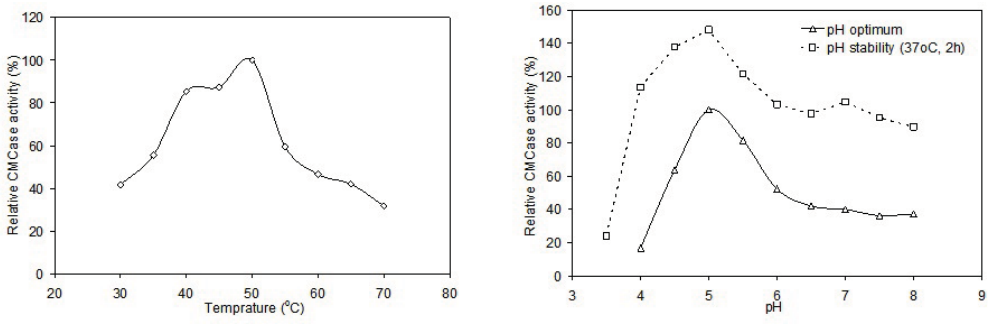
Temperature and pH Optima:

The EG from *A. awamori* VTCC-F099 had an optimum temperature of 50°C and an optimum pH of 5 (Fig. 2).

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, A.M. *et al*., 2009), from *A. aculeatus* SM-L22: 55-70°C and pH 3.5-4.0 (Chen, G., 2001), from *A. terreus* M11: 60°C and pH 2 (Gao, J., 2008), from *A. terreus* strain AN_1: 60°C and pH 4 (Nazir, A., 2009), from *A. niger* IFO31125: 70°C and pH 6-7 (Akiba, S., 1995).

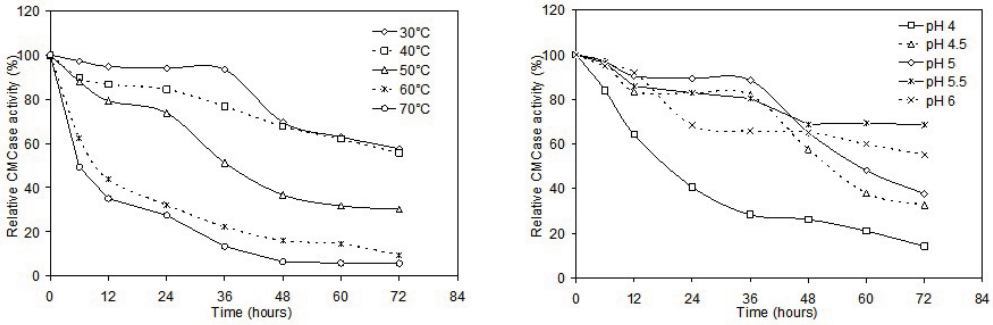
Temperature and pH Stability:

The *A. awamori* EG was temperature stable up to 40°C and pH stable in pH range of 4.5-5.5, the residual activity was over 80% after 36 hours treatment at 30-40°C (Fig. 3A) and pH 4.5-5.5 (Fig. 3B). Interestingly, the enzyme, incubated at 37°C, in pH range of 4-8 for a short time of 2 h, showed no loss of activity, but an increase by 48% at pH 5 (Fig. 2B). The purified endoglucanase from *A. terreus* DSM 826 could stand heating up to 50°C for 1 h without apparent loss of activity (Elshafei, A.M. *et al*., 2009). However, the



(A) (B)

Fig. 2: Temperature (A) and pH (B) optima curves of purified EG from *A. awamori* VTCC-F099.



(A) (B)

Fig. 3: Temperature (A) and pH (B) stability curves of purified EG from *A. awamori* VTCC-F099.

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, J., 2008). The EG from *A. terreus* strain AN_1 was stable over a broad range of pH (3-5) at 50°C (Nazir, A., 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, S., 1995).

Effect of Metal Ions:

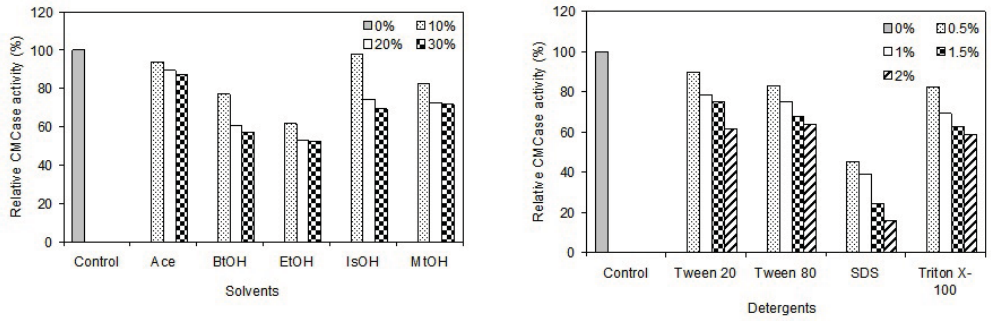
The addition of Cu^{2+}, Fe^{2+} and EDTA increased EG activity by up to 55% (Table 3) whereas the addition of other metal ions showed an obviously inhibitory effect on EG activity (Table 3). Chen et al. (2001) also reported that the activities of all components from *A. aculeatus* SM-L22 were stimulated 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, A.M. *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, J., 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, S., 1995).

Effect of Organic Solvents and Detergents:

The addition of organic solvents tested (acetone, ethanol, isopropanol, methanol and n-butanol) at the final concentration of 10% lead to slight 6-38% reduction in EG activity **(**Fig. 4A**)**. EtOH strongly showed an decrease in EG activity by one half (Fig. 4A). All tested detergents inhibited EG, especially, SDS at 2% concentration strongly reduced EG activity to 16% **(**Fig. 4B**).**

Table 2: Effect of metal ions on EG activity from *A. awamori* VTCC-F099.

Metal ions (mM)	Residual activity (%)		
	5	10	15
Ca^{2+}	97	95	90
Co^{2+}	99	96	79
Cu^{2+}	126	137	148
EDTA	146	153	155
Fe^{2+}	129	120	107
Mn^{2+}	70	63	54
Ni^{2+}	96	93	74
Zn^{2+}	94	79	78
Ag^{+}	74	70	43
K^{+}	92	86	87



(A) (B)

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

Conclusion:

An extracellular endoglucanase from *A. awamori* VTCC-F099 was purified 3.08 folds through a two-step purification scheme involving Sephadex G-100 gel filtration chromatography, and DEAE-Sephadex A-50 ion exchange chromatography. The purified EG had a specific activity of 65.3 U/mg protein and was a monomeric protein with a molecular mass of 32 kDa. K_m and V_{max} were 5.83 mg CMC/ml and 333.33 U/mg protein, respectively. The optimal temperature and pH were at 50°C and 5, respectively. This enzyme was stable at pH 4.5-5.5 and at 30-50°C. EG was activated by Fe^{2+}, Cu^{2+} and EDTA but inhibited by other tested metal ions, organic solvents and detergents. These results suggested that EG from *A. awamori* VTCC-F099 could potentially be used as an additive in the feed for monogastric animals.

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