Cloning, sequence analysis and characterization of a novel β-glucosidase-like activity, MUGA from *Pichia etchellsii*

By

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Dedicated to my beloved parents

CERTIFICATE

This is to certify that the thesis entitled **"Cloning, Sequence analysis and characterization of a novel β-glucosidase MUGA from *Pichia etchellsii*"**, being submitted by Ms. Pranita Roy to the Indian Institute of Technology, Delhi, for the award of the degree of "Doctor of Philosophy", is a record of the bonafide research carried out by her, which has been prepared under my supervision in conformity with rules and regulations of the "Indian Institute of Technology, Delhi". The research reports and results presented in the thesis have not been submitted for any degree or diploma in any other University or Institute.

Saroj Mishra

Prof. Saroj Mishra

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ABSTRACT

β-glucosidases (*β*-D-glucoside glucohydrolase, EC 3.2.1.21) comprise a heterogenous group of enzymes that are able to cleave the *β*-glucosidic linkages of di-and/or oligosaccharides and other glucose conjugates. These enzymes are widely distributed in the living world and play pivotal roles in many biological processes. To study the biological role and useful biotechnological applications of these enzymes, we have reported on a number of β-glucosidases isolated from the thermo-tolerant yeast *Pichia etchellsii*. This organism grows optimally at 40-45^{0}C and produces multiple *β*-glucosidases. Two enzymes namely, Bgl I and Bgl II were identified by way of expression in *Escherichia coli*. Two *β*-glucosidases have been purified from the cell wall of the yeast, namely BGLI and BGLII and detailed analysis indicated these to be different from each other and from the other *E. coli* expressed enzyme. Our continued search in this yeast system has lead to identification of a novel hydrolytic activity. It resembled the reported β-glucosidases in terms of its ability to hydrolyse MUG (methyl umbelliferyl β-D-glucoside) but did not hydrolyze *p*NPG (*p*-nitrophenyl β-D-glucoside), which is a commonly used substrate for assay of these enzymes. The present work was undertaken to determine and analyze the nucleotide sequence encoding this activity, characterize the purified protein (biochemically and structurally), and evaluate its function in the yeast.

Genomic DNA fragment encoding a novel *β*-glucosidase-like activity of the yeast *P. etchellsii* was cloned and expressed in *E. coli*. Two MUG hydrolyzing clones were isolated, namely, pMG8: DH5*α* and pMG16: DH5*α* containing the same gene on different insert lengths. The sequencing of the 6.35 kbp yeast insert in pMG8 plasmid showed multiple ATG's with a single termination codon (TAG). An open reading frame

(ORF) of 1515 bp termed *mugA* was confirmed by sub-cloning the pMG8 plasmid which codes for a protein of predicted molecular mass of 54.1 kDa. The sequence homology search of the ORF did not show homology with any of the reported β-glucosidases, however, a very significant identity was seen with several Ser (S)-Asp (D) rich cell surface proteins. The secondary structure prediction program 3D-PSSM indicated the protein to be composed of largely helical and coiled structures, quite characteristic of cell-surface associated proteins which was confirmed by circular dichroism spectroscopy. The MUGA protein was found to be localized in the intracellular space of re-*E. coli*. It was released by sonication and used as starting material for purification. The protein was purified to homogeneity by a combination of DEAE-Sepharose, hydroxyapatite and Sephadex G-25 column chromatography to 53 fold purity. The biochemical properties of purified MUGA were investigated in detail. The molecular mass of the protein determined from SDS-PAGE gel was around 50.1± 5.5 kDa while the mass was estimated to be 52.1 kDa by MALDI-TOF. The protein was optimally active at 45^0C and in the pH range of 6-11. The protein displayed high hydrolytic activity on MUG but relatively very little hydrolysis of *p*NPG and gentiobiose, characteristic substrates for β-glucosidases. Kinetic measurements indicated that the best substrate was MUG, with highest value of specificity constant (k_{cat}/K_M) of 0.083 x 10^{-3} $\mu mol^{-1}min^{-1}$. However, the protein displayed higher affinity towards *p*NPG. The protein did not show any stimulation or inhibition in activity in the presence of divalent ions, denaturants and group specific reagents. However, slight increase in hydrolytic activity was obtained in the presence of methanol.

The binding experiments performed between *P. etchellsii* cells and the purified *E. coli* expressed MUGA indicated binding with the cell surface which was monitored by fluorescence microscopy. In competition experiments with the SD dipeptide, less protein was shown to bind to the cell surface, in a concentration dependent manner indicating the binding of MUGA protein to the cell-surface. The expression of the *mugA* gene in BL21 (DE3) was significantly improved by its positioning under control of the T7 promoter in the pET29a expression vector.

CONTENTS