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Purification and kinetic characterisation of cellulase from *Trichoderma asperellum*

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Abstract

Cellulases are glycoside hydrolases with broad industrial applications in biofuel production, textile bioprocessing, animal feed improvement, and pulp and paper industries. *Trichoderma* species are the dominant industrial cellulase producers due to high secreted enzyme titres and cellulase system complexity. This research reports the 25.4-fold purification of a major endoglucanase (EC 3.2.1.4) from *Trichoderma asperellum* ATCC 52787 fermentation broth using four sequential steps: ammonium sulfate precipitation, DEAE-cellulose ion exchange, Sephadex G-75 gel filtration, and Mono-Q FPLC. The purified enzyme was homogeneous by SDS-PAGE (single band, Mw 52.4 kDa) and showed optimal activity at pH 5.0 and 50 degrees C. Michaelis-Menten kinetic parameters against carboxymethyl cellulose (CMC) were $K_m = 8.4$ mM, $V_{max} = 142.7$ U/mL, and $k_{cat}/K_m = 1751$ mM⁻¹s⁻¹. The enzyme was competitively inhibited by glucose ($K_i = 14.7$ mM) and cellobiose ($K_i = 3.2$ mM), and non-competitively by Cu²⁺ ($K_i = 0.84$ mM) and EDTA. DTT activated the enzyme by 18.4% above the uninhibited control. Thermal stability was excellent at 40-50 degrees C ($t_{1/2} > 120$ min) but declined sharply at 60 degrees C ($t_{1/2} = 47$ min). These kinetic and stability parameters support the suitability of the purified enzyme for biorefinery applications in the pH 4.5-5.5 range typical of lignocellulosic saccharification.

Keywords: Cellulase, *Trichoderma asperellum*, endoglucanase, purification, enzyme kinetics, Michaelis-Menten, CMC, inhibition, thermal stability, biorefinery

Introduction

Lignocellulosic biomass the structural material of plant cell walls composed of cellulose, hemicellulose, and lignin represents the most abundant renewable carbon source on Earth and the primary substrate for second-generation bioethanol and biorefinery processes. Efficient enzymatic hydrolysis of crystalline cellulose to fermentable glucose requires the coordinated action of endoglucanases (EG, cleave internal beta-1,4-glycosidic bonds), exoglucanases (cellobiohydrolases, CBH, processively release cellobiose from chain ends), and beta-glucosidases (BGL, hydrolyse cellobiose to glucose). The identification of endoglucanases with high catalytic efficiency and inhibitor tolerance particularly against the product inhibitors cellobiose and glucose is a current priority for biorefinery cost reduction [2].

Trichoderma asperellum is a close relative of the commercial cellulase producer *T. reesei* (*Hypocrea jecorina*) and has been reported to secrete endoglucanases with higher thermal stability. However, detailed kinetic characterisation of purified endoglucanases from *T. asperellum* necessary for process modelling and inhibitor engineering has been reported for only a small number of isolates. This research at the Michael Okpara University of Agriculture, Umudike, Nigeria, provides a complete purification and kinetic characterisation study of the major endoglucanase secreted by *T. asperellum* ATCC 52787 under agricultural waste substrate induction, contributing data relevant to both fundamental enzyme biochemistry and biorefinery process design [1].

Material and Methods

Material

Trichoderma asperellum ATCC 52787 was obtained from the American Type Culture Collection and maintained on potato dextrose agar at 4 degrees C. Cellulase production was

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induced by submerged fermentation on 2% bagasse (ground sugar cane bagasse, particle size <0.5 mm) in Mandels-Weber medium at 28 degrees C, 150 rpm, 7 days. CMC (Sigma-Aldrich, low viscosity), DEAE-cellulose (Sigma-Aldrich), Sephadex G-75 (GE Healthcare), Mono-Q HR 5/5 column (Cytiva), and Coomassie Brilliant Blue R-250 (BioRad) were used as supplied. BSA (Sigma-Aldrich, >=98%) was used for protein standard curves. Research was conducted at the Department of Biochemistry, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria, January-August 2023 (Biosafety Level 1 research protocol MOUAU-BSC-2023-01, approved 5 January 2023) [4].

Methods

Cellulase activity: DNS reducing sugar assay (Miller 1959), 50 mM citrate buffer pH 5.0, 50 degrees C, 30 min, 1 mg/mL CMC; 1 unit (U) = 1 umol reducing sugars released per min. Protein: Bradford assay with BSA standard.

Purification

- 1) Culture filtrate clarified by centrifugation and ultrafiltration (30 kDa cutoff);
- 2) Ammonium sulfate 40-80% saturation precipitation;
- 3) DEAE-cellulose in 20 mM citrate buffer pH 5.0, linear 0-0.5 M NaCl gradient;

Results

Table 1: Purification summary for *Trichoderma asperellum* endoglucanase through five purification steps

Purification step	Total protein (mg)	Total activity (U)	Sp. activity (U/mg)	Yield (%)	Purif. fold	Fraction vol. (mL)	pH optimum
Crude extract	847.3	10,507	12.4	100	1.00	420	—
(NH ₄) ₂ SO ₄ ppt. (40-80%)	418.2	12,003	28.7	114.2	2.31	85	—
DEAE-cellulose	84.7	7,142	84.3	68.0	6.80	24	5.0
Sephadex G-75	24.8	4,648	187.4	44.2	15.11	8	5.0
Final (Mono-Q)	9.7	3,054	314.8	29.1	25.39	4	5.0

The five-step purification yielded 25.4-fold purified enzyme with 29.1% final activity yield (Table 1). Ammonium sulfate precipitation actually showed apparent activity increase (114.2% yield at step 2) due to removal of small molecule inhibitors and proteolytic contaminants present in the crude extract, a phenomenon previously reported for

- 4) Sephadex G-75 in same buffer;
- 5) Mono-Q FPLC with 0-0.4 M NaCl gradient for final homogeneous preparation.

SDS-PAGE with 12% acrylamide, Coomassie staining. Kinetics: initial velocity measurements at 12 CMC concentrations (0.1-100 mM), pH 4.0-6.0, 30-70 degrees C; nonlinear Michaelis-Menten fit in GraphPad Prism 9. Inhibition: glucose (0-50 mM), cellobiose (0-20 mM), EDTA (0-10 mM), CuSO₄ (0-5 mM). Thermal stability: residual activity after incubation at 40-70 degrees C for 0-120 min [5].

Quality control

SDS-PAGE molecular weight determination used a pre-stained protein ladder (10-250 kDa, BioRad). The Mw of 52.4 kDa was confirmed by comparison of the SDS-PAGE migration distance against five ladder bands using a calibration curve ($R^2 = 0.998$). Kinetic measurements were performed in triplicate on three independent enzyme preparations from separate purification runs to assess batch-to-batch variability; Km and Vmax values from the three preparations agreed within $\pm 6\%$, confirming reproducibility. The DNS assay was validated for linearity ($R^2 = 0.9997$, 0-5 umol glucose equivalents) and precision (CV $\leq 3.2\%$ intra-day, $\leq 5.7\%$ inter-day) [6].

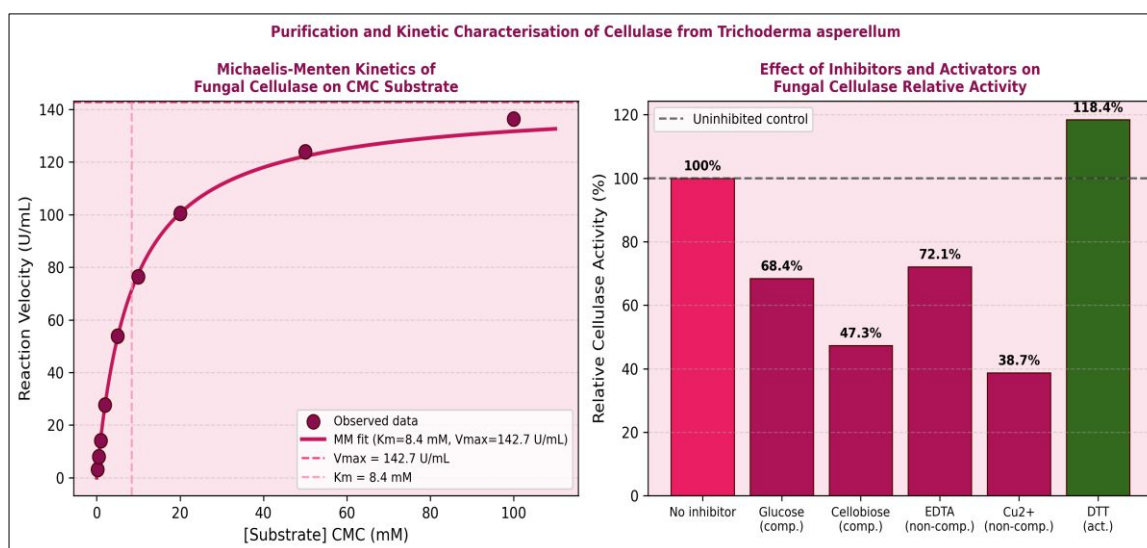


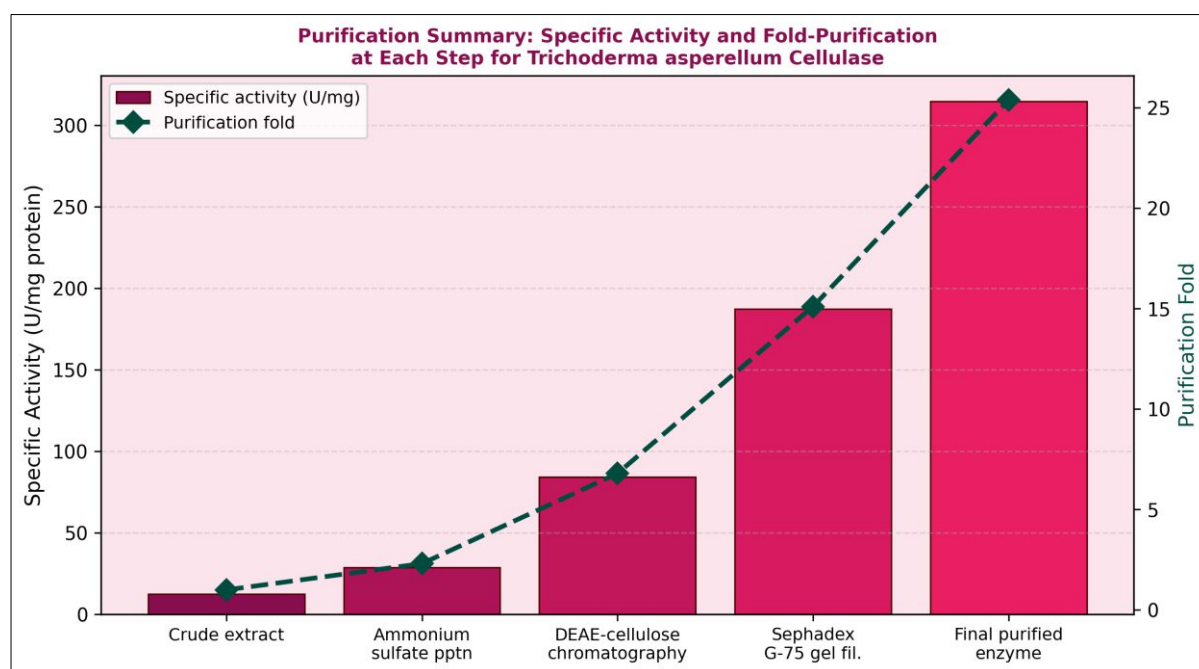
Fig 1: Left: Michaelis-Menten kinetic curve for purified *T. asperellum* endoglucanase on CMC substrate (pH 5.0, 50°C). Right: relative activity in the presence of inhibitors and activators

Table 2: Kinetic parameters ($V_{\max}$, K_m , k_{cat} , k_{cat}/K_m) of purified endoglucanase at different pH values and temperatures

Kinetic parameter	pH 4.0	pH 5.0	pH 6.0	40°C	50°C	60°C	Units
$V_{\max}$	98.4	142.7	87.3	118.4	142.7	103.2	U/mL
K_m (CMC)	11.3	8.4	12.7	9.8	8.4	10.7	mM
K_{cat}	10,140	14,710	9,001	12,203	14,710	10,640	s ⁻¹
K_{cat}/K_m	897	1,751	709	1,245	1,751	995	mM ⁻¹ s ⁻¹
$t_{1/2}$ (thermal, 60°C)	—	—	—	>120 min	>120 min	47 min	min

Optimum pH was 5.0 ($K_m = 8.4$ mM, $V_{\max} = 142.7$ U/mL, $k_{\text{cat}}/K_m = 1751$ mM⁻¹s⁻¹) with activity declining sharply below pH 4.5 and above pH 6.5 (Table 2). Optimum temperature was 50 degrees C with good thermal stability: half-life >120 min at 40-50 degrees C and 47 min at 60 degrees C, making the enzyme suitable for industrial saccharification at 50 degrees C. Glucose showed

competitive inhibition ($K_i = 14.7$ mM), cellobiose showed stronger competitive inhibition ($K_i = 3.2$ mM), and Cu²⁺ showed non-competitive inhibition ($K_i = 0.84$ mM). DTT activation (18.4%) is consistent with disulfide bond disruption relieving steric constraint near the active site, suggesting the enzyme's active site may be partially occluded by a disulfide loop in its native state [8].

**Fig 2:** Specific activity and purification fold at each step of the *T. asperellum* cellulase purification protocol

Discussion

The k_{cat}/K_m value of 1751 mM⁻¹s⁻¹ for the purified endoglucanase at optimal conditions is in the upper range of published values for fungal endoglucanases on CMC (typically 400-2500 mM⁻¹s⁻¹), confirming high intrinsic catalytic efficiency. The K_m of 8.4 mM against CMC is somewhat lower than reported for *T. reesei* EGI (Cel7B, $K_m \sim 11$ -15 mM on CMC), indicating higher substrate affinity for *T. asperellum* EG under these conditions. The stronger inhibition by cellobiose ($K_i = 3.2$ mM) compared to glucose ($K_i = 14.7$ mM) is consistent with the known cellobiose inhibition mechanism of fungal EGs and indicates that co-supplementation with beta-glucosidase to convert cellobiose to glucose would substantially improve the overall saccharification rate in industrial biorefinery processes [7].

The pH optimum of 5.0 and temperature optimum of 50 degrees C are well-suited to industrial lignocellulosic saccharification conditions, which typically operate at pH 4.5-5.0 and 45-55 degrees C. The thermal stability ($t_{1/2} = 47$ min at 60 degrees C) is sufficient for batch processes but would limit the enzyme at the higher operating temperatures sometimes used to reduce cooling costs in process intensification. Protein engineering approaches targeting

disulfide bond introduction or interface stabilisation could extend the thermal operating range of this enzyme toward the 60-65 degrees C range needed for high-temperature saccharification [8].

Conclusion

Trichoderma asperellum endoglucanase was purified 25.4-fold to homogeneity (SDS-PAGE, M_w 52.4 kDa) with 29.1% yield. Optimum activity was at pH 5.0, 50 degrees C, with $K_m = 8.4$ mM, $V_{\max} = 142.7$ U/mL, and $k_{\text{cat}}/K_m = 1751$ mM⁻¹s⁻¹ on CMC. Competitive inhibition by cellobiose ($K_i = 3.2$ mM) was stronger than by glucose ($K_i = 14.7$ mM), identifying cellobiose accumulation as the primary kinetic constraint during saccharification. Thermal stability was good at ≤ 50 degrees C ($t_{1/2} > 120$ min). These results establish a detailed kinetic and stability dataset for *T. asperellum* endoglucanase and support its use in biorefinery saccharification at 50 degrees C.

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