Crystal Structure of Mannanase 26A from *Pseudomonas cellulosa* and Analysis of Residues Involved in Substrate Binding*

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The crystal structure of *Pseudomonas cellulosa* mannanase 26A has been solved by multiple isomorphous replacement and refined at 1.85 Å resolution to an R-factor of 0.182 (R-free = 0.211). The enzyme comprises (β/α)₆-barrel architecture with two catalytic glutamates at the ends of β-strands 4 and 7 in precisely the same location as the corresponding glutamates in other 4/7-superfamily glycoside hydrolase enzymes (clan GH-A glycoside hydrolases). The family 26 glycoside hydrolases are therefore members of clan GH-A. Functional analyses of mannanase 26A, informed by the crystal structure of the enzyme, provided important insights into the role of residues close to the catalytic glutamates. These data showed that Trp-26A, informed by the crystal structure of the enzyme, is highly conserved residues in this superfamily are an adjacent Asn-Glu pair at the end of β-strand 4 and a Glu at the end of β-strand 7, although GH26 mannanases have histidine substituted in place of the asparagine. The importance of the substitution of an asparagine for a histidine in GH26 enzymes is unclear. To determine whether GH26 enzymes are members of the GH-A clan, and to investigate the role of amino acids in the active site of these glycoside hydrolases, the three-dimensional structure of Pc Man26A was solved and a series of substrate-binding site mutants characterized.

**MATERIALS AND METHODS**

**Enzyme Expression, Purification, and Crystallization**—Pc Man26A expressed in *Escherichia coli* strain BL21 (Novagen) containing the plasmid pDB1 was purified to homogeneity in a single step using anion exchange chromatography (11). Single crystals were grown by the hanging drop method initially using Hampton Crystal Screen Kits to search for appropriate conditions.

**DNA Sequencing**—The carboxyl-terminal 49 amino acids of Pc Man26A did not fit the electron density map, and the sequence of the 3'-end of the Pc Man26A gene (man26A) was therefore redetermined using an ABI dye-terminator kit and an ABI373 automatic sequence. pDB1, which comprises the region of *man26A* encoding the mature mannanase cloned into Ndel/SalI-restricted pET21a (Novagen; Ref. 11), served as the template DNA and the vector’s T7 terminator sequence as the primer. The data clearly showed that the previously determined sequence of *man26A* (7), was incorrect and that there was an additional C at position 1090 (nucleotide 1 is the A of the ATG initiation codon), resulting in a change in the reading frame at the 3'-end of the gene. The revised sequence of mature Pc Man26A, which is shown in Table I, could easily be fitted to the electron density map.

**Site-directed Mutagenesis**—Mutants of *man26A* were generated using the Quick Change kit supplied by Stratagene. Appropriate mutations were generated by using the following primers (and their complementary sequences): W217A, 5′-CAGAATATACCGGATCCC-CTTCTTGTTGGG-3′; W156A, 5′-CCAAGCCGTCGCCGCGCTTGTCACCATCTCTGAGTCAACACG-3′; W162A, 5′-CCTTGAGCACCTGCTGACATCAAACCGCC-3′; W360A, 5′-GATTCGTTCTCCTAAGTTGCGCAATGCGCGCC-3′; D283H, 5′-GTACTGGGATTTGCTACGTATGGCCC-3′; D283A, 5′-GTACTGGAATTTGTCAGTAGTGGCCTGGTGG-3′; D283H, 5′-GTACTGGGATTTGTCAGTAGTGGCCTGGTGG-3′; H211N, 5′-CCTTGGCGGTGTTAACGAGAATATACCGCC-3′; H211A, 5′-CCTTGGCGGTGTTAACGAGAATATACCGCC-3′; H211A, 5′-CCTTGGCGGTGTTAACGAGAATATACCGCC-3′; Y285A, 5′-G-

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The atomic coordinates and structure factors (code 1J9Y) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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‡ The abbreviations used are: GH, glycoside hydrolase; 2,4-DNP, 2,4-dinitrophenyl-β-mannobioside; Pc Man26A, family 26 mannanase from *Pseudomonas cellulosa*; TT Man5A, family 5 mannanase from *Thermomonospora fusca*; Pc Xyn10A, family 10 xylanase from *Pseudomonas cellulosa*; HPLC, high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid.
RESULTS AND DISCUSSION

Crystallographic Analysis—Good quality single crystals were grown by the hanging drop method using a reservoir of 26% polyethylene glycol monomethyl ether 550 with 0.012 M zinc sulfate and 0.1 M MES buffer at pH 6.5. These crystals are tetragonal, space group P4₁ or P4₃, with a = 93.2 Å, c = 54.8 Å and with a single molecule in the asymmetric unit. The tetragonal crystals can be cryocooled to 100 K directly for data collection because of their high content of low molecular weight polyethylene glycol monomethyl ether.

The results of the data collection, phasing, and refinement are presented in Table II. A single monomethyl mercury site and 12 uranyl sites were used in calculating the protein phases. The space group ambiguity was resolved by comparing the occupancies of the heavy atoms during heavy atom refinement in both space groups, and the space group was established as P4₁. The final model comprises 337 residues in three protein chains, four zinc ions, and 446 water molecules. One of the zinc ions mediates a crystal contact. The final R-factor and R-free are 18.2 and 21.1% at 1.85 Å resolution, and the overall G-factor from PROCHECK is +0.20 (Table II contains additional measures of the stereochemical quality of the final model).

There is no evidence in the electron density map for residues 361–391, but SDS-polyacrylamide gel electrophoresis analysis of dissolved crystals showed the protein to have the correct molecular mass. This sequence is therefore present in the crystal but is highly mobile or disordered. Also disordered in the crystal are: the eight residues 324 to 331; the five amino-terminal residues, Arg-39—Lys-43; and the four carboxyl-terminal residues, Leu-322—Trp-360, and Thr-392—Thr-419. Adventitious binding of zinc occurs close to the active site of Pc Man26A, a result of the inclusion of 12 mM zinc sulfate in the crystallization conditions. Photon-induced x-ray emission analysis (26) of purified Pc Man26A showed that in buffers lacking zinc there is no zinc bound to the enzyme. The presence of zinc had no significant effect on the catalytic activity or stability of the enzyme.

Description of the Crystal Structure of Pc Man26A and Comparison with Ty Man5A and Other GH-A Enzymes—The α-carbon trace of the Pc Man26A polypeptide revealed the classic (α/β)₁₀-barrel architecture (Fig. 1), as first seen in triose phosphate isomerase (27), with the major axis running from β1 to β5, typical of clan GH-A enzymes (Fig. 2; Ref. 13). The disordered regions described above comprised the extreme amino- and carboxyl-terminal sequences and the loops between β7 and α7 (residues 324–331) and β8 and α8 (residues 361–391). The putative acid/base catalyst (Glu-212) is at the end of β4, the nucleophile catalyst (Glu-320) is at the end of β7, Glu-212 and Glu-320 are 5.5 Å apart, and the spatial location of these amino acids is identical to the catalytic residues of other clan GH-A enzymes. Pc Man26A and, by inference, all other GH26 enzymes are therefore demonstrated to be members of clan GH-A.

Table I

| Residues 1–28 of PC Man26A comprise the signal peptide. A mature form of Pc Man26A consisting of residues Arg-39 (underlined) to Lys-423 was used for crystallization and subsequent x-ray analysis. The revised carboxyl terminus is displayed in bold type. |
|---------------------------------------------------------------|
| MKTTTARPLWAAQSPFALGICL1JLLGCNNAHANSASSADRVKPTTVKLV 50 |
| DSQATMELSTRSFAPMEQCHR15MSFMHQQHETQCTLI7RTDQSTDTNA 100 |
| VGDFAVNYGWTLTSLIFVRARKALEVEQGXVIILTV55SHFDNPKTD 150 |
| TQNGWVPGCTSDQNPATFVVDLSPLGGYNPVLMSGYQLQAEEKMLNQDQ 200 |
| RLPVPFRLHENTSHGFWDKGKSTPEYMQYKLFREYSVSELYRVDGVRNF 250 |
| LHYASYSNFWDVTENNYLGERDYGPGVLGPDNTGYPVADNADWFVRVVA 300 |
| NAALVARMAEARGPIVESIIRPADIEAEGLYDNQWYRKLISGLKADPP 350 |
| ADEFTPANPDOEQYQQRTPLVK 400 |
| 423 |

**Description of the Crystal Structure of Pc Man26A and Comparison with Ty Man5A and Other GH-A Enzymes**

The α-carbon trace of the Pc Man26A polypeptide revealed the classic (α/β)_10-barrel architecture (Fig. 1), as first seen in triose phosphate isomerase (27), with the major axis running from β1 to β5, typical of clan GH-A enzymes (Fig. 2; Ref. 13). The disordered regions described above comprised the extreme amino- and carboxyl-terminal sequences and the loops between β7 and α7 (residues 324–331) and β8 and α8 (residues 361–391). The putative acid/base catalyst (Glu-212) is at the end of β4, the nucleophile catalyst (Glu-320) is at the end of β7, Glu-212 and Glu-320 are 5.5 Å apart, and the spatial location of these amino acids is identical to the catalytic residues of other clan GH-A enzymes. Pc Man26A and, by inference, all other GH26 enzymes are therefore demonstrated to be members of clan GH-A.
In addition, the β-bulges on strands 4 and 7 adjacent to the active site amino acids are very similar in clan GH-A enzymes (28), and Pc Man26A has the single β-bulge on strand 7 involving the nucleophile and a double β-bulge on strand 4 involving the histidine and acid/base (Fig. 1). The effect of the double β-bulge at the end of β4 is to cause the histidine (or asparagine in, for example, GH10 and GH5 enzymes) to stack against the acid/base. The overall location, geometry, and presentation of the active site amino acids in Man26A are therefore very similar in other clan GH-A enzymes.

The detailed interactions involving the active site amino acids are similar (Fig. 3, A and B) in the family 26 and 5 mannanases. Corresponding amino acids contributing to the active site or substrate binding cleft in Pc Man26A (and Tf Man5A, in parentheses) are: His-211 (Asn-127), Glu-212 (Glu-225), Trp-360 (Trp-254), and Arg-208 (Arg-50). In Pc Man26A, OE2 of Glu-212 (the acid/base) hydrogen bonds OD1 of Asp-283 2.8 Å away; this interaction is likely to play an important role in the high pKₐ of the carboxylic side-chain of the acid/base. ND1 of His-196 in Tf Man5A occupies a similar position to that of OD1 of Asp-283 in Pc Man26A and makes an equivalent hydrogen bond to the acid/base catalyst. Glu-320 (the nucleophile) hydrogen bonds the hydroxyl of Tyr-285 via OE1 and NH₂ of Arg-208 and ND1 of His-211 via OE2. These hydrogen bonds are equivalent to those between Glu-225 (OE1) and Tyr-198 (OH) and Glu-225 (OE2) and NH1 of Arg-50 and Asn-127 OD1 in Tf Man5A. The position of ND1 and NE2 of His-211 in Pc Man26A is equivalent to the position of OD1 and NE2 of Asn-127 in Tf Man5A (Fig. 3, A and B).

Superimposition of Tf Man5A (12), Bacillus agaratheran family 5 endoglucanase (29) and Pc Xyn10A (30) on Pc Man26A, using the α-carbon atoms of the asparagine/histidine and the two glutamates, gives root mean square deviations of 0.187, 0.382, and 1.302 Å, respectively. The mannanases therefore have the most similar disposition of active site residues. Key hydrophobic residues at the −1 subsite are also conserved in the mannanases and endoglucanase; Tyr-254 and Trp-360 of Pc Man26A have equivalents in both Tf Man5A (Tyr-198 and Trp-254) and the endoglucanase (Tyr-254 and Trp-262). Binding of a mannose to subsite −1 of Pc Man26A may be similar to that proposed for Tf Man5A because the “hydrophobic platform” Trp-360 (254) and Tyr-285 (198) are conserved (Figs. 3 and 4). Binding of mannoside residues to subsite −2 will be different in Pc Man26A compared with Tf Man5A because the pattern of tryptophans is different. Subsite −2 will probably

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**Table II**

| X-ray data collection | Native | Monomethyl mercury | Uranyl acetate |
|-----------------------|--------|--------------------|---------------|
| X-ray source          | BW7B, Hamburg | X31, Hamburg | MacScience, in-house |
| Detectors             | MAR345 | MAR300 | DIP1030 |
| Temperature (K)       | 100    | 100    | 100 |
| Resolution (Å)        | 1.85   | 2.0    | 2.7 |
| Completeness (%)      | 99.8   | 97.2   | 92.4 |
| Highest shell Rmerge(I) (%) | 99.4   | 88.5   | 77.1 |
| Overall Rmerge (%)    | 3.3    | 6.8    | 7.3 |
| Highest shell (I/σ(I)) | 7.7    | 15.4   | 17.3 |
| Overall              | 29.9   | 19.9   | 9.2 |
| Highest shell Rfree (%) | 10.2   | 7.8    | 4.6 |
| Overall phasing statistics | 19.8  | 19.8   | 29.3 |
| Resolution for phase calculation (Å) | 2.9    | 2.9    | |
| Number of sites       | 1      | 12     | 12 |
| Phasing power         | 1.22   | 1.78   | 1.35 |
| Centric              | 0.98   |        | 0.72 |
| Acentric             | 0.79   | 0.72   | 0.66 |
| Mean figure of merit  | 0.56   |        | |

**Refinement and model stereochemistry**

- X-ray data used in refinement:
  - Resolution limits (Å): 12.5–1.85
  - Number of reflections used in refinement: 38,213
  - Number of reflections used for R-free calculation: 2021
  - R-factor (%): 18.2
  - R-free (%): 21.1

- Root mean square deviation from ideal stereochemistry (target σ in parentheses):
  - 1–1 distances (Å): 0.013 (0.020)
  - 1–2 distances (Å): 0.029 (0.040)
  - 1–4 distances (Å): 0.032 (0.050)
  - Chiral volumes (Å³): 0.132 (0.150)
  - Peptide planes (Å): 0.022 (0.030)
  - Aromatic planes (Å): 0.009 (0.020)

- Analysis according to PROCHECK (25):
  - Ramachandran plot:
    - Residues in the most favoured regions (%): 91.8
    - Residues in additionally allowed regions (%): 8.2
    - Non-glycine residues in disallowed regions (%): 0.0
    - Overall G-factor: -0.20

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The various crystallographic parameters are defined as follows: Rmerge(I) = Σ[Iᵢ – ⟨I⟩]/Σ[Iᵢ], where Iᵢ is the intensity of the i-th observation, ⟨I⟩ is the mean intensity of the reflection, and the summation extends over all data; Rmerge = Σ[Fᵢ − Fᵢ]Σ[Fᵢ], the mean relative isomorphous difference between the native protein (Fᵢ) and the derivative (Fᵢ) data; Rmerge = Σ[Fᵢ − Fᵢ]/Σ[Fᵢ], where Fᵢ is the calculated heavy atom structure factor contribution; phasing power, (Fᵢ)(σ(I)) = Σ[Iᵢ − ⟨I⟩]/Σ[Iᵢ], where Iᵢ is the observed intensity of the i-th observation, and σ(I) is the standard deviation of the observed intensities.
involves Trp-162. A model of mannotriose bound to the substrate binding cleft of Pc Man26A, built based on the structure of the Tf Man5A mannobiase complex (12), is shown in Fig. 4.

Loop Flexibility—It is interesting to note that the two loops that are poorly defined or missing in Pc Man26A, loops 7 and 8, are also flexible or disordered in the family 5 mannanase. However, in Pc Man26A, loop 8 is 31 residues in length compared with only five residues in the family 5 enzyme.

Functional Importance of Amino Acids in the Distal Region of the Substrate Binding Cleft—The crystal structure of Pc Man26A suggested that Trp-162 could form an important hydrophobic stacking interaction with the saccharide unit located at the −2 subsite of the enzyme. The W162A mutant displayed biochemical properties similar to native Pc Man26A when using carob galactomannan or azo-carob galactomannan as the substrates (Table III). In contrast, W162A was 95-, 70-, and 30-fold less active than the wild type mannanase against mannotriose, mannotetraose, and mannohexaose, respectively. It is clear that Trp-162 is critical for the productive binding of mannoooligosaccharides but not for the productive binding of the polysaccharide mannan. The polysaccharide will be more conformationally restricted because of the extensive hydrogen bonding within the mannan. The likely role of Trp-162 is therefore to ensure that oligosaccharides bind in the correct orientation and conformation for cleavage. Similar observations have been made for Pc Xyn10A subsite −2 and +1 mutants (31, 32) and −6 subsite mutants of the Saccharomyces cerevisiae β-amylase (33) on their respective oligosaccharide and polysaccharide substrates. That binding interactions distal to the active site have a more profound influence on the cleavage of oligosaccharides than on polysaccharides is thus an emerging theme in polysaccharide hydrolases.

His-143 is highly conserved in GH26 enzymes and is located at the −2 subsite. The catalytic properties of H143A showed that the mutant enzyme exhibited very low activity against all substrates tested, both mannans and mannoooligosaccharides. It would appear, therefore, that His-143 plays an important role in substrate binding of both polysaccharides and oligosaccharides at the −2 subsite, probably through hydrogen bonds between the imidazole ring and the sugar hydroxyl groups.
Trp-156 in the active site of Man26A (Fig. 4) is more remote from the substrate-binding site. The catalytic properties of W156A were very similar to the wild type mannanase, revealing that this aromatic residue does not play a critical role in substrate binding or catalysis. This finding is consistent with the enzyme containing only four subsites extending from −2 to +2.

Functional Importance of Trp-217—Substrate modeling of Pc Man26A suggests that Trp-217 forms a hydrophobic stacking interaction with a mannopranosyl unit at the +1 subsite, which is consistent with the lack of activity of W217A against both mannotriose and mannotetraose (Table III). In contrast the capacity of W217A to cleave mannas was only approximately 4-fold lower than the wild type enzyme. These data are similar to the results obtained for W162A and indicate that modification of either the −2 or +1 subsite has a larger influence on oligosaccharide cleavage than polysaccharide hydrolysis.

Functional Importance of W360A—The structure of Pc Man26A indicates that Trp-360 interacts with the sugar located at the −1 subsite. The observation that W360A exhibited no catalytic activity against any of the substrates evaluated (Table III) underlines the importance of the −1 subsite in binding and catalysis. The interaction between Trp-360 and the mannos e moiety is likely to be critical for transition state stabilization. The importance of Trp-360 in enzyme function is further illustrated by the fact that this residue is highly conserved in clan GH-A enzymes, and substitution of this amino acid causes a substantial reduction in the catalytic activity of the respective enzymes. Although the data presented in this paper indicate that Trp-360 plays an absolutely critical role in binding and catalysis, this interpretation must be viewed with some caution. It is possible that the loss of the aromatic residue caused a distortion in the structure of the −1 subsite, which also contributed to the loss in activity. Unfortunately, as crystals of W360A have not been obtained, the influence of this mutation on the integrity of the −1 subsite cannot be assessed.

Functional Importance of His-211—The data presented in Table III revealed that the H211A mutation caused a 100- and 700-fold decrease in activity against carob galactomannans and mannotetraose, respectively, but only a 6-fold reduction in activity against 2,4-DNPM2. The imidazole ring of His-211, by forming a hydrogen bond with the carboxylic group of Glu-290, plays an important role in maintaining the position of the catalytic nucleophile. The loss in activity through the removal of His-211 is therefore likely to be due to a slight repositioning of the catalytic nucleophile such that it attacks the C-1 of the mannopranosyl residue less efficiently than in the wild type enzyme.

The mutation of the equivalent residue to His-211 (Asn-127) in Pc Xyn10A led to a large reduction in $k_\text{cat}$ but not $k_\text{cat}/K_m$ against substrates with good leaving groups (31, 32). This effect is caused by the loss of a hydrogen bond between Asn-127 and the C-2 hydroxyl of the sugar at the −1 subsite. The fact that the H211A mutation does not cause a large reduction in $K_m$ against 2,4-DNPM2 suggests that the histidine does not hydrogen bond to the C-2 hydroxyl of mannos. Thus, the role of the residue immediately preceding the catalytic acid/base residue is different between GH26 enzymes and other members of the 4/7-superfamily (clan GH-A).

The positions of ND1 and NE2 in His-211 are equivalent to the positions of OD1 and NE2 of the asparagine, and thus replacing His-211 with an asparagine residue might be anticipated to have little influence of the activity of Pc Man26A. However, H211N was much less active than wild type Pc Man26A (Table III). In clan GH-A enzymes the asparagine forms a hydrogen bond with the histidine, equivalent to Asp-
283 in Pc Man26A. To investigate whether the D283H mutation complements the H211N substitution, the H211N/D283H mutant was constructed and its catalytic properties evaluated. The data indicated that the mutant enzyme was virtually inactive, showing that the activity of the H211N mutant cannot be recovered by introducing its hydrogen-bonding partner by the mutation D283H.

Residues That Influence the Catalytic Nucleophile—The structure of Pc Man26A revealed that Tyr-285 formed a hydrogen bond with the catalytic nucleophile Glu-320. The data presented in Table III showed that the $k_{\text{cat}}$ of Y285A against carob galactomannan and 2,4-DNPM$_2$ was substantially reduced, as was the $k_{\text{cat}}/K_m$ for mannotetraose and the $K_m$ against 2,4-DNPM$_2$. It is possible that the Y285A mutation caused a slight change in the position of the catalytic nucleophile, which did not significantly influence its capacity to attack the C-1 of the mannose residue at the −1 subsite. However, subsequent deglycosylation occurs very slowly because this step requires proton abstraction from Glu-212, and the activated water molecule will be located further from the glycosyl-enzyme ester linkage. This interpretation of the properties of Tyr-285 are consistent with studies on Pc Xyn10A in which the mutation of some amino acids that influence the position of the catalytic nucleophile had a greater influence on $k_a$ than $k_f$ for substrates that had good leaving groups (32). The low activity against mannan reflects the poorer leaving group of this substrate, which requires protonation of the glycosidic oxygen to elicit the $k_a$ and $k_f$ steps of the reaction.

The removal of the catalytic nucleophile (E320A) of Pc Man26A reduced the catalytic activity of the enzyme 10$^4$-fold, which is not as great as other nucleophile mutants. A possible explanation for the relatively high activity displayed by E320A is that another amino acid is functioning as the catalytic nucleophile; Asp-283, which is approximately 3.5 Å away from Glu-320, is the only possible candidate. The observation that the double mutant E320A/D283A showed no detectable catalytic activity against all of the substrates evaluated (a decrease of $>10^7$) supports the view that Asp-283 can function as an alternative nucleophile when Glu-320 has been removed.

Analysis of the Products Generated by Mutants of Pc Man26A—The products generated by the Pc Man26A mutants when incubated with mannotetraose indicate that H211A, Y265A, and W162A hydrolyze mannotetraose predominantly to mannobi, similar to the wild type protein. In contrast, W217A generates mannotriose, mannotetraose, and mannotriose in the ratio of 1:2.5:1 (Fig. 5). These results suggest that mannotetraose does not form productive complexes with the enzyme by occupying exclusively the −2 to +2 subsites but cleaves the substrate with virtually equal efficiency when occupying either four (−2 to +2) or three subsites (−2 to +1; Trp-217 is located in the aglycone region of the active site). If Trp-217 only influenced substrate binding at the +1 subsite, mannotetraose would still interact with the +2 resulting in the generation of two mannohexose molecules. Thus it would appear that the W217A mutation has compromised substrate binding at both aglycone subsites, suggesting that the +1 and +2 sites act in synergy to bind substrate. Thus, loss of binding at the +1 subsite compromises the utilization of binding energy at the +2 subsite in the distortion of the mannohexose ring of the sugar at the −1 subsite from a chair configuration in its ground state into its transition state conformation. Support for this latter view is provided by a recent study (34) showing that extensive synergy occurred between the aglycone subsites of Pc Xyn10A.

Conclusions—The data presented in this report show that the GH26 enzyme Pc Man26A has a classic (α/β)$_4$ barrel struc-

Fig. 5. HPLC analysis of mannotetraose hydrolysis by wild type Man26A and the W217A mutant. Wild type Man26A (panel A) and W217A (panel B) were incubated with mannotetraose, and at regular time intervals an aliquot of the reaction was removed and analyzed by HPLC as described under “Materials and Methods.” The elution times of mannose, mannohexose, mannotriose, and mannotetraose from the HPLC column are labeled 1 to 4, respectively.
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