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Neutral α-mannosidase from fungus *Aspergillus fischeri* (NCIM 508) belongs to Class IIC subfamily

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Abstract

BACKGROUD & OBJECTIVE: The α-mannosidase gene from *Aspergillus fischeri* was isolated by DNA and RNA method. **METHODOLOGY:** Cloning of gene, isolation of recombinant plasmid and sequencing of gDNA gene was carried out and bioinformatic analysis was done for gene to get intron-exon information and for sequence homology. **RESULTS:**The α-mannosidase gene from *Aspergillus fischeri* is 1990 base pair long and sequence analysis showed presence of three introns and four exons in the gene. BLAST analysis revealed 100% similarity to the *Neosartorya fischeri* (*A. fischeri*) and 94% with *Aspergillus fumigatus* class I α-mannosidase reported in NCBI Gene Data Bank. **CONCLUSION:** The neutral α-mannosidase from *Aspergillus fischeri* could belong to the Class IIC subfamily.

Keywords: α-mannosidase gene, *Aspergillus fischeri*, Cloning, Sequencing, gDNA.

1. Introduction

Mannosidases are Glycohydrolases involved in the processing of mannose containing glycans *in vivo*. They are involved in the maturation and degradation of glycoprotein-linked oligosaccharides. Use of natural substrates for specificity determination, antibodies from sub cellular localization, specific enzyme inhibitors and mutant cell lines, has revealed the presence of different α-mannosidase isoenzymes. This suggests that α-mannosidases have multiple functions in glycoprotein metabolism [1]. There are two classes of processing α-mannosidase class I and class II, based on their distinctive substrate specificity, responses to inhibitors, cat ion requirements, protein molecular weights, sub cellular localization, enzyme mechanisms and characteristic regions of conserved amino acid sequences[1,2]. They belong to family 47 and family 38 glycosidase, respectively in Henrissat's glycosidase classification[2,3].

There is significant correlation of biochemical and physiological roles of the various member genes for each of these two classes. Processing mannosidases are located in both the ER and Golgi of mammalian cells. The differential activity of Class II α-mannosidase towards artificial substrates has important practical ramifications. Because Class II enzymes are readily assayed with aryl mannosides, they are almost always employed for the initial screening of new potential inhibitors. Class I enzymes are not as easily assayed, so good inhibitors of these may be missed or incompletely characterized[1].

The purification, characterization, chemical modification and tryptophan environment of α-mannosidase from *A. fischeri* has already been reported[4-8]. The enzyme is a Class II α-mannosidase as revealed by the substrate specificity and independency on the metal ion/s for activity[4]. The present study describes the cloning of a high molecular weight α-mannosidase from *Aspergillus fischeri* (*Neosartorya fischeri*) and its characterization and proves that the enzyme belongs to Class IIC subfamily of α-mannosidase.

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2. Material and Methods

2.1 Material

Ampicillin, Kanamycin, Tris, IPTG, X-gal, SDS, BSA, EDTA and Ethidium bromide were purchased from Sigma-Aldrich, USA. Agarose, restriction enzymes, T4 DNA ligase, RNase A and lysozyme were obtained from GIBCO-BRL (USA), Promega (USA) and Amersham (UK). Taq DNA polymerase was obtained from Bangalore Genei (India). Plasmid vectors pGEM-T Easy Vector is purchased from Stratagene (USA). All other chemicals and solvents of analytical grade were purchased from HIMEDIA, Qualigens Fine Chemicals and E-Merck Laboratories, India. Pipette tips and micro centrifuge tubes were purchased from Axygen (USA).

2.2 Isolation of genomic DNA

The fungal culture *Aspergillus fischeri* was grown in 500 ml of Basal medium (pH 5.0) at 28 °C for 7 days with continuous shaking at 180 rpm. DNA was isolated according to the method described by Hopwood *et al.*,1985[9] with slight modifications.

2.3 Total RNA Isolation

Total RNA from fungal mycelium was isolated using Trizol reagent. RNase free environment was created and maintained as described by Blumberg (1987)[6]. All glass and plasticware was DEPC (0.1% in water) treated overnight and autoclaved. The pestle and mortar were also DEPC treated and then baked at 300 ºC for 6 h. All materials were dried in a vacuum oven.

2.3 cDNA first strand synthesis by Reverse Transcription

Complementary DNA (cDNA) is synthesized from a mature mRNA template in a reaction catalyzed by the enzyme reverse transcriptase. In the present study cDNA first strand was synthesized using ImProm-II™ Reverse Transcription System (Promega, USA). The reactions were set up as per the

manufacturer's guidelines.

2.4 Extraction and purification of DNA from agarose gels

The restriction digested DNA or PCR amplified products were run on an agarose gel in 1X TAE buffer. The gel was stained with ethidium bromide (0.5 µg ml^{-1}) and viewed using a hand held long wavelength UV illuminator. The fragment of interest were excised from the gel and weighed. A 50-200 mg gel slice was transferred to a 1.5 ml micro centrifuge tube and 0.5 ml GEX buffer (AuprepTM GEL^{X} kit, Life Technologies, USA) added and further processed to load DNA on column and eluted. The eluted DNA was stored at -20 °C.

2.5 Ligation reaction

The pGEM-T vector and PCR amplified product were mixed in a molar ratio of 1:2 and suspended in 5 µl of DNA ligase buffer. To the mixture, 1unit of ligase was added and incubated for 4 h at 16 °C.

2.6 *Escherichia coli* transformation and selection

LB medium (50 ml) was inoculated with 1% of the overnight grown *E. coli* culture and allowed to grow till 0.5 O.D. at 600 nm. The competent *E. coli* cells are made and were transformed according to Sambrook *et al*,1989[10].

In order to differentiate between the colonies containing recombinant plasmids from the non-recombinant colonies, blue white selection of the colonies was performed using ampicillin (40 µg/ml), 40 ml of a stock solution of X-gal (20 µg/ml in dimethylformamide) and 4 ml solution of isopropylthio-β-D-galactoside (IPTG) (200 µg/ml).

2.7 Isolation of plasmid DNA from *E. coli* cells

The alkaline lysis method of Sambrook *et al.*[10] was improvised upon so that 12-24 samples could be processed conveniently for plasmid DNA extraction within 3 h, with yields of 5-30 µg per 1.5 ml culture depending on the host strain and the plasmid vector.

2.8 Restriction digestion of DNA

Plasmid DNA restriction digestion was set up as per recommendations of manufacturer.

2.9 Primer design for the α-mannosidase gene and its amplification

The whole genomic sequence of *Neosartorya fischeri* (*Aspergillus fischeri*) fungus is available in the NCBI website. The information on α-mannosidase gene reveals that it is Class I α-mannosidase. As reported in our earlier manuscripts[6-8], it is proved that the α-mannosidase of molecular weight 69200 Da (on SDS PAGE) from *Aspergillus fischeri* is a Class II enzyme. To confirm whether this is the same enzyme as reported in genome sequence, the tryptic digest of enzyme and peptide mass fingerprinting was done with MALDI-TOF. The peptide masses so obtained were compared with the peptide masses of theoretical tryptic digest of the protein sequence of reported α-mannosidase. 11 peptide masses were exactly matched with each other. Again, the molecular mass of the enzyme (69200 Da) studied is same as the reported. Based on this information, the reported α-mannosidase in website was confirmed same as the enzyme under study and the gene (cDNA) was used for the primer design. The genomic DNA was used as a template for the amplification of the α-mannosidase gene of interest. The following primers were designed based on the cDNA sequence available from the NCBI Gene Bank database. The NCBI Gene ID, ACCESSION XM, VERSION XM, GI are 4594076. 001267006, 001267006.1, 119500499 respectively. Primers with restriction site to be incorporated in the amplified product were flanked with restriction sites on the 5' end of the respective primers. The forward primer with Nde site (**5' CAT ATG ATT CTA GGT CGC AGG CGC 3'**) and reverse primer with Hind III site (**5'AAG CTT TGT ACG AGC AGA GGG ACG CTT 3'**) were used for the study.

2.10 Bioinformatic analysis

Nucleotide and amino acid sequence analysis was done using software **pDRAW 32** and online bioinformatics analysis facility available at ***www.justbio.com***, ***www.expasy.org*** *and* ***www.ncbi.nlm.nih.gov.***

3. Results and Discussion

3.1 **Dissection of the α-mannosidase gene from *A. fischeri***

Genomic DNA was extracted and Primers were designed based on the gene sequence available in NCBI Gene data Bank as described in materials and methods. PCR reactions were set up with *A. fischeri* genomic DNA using all possible combinations of the forward and the reverse primers. A DNA fragment of ~2.0 Kb was amplified (Figure I) with

Forward primer

AfManF [5'ATG ATT CTA GGT CGC AGG CGC 3']

Reverse primer

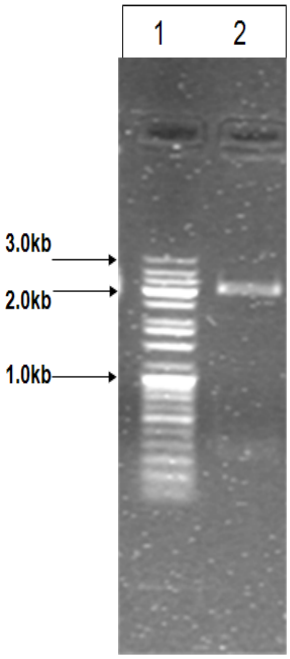
AfManR-2 [5' TGT ACG AGC AGA GGG ACG C 3']

3.2 Cloning of α-mannosidase gene

The amplicon obtained by primers AfManF forward and AfManR-2 reverse was cloned in to pGEM-T Easy Vector (Promega, USA) by TA cloning and transformed in to *E. coli*. The fact that *Taq* polymerase adds up poly A tail to the amplicon has been exploited in TA cloning. The pGEM-T has poly T at the cloning sites which make it easy to bind to the poly A of the PCR amplified fragment with poly A tail. The positive clones were selected based on the blue-white selection using IPTG and X-Gal. The positive clones were then cross checked for the presence of the gene by isolating the plasmid and sequencing it (Figure II).

The nucleotide sequences of α-mannosidase genomic clones were searched for sequence similarity in NCBI Gene Bank database and they showed sequence similarity with reported *Neosartorya fischeri* (*Aspergillus fischeri*) (100%) and *Aspergillus fumigatus* (94%) Class I α-mannosidase fungal genes. Sequencing data analysis of gDNA of α-mannosidase showed that the clone was of 1990 bp long. The sequences upon alignment showed 100% sequence similarity with *Neosartorya fischeri* (*A. fischeri*) class I α-mannosidase. The calculated GC content (www.justbio.com) of the gene was 51.0 %. Manually, the intron / exon junctions in the clone were identified after doing BLAST search in gene bank. The α-mannosidase gDNA nucleotide sequences show presence of putative four exons and three introns. In the α-mannosidase gene clone the four exons spanned the nucleotide positions 4 - 719, 773 - 1566, 1633 - 1892 and 1942 - 1984. The putative introns spanned nucleotide positions 720 - 772, 1567 – 1632 and 1893 – 1941 (Suppl material S1).

Figure I: PCR with *A. fischeri* genomic DNA template and primers **AfManF** and **AfManR-2**. DNA size marker (lane 1), PCR amplified product (lane 2)



Restriction analysis of α-mannosidase gDNA was done using bioinformatic software **pDRAW32**. Analysis was limited to enzyme site cutting not more than two times in the sequence.

3.3 α-mannosidase gene isolation from *A. fischeri* by cDNA method

Total RNA was extracted from the *A. fischeri* and cDNA was synthesized as described in materials and methods. PCR reactions were set up with *A. fischeri* cDNA

Figure II: Nucleotide sequence of GDNA amplicon of α-mannosidase (1990 bp)

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   1 CATATGATTC TAGTCGCAGG CGCTATCGCT ATGCCTTACC AACCTTTATT
  51 CTAATTCTCA TTACCTTCTT CCTTTTCTCG AGTCTACCGC CTGGCGCGGA
 101 ACCTCCCCTT GTCGACGACC ACTCATACTA CGCAGAATAC CATCCCCAAC
 151 CCCCGCAGAA CGATAACCGT ATCCATTGGA CCAAACATAC CGAAAGACAT
 201 CCTGTCCCCA GTTTTATTCC TCTCCCTACT CCCGCGCCAT CCGCAATTCC
 251 TCGTGTCCAG TACGACTTCG CGAAGGAATC ATGGCTTGGC CGGAGAAAAA
 301 GAGTGAACAG ACAGAAGGCC GTCAAGGAGG CCTTCACGCA TGCTTGGAAA
 351 GGGTACAAGC AGCATGCCTG GATGCGCGAT GAACTTTCGC CCCTCAGTGC
 401 TCGTTACAGA ACCACATTTG CTGGCTGGGC TGCCACTCTG GTGGACGCAC
 451 TTGACACTCT TGTCATTATG GGTATGGAAG ACGAGTTCAA GGATGCGTTG
 501 CATGCCATTG AGAGCATTGA TTTTACTACC ACCGATGCCA CCCAGATCAA
 551 TGTATTCGAG ACTACTATCC GATATGTAGG AGGGTTACTT GGCGCCTACG
 601 ACCTGACCGA CGGAAAACAC CCGATCCTTC TGAAGAAGGC TGTGGAACTG
 651 GCCGACGTGA TCTACGATGC TTTTGATACT AGCAAATAGG ATGCCCCAGT
 701 CTCGCTGGCA ATGGAGCAGT AAGCCGGCCA TTTGTTTCTG GAACCACTTA
 751 AAGCTGCTGT TAACTAGGCC ATAGATCTGC GCGAGGCCTG AGCATTCAAC
 801 CAAGCAGACA AACTATCCTC GCAGAATTGG GTTCATTGAA TCTAGAATTC
 851 ACCAGGCTGT CCCAGCTGAC TCATGATCCC AAGTACTTTG ATGCGGTCCA
 901 ACGAATTACA GACGTTCTCG ACGACGCTCA GAATAAGACC AAAATCCCTG
 951 GGCTGTGGCC TATGATGGTC AACGCTCAGG ATTTAGAGTT CACAGACCCT
1001 CGCTTTACTG TGGGTGGAAT GGCCGACTCA ACATATGAGT ATTTGCCGAA
1051 AGAGCATATG CTACTGGGAG CTCAGACGGA TCAATACCGC AAGATGTATG
1101 CCGCCGCCAT GGAGGCGATC AAGAAGCGCT TATTGTTTCG ACCCATGACC
1151 AAAAACGGCG AGGATCTCCT CTTCGCCGGG AACACCCACA CGAGTCTCGC
1201 AACTAACGCG CCCCCGGAGC CTCAATGGGA ACATCTGAAA TGCTTTTTTG
1251 GGGGCACTGT GGGCATCGGG GCCAAGGTTT TCAACCGCCC GGAGGAGCTC
1301 TCAATTGCAC GAAAGCTAAC AGATGGCTGT ATCTGGGCAT ACGATGTCAT
1351 GCCCACCGGA ATCATGCCGG AAATCATGCA TCTCAGCGCT TGCCAGAGTA
1401 TGGATAGTTG TGAATGGGAC GAGCAGAAGT GGTACCAAGA TGTCCGGCGG
1451 CGACTGGCCA AGGGATCAAA GGAGAGAGAC ACTGTTCAAG AAGCCAAGAC
1501 AATTATCCAG GAGAGTGGGC TTCAACCTGG CCTAACTGAG ATATCGGATC
1551 CGAGATATTT GCTGCGGTAT GTGATTTGCT GACAATTGAT TTTCGTCTTG
1601 CATCGGGTGT AGTAGTGCTG ATATTGGTTC TAGACCCGAG GCTATCGAGT
1651 CAGCTTTCAT CATGTACCGC ATCACTGGGG ACAAGAAATT GCAGGACGAT
1701 GCATGGAGAA TGTTTCAGAG CATTGAGAAA GCAACTCGAA CCAATCATGC
1751 CCATGCCGCG ATAGACGACG TCCGGGACGT GAAGACAACC CAGTTAGATT
1801 ACATGGAGAG TTTCTGGCTG GCAGAGACCT TGAAGTACTT CTATCTCGTC
1851 TTCTCCGAAC CAGGGCTTGT CAGCTTGGAT GATTACGTAC TGTATGTGCT
1901 GTTGTCTTGT GAGGCTCTCG CCAAAGCTAA CGATTAAGCA GGAACACGGA
1951 AGCACATCCA TTCAAGCGTC CCTCTGCTCG TACAAAGCTT
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using all possible combinations of the forward and the reverse primers. A DNA fragment of ~1.8 Kb was amplified (Figure III) with Forward primer

AfManF [5'ATG ATT CTA GGT CGC AGG CGC 3']

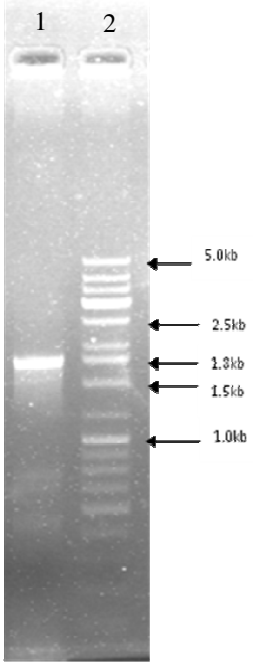
Reverse primer

AfManR-2 [5' TGT ACG AGC AGA GGG ACG C 3']

4. DISCUSSION

The α-mannosidase gene from *Aspergillus fischeri* was isolated by both genomic DNA and RNA method. Cloning of gene, isolation of recombinant plasmid and sequencing of genomic DNA gene showed that it is 1990 base pair long and its bioinformatic's analysis showed presence of three introns and four exons in the gene.

Figure III: PCR with *A. fischeri* cDNA template and primers **AfManF** and **AfManR-2**. DNA size marker (lane 1), PCR amplified product (lane 2).



BLAST analysis revealed 100% similarity to the *Aspergillus fischeri* and 94% with *Aspergillus fumigatus* class I α-mannosidase reported in NCBI Gene Data Bank. But this sequence is not matching with any Class II α-mannosidase at gene level. Our previous studies have revealed that the enzyme is class II α -mannosidase.

So far, there are three subfamilies of genes known, Class IIA, IIB and IIC [1,11,12]. Class II A α-mannosidase occurs only in higher eukaryotes, involved in N-glycan synthesis in golgi and removes α, 1-3, 1-2, 1-6 linked mannose residues from N-glycans during their synthesis. Class II B α-mannosidase found only higher eukaryotes and involved in N-glycan breakdown, removal and recycling in the cytoplasm, lysosome and vacuole. Class II C α-mannosidase found in higher and lower eukaryotes and involved in N-glycan breakdown, removal and recycling in the cytoplasm, lysosome and vacuole. They are heterogeneous enzymes with a diversity of functions and cellular location and show low sequence similarity with the other two groups of enzymes[12]. Phylogenetic analysis of the sequences shows that the Class IIA and Class IIB subfamilies have diverged more recently than the Class IIC subfamily. A likely scenario is that a single common ancestor was duplicated after the divergence of lower eukaryotes, such as fungi, from the higher eukaryotes. The lower eukaryotes thus only contain the orthologue of the common ancestor[13]. As Class IIC found in higher and lower eukaryotes and has very low sequence similarity to the other two subfamilies and the present α-mannosidase from *Aspergillus fischeri* shows similar properties, hence the *Aspergillus fischeri* α-mannosidase could belong to the Class IIC subfamily.

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