Molecular Characterization of a Rhodotorula-lytic Enzyme from Paecilomyces lilacinus Having β-1,3-Mannanase Activity

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We cloned the gene and corresponding cDNA for an extracellular Rhodotorula-lytic enzyme which has β-1,3-mannanase activity, tentatively named MAN5C, from Paecilomyces lilacinus. MAN5C showed a high homology score with the members of glycoside hydrolase family 5 in a domain search with the Pfam database, indicating that MAN5C is a novel and unique member of glycoside hydrolase family 5.

Key words: β-1,3-mannanase; glycoside hydrolase family 5; Paecilomyces

Glycoside hydrolases include a wide variety of enzymes concerned with carbohydrate metabolism and possess broad substrate specificities. Primary structures of glycoside hydrolases from many organisms have been identified and many of their high-order structures have also been clarified. A classification based on their conserved domains and/or motifs has been proposed. According to the classification, the enzymes are grouped into 90 glycoside hydrolase families1) and 13 superfamilies, clans GH-A to -M.2) No enzyme lysing the Rhodotorula cell wall from commercial and natural sources had been known until Murao et al. succeeded in the isolation of a strain, Paecilomyces lilacinus ATCC 36010, able to use Rhodotorula cells as carbon sources.3) They purified an extracellular lytic enzyme from the culture of P. lilacinus.4) The enzyme has the activity of a β-1,3-mannanase, tentatively named MAN5C, which recognizes a β-1,3 bond adjacent to the β-1,4 bond of the exocellular mannan from Rhodotorula.4) Considering the substrate specificity and the reactivity, the enzymes might be a novel and unique mannanase. The homology and phylogenetic relationship of MAN5C to the well documented β-1,4-mannanases are, however, unknown as yet because the primary structure has not been determined. The enzyme is not assigned to any family of glycoside hydrolases.

In this study, we cloned and sequenced the gene and corresponding cDNA for MAN5C from P. lilacinus, deduced the primary structure of the protein and unveiled the molecular features. MAN5C was purified according to the method of Murao et al.5) The purified MAN5C had a specific activity of 14.5 units/mg protein. SDS-PAGE analysis of purified MAN5C showed a single dense band with a molecular mass of 38 kDa, which is consistent with the value previously reported.5) The N-terminal amino acid sequence of the purified MAN5C was analyzed with a Procise 490 protein sequencer (Applied Biosystems). The purified enzyme was also digested to polypeptides with cyanogen bromide.6) The resulting polypeptides (polypeptides-1 to 6) were separated by tricine SDS-PAGE and their N-terminal amino acid sequences were analyzed. Polypeptide sequences obtained are shown in Fig. 1.

We amplified the genomic DNA coding sequence between the N-terminal and polypeptide-5 by PCR. The PCR product carried a significant sequence between the N-terminal and polypeptide-5 sequence, and used it as a probe for Southern hybridization of P. lilacinus chromosomal DNA. The positive 2.5-kb band was detected from SalI-digested chromosomal DNA and cloned. The cloned DNA fragment was long enough, 2551 bp, to code for MAN5C, and harbored the N-terminal amino acid sequence and polypeptide-4, 5, and 6 despite the absence of a continuous open reading frame. Oligonucleotide primers corresponding to the 5'- and 3'-flanking sequences of the predicted coding region of the MAN5C gene were prepared and used for PCR amplification of the MAN5C-cDNA from double-stranded cDNAs. The

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amplified cDNA was directly sequenced.

Comparison of the sequences of the MAN5C-cDNA and the genomic MAN5C made it possible to estimate the sites of introns and exons, and to confirm the boundaries between the coding and the 5'- and 3'-flanking regions in the gene. MAN5C had two introns and three exons, the boundaries of which conformed to the canonical GT-AG rule. The introns were 46 bp and 74 bp long (Fig. 1). The eukaryotic β-mannanase gene is known only in a blue mussel,7) which contains five introns spread over in the entire coding sequence. The locations of introns in the two genes were not conserved. We found consensus promoter sequences of a TATA box and CAAT box8) in the 5'-flanking region. A deduced polyadenylation signal (AATAA) existed downstream from the translational stop codon.

The cDNA of MAN5C demonstrates that it had an ORF which coded for a protein of 362 residues with a calculated molecular mass of 39,268 Da. Excluding a deduced signal peptide described below, the ORF was comprised of 341 residues with a calculated molecular mass of 36,960 Da. This value was well consistent with that determined by SDS-PAGE analysis for the purified enzyme. The amino acid sequence for the deduced signal peptide had the same features as those of secretion proteins of eukaryotes, i.e., a positively charged amino acid, arginine in the N-terminal and alanine in the C-terminal position, and high content of hydrophobic amino acids.7) This conformed to the fact that MAN5C is secreted into the extracellular fraction of the culture of P. lilacinus.

A homology search of MAN5C in their whole region was done with the BLAST and GENETYX (GENETYX Corporation, Tokyo) programs. The results from BLAST program showed homology only in their N-terminal half (amino acid residues 43-201) with β,1,4-mannanases (E.C.3.2.1.78), which is derived from Thermotoga neapolitana (27% identity),9) Arabidopsis thaliana (30%, accession no. CAB82763), Thermotoga maritima (26%),9) and Aspergillus aculeatus (24%).10) The results with the GENETYX program showed lower homology of MAN5C with the proteins described above. The identities of the amino acid sequences with that of T. neapolitana, A. thaliana, T. maritima, and A. aculeatus were 15%, 14%, 15%, and 18%, respectively.

We examined the domain structure of MAN5C using the database, Pfam, with the profile HMMs program. Although the primary structure of MAN5C was not significantly conserved, the search result demonstrated that domain structure of MAN5C had a high homology (score, 48.8 bits) with that of glycoside hydrolase family 5. All of the mannanases and mannosidases previously reported are grouped in either family 5 or 26, and in any of family 1, 2, or 5 of glycoside hydolases, respectively, based on domain structure. MAN5C might be a unique enzyme belonging to glycoside hydrolase family 5, given its substrate specificity, the reactivity of the cleaving end-β,1,3 mannoside linkage, and the primary structure.

Multiple alignment of MAN5C and the four β,1,4-mannanases described above was then performed using the ClustalW program (Fig. 2). The conserved amino acids, R, H, and Y, which designated a closed triangle in Fig. 1.

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Fig. 2, in \( /C12\)-1,4 mannanase were not conserved in MAN5C except for the R residue. The enzymes belonging to GH-A, including family 5 enzymes, are considered to have a \((/C11=/C12)\) \(8\) barrel structure as the \(/C11\) helix and \(/C12\) strand are repeated \(8\) times in the molecule. Two glutamate residues located close to the carboxy-terminal end of \(/C12\) and at the carboxy-terminal end of \(/C12\) in the \((/C11=/C12)\) \(8\) barrel structure are conserved and function as acid/base and nucleophile catalysts. 11) There was an E178 residue regarded as an acid/base catalyst in MAN5C despite the conserved sequential residues of NEP in the other mannanases being changed to NEA (boxed in Fig. 2). However, the E residue which might function as a nucleophile is not conserved and converted to a D295 residue (open triangle in Fig. 2). This D295 residue may work as a nucleophile and give MAN5C the

![Fig. 2. Comparison of MAN5C with Other \(-/C12\)-1,4-Mannanases.](image-url)
distinctive capability to catalyze the cleavage of β-1,3 mannann.

Analysis of the three-dimensional structure of MAN5C would make clear how the enzyme cleaves the β-1,3 linkage of mannan in detail.

References