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Characterization of GDP-mannose pyrophosphorylase from *Escherichia coli* O157:H7 EDL933 and its broad substrate specificity

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Abstract

GDP-mannose pyrophosphorylase gene (ManC) of *Escherichia coli* (E. coli) O157 was cloned and expressed as a highly soluble protein in *E. coli* BL21 (DE3). The enzyme was subsequently purified using hydrophobic and ion exchange chromatographies. ManC showed very broad substrate specificities for four nucleotides and various hexose-1-phosphates, yielding ADP-mannose, CDP-mannose, UDP-mannose, GDP-mannose, GDP-glucose and GDP-2-deoxy-glucose.
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1. Introduction

Sugar-nucleotide diphosphate (NDP-sugar) donors are key substrates for glycosyltransferase reactions that can catalyze the synthesis of oligo and polysaccharides and glycoconjugates. Since genetic information of the sugar sequence is not encoded in the cell chromosome, the substrate specificities of glycosyltransferases ultimately determine the sugar composition and its sequence in the glycoconjugates [1]. GDP-mannose is an activated form of mannose used for mannosylation of N-linked glycoproteins and glycosylphosphoinositol anchors [2], and becomes also a key metabolic intermediate for the synthesis of various glycoconjugates, such as GDP-β-L-fucose, GDP-colitose, GDP-talose, GDP-perosamine and GDP-D-rhamnose [3–6]. Different forms of NDP-mannose other than GDP-mannose, i.e. ADP-mannose, UDP-mannose, and CDP-mannose, are rarely found in nature, but often used as substrates for structural study of glycosyltransferases [7,8], hence become alternative sugar donors for mannosyltransferase [9]. Likewise, variation of the sugar part of the NDP-sugar, such as NDP-glucoses (ADP-, CDP-, UDP- and GDP-glucose), NDP-galactose, etc. [10–12] are possible. Then the NDP-hexoses become important as activated or precursor compounds for the synthesis of other glycoconjugates and polysaccharides. Until now, because rare NDP-sugars are not easily available on the market, their roles and functions are not thoroughly studied yet, but their economic synthesis is in great demand.

GDP-mannose pyrophosphorylase is an important enzyme in eukaryotic and prokaryotic cells, doing the synthesis of GDP-α-D-mannose (GDP-mannose) from mannose-1-phosphate and GTP. Although the enzyme favors for the transferring PPi to the GMP moiety of GDP-mannose, which yields mannose-1-phosphate and GTP [2], its reverse enzyme reaction is relatively fast and efficient in generating GDP-mannose and PPi in the open system of cell metabolism. In this reversible reaction, the substrate and product inhibition

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levels of PPi to the enzyme are relatively high, and critical to determine its maximum activity [13].

GDP-pyrophosphorylase would surely become the key enzyme in in vitro synthesis of GDP-mannose. However, we recently found an interesting property of this enzyme, which shows very broad substrate specificities for sugar and nucleotide moieties, enabling production of various NDP-sugars. Thus, detailed characterization of the GDP-mannose pyrophosphorylase has drawn our attention and interest. In addition, since current ample microbial genome databases are becoming rich source of selecting better enzymes satisfying our needs, there is a great possibility of selecting better NDP-sugar pyrophosphorylase from the databases.

From the completed *E. coli* O157 genome database, the gene cluster involved in the synthesis of GDP-mannose has been revealed [14], but the functions of each gene in the cluster have not been elucidated by experiments.

In this paper, we present a notable broad substrate specificity of ManC [15] from *E. coli* O157 for various nucleotides and hexoses with epimeric carbon at C-2 position. Using such a broad substrate specificity, we could demonstrate efficient enzymatic synthesis of NDP-mannose except dTDP-mannose. In addition, our result suggests that using only this enzyme, we can possibly make 16 kinds of NDP-sugars.

2. Materials and methods

2.1. Bacterial strains, and materials

Escherichia coli (*E. coli*) O157 was obtained from ATCC (ATCC 700927) for ManC cloning, and *E. coli* DH5α and BL21 (DE3) were used for cloning and protein over-expression, respectively. Kanamycin and IPTG, mannose-1-phosphate, glucose-1-phosphate, 2-deoxy-glucose-6-phosphate, galactose-1-phosphate, *N*-acetyl-D-glucosamine-1-phosphate, glucose-1,6-diphosphate, guanosine 5′-triphosphate (GTP), uridine 5′-triphosphate (UTP), thymidine 5′-triphosphate (dTTP), adenosine 5′-triphosphate (ATP), cytidine 5′-triphosphate (CTP), GDP-mannose, and GDP-glucose were purchased from Sigma (Deisenhofen, Germany).

2.2. Construction of plasmid pYH0302

The coding region of ManC was amplified by PCR using following primers (forwarding-5′-TTAGGAGGATCCA-TGTCTGATGCGCCAATA-3′/backwarding-5′-TTAGGA-AAGCTTTTAATCTTCATGTTTGTAACG-3′) from the genomic DNA of *E. coli* O157. The PCR fragments of ManC were digested with BamHI/HindIII, and inserted into the IPTG-inducible expression vector pET24ma, which was kindly donated by Dr. Hiroshi Sakamoto (Pasteur Institute, Paris). The plasmids were introduced into *E. coli* BL21 and the transformants were grown in Luria-Bertani (LB) broth containing 50 mg l^{-1} kanamycin at 37 °C. When the cell OD_{600} reached 0.6, 0.1 mM IPTG was added to the cell broth. The cells were harvested after 8 h of induction at 30 °C. Other DNA manipulations, including preparation of plasmids, restriction enzyme digestion, ligation, and transformation of *E. coli* were followed the methods of Sambrook et al. [16].

2.3. Enzyme preparation and purification

For the preparation of ManC, the cells harvested from 100 ml of culture broth of recombinant *E. coli* BL21 were washed and suspended in 5 ml of 50 mM ammonium bicarbonate buffer (pH 7.5) containing 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.01% (v/v) 2-mercaptoethanol, and 1 mM dithiothreitol and then were subjected to ultrasonic disruption for 10 min alternating on and off for 3 and 8 s, respectively. The supernatant solution was obtained after centrifugation (17,000 × *g*, 20 min). For the purification, butyl-sepharose (10 mm by 30 cm; Pharmacia) with 500 mM $(NH_4)_2SO_4$ gradient–25 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 7.2) and Q-sepharose (16 mm by 60 cm; Pharmacia) with 500 mM NaCl gradient–20 mM Tris–HCl buffer were used by FPLC (Amersham pharmacia biotech. USA). The fractionated protein solutions were pooled and concentrated using an Amicon PM-30 ultrafiltration unit.

2.4. Enzyme reactions

The enzyme (0.4 U of ManC) was assayed with 5 mM $MgCl_2$, 50 mM ammonium bicarbonate buffer (pH 7.5), 5 mM GTP, 20 mM mannose-1-phosphate for 5 min at 37 °C [17]. One unit of ManC activity corresponds to the amount of enzyme which converts 1 μmol of GTP into GDP-mannose during 1 min at pH 8 at 37 °C. To examine substrate specificity on nucleotides, the same nucleotide concentration and reaction condition were used for all other NDPs, i.e. ATP, CTP, dTTP, and UTP, except GTP for 2 h at 37 °C. For sugar moiety specificity, 20 mM mannose-1-phosphate was replaced with glucose-1-phosphate, galactose-1-phosphate, 2-deoxy-glucose-1-phosphate, glucosamine-1-phosphate, and *N*-acetyl-*D*-glucosamine-1-phosphate, respectively. To make 2-deoxy-glucose-1-phosphate, 20 mM 2-deoxy-glucose-6-phosphate was incubated with phosphomannomutase (PMM) [18] from *E. coli* K12 using glucose 1,6-diphosphate as a cofactor.

2.5. Analytical methods

Analysis of NDP-sugars was carried out by HPLC [19] using strong anion exchange (4.5 × 250 mm) column with isocratic elution of the solvent mixture of 100 mM potassium dihydrogen phosphate (pH 3.5) and acetonitrile (9:1 volume ratio). The flow rate was set at 0.7 ml min^{-1}. For the analysis of UDP-glucosamine and UDP-*N*-acetylglucosamine, the solution of 25 mM potassium dihydrogen phos-

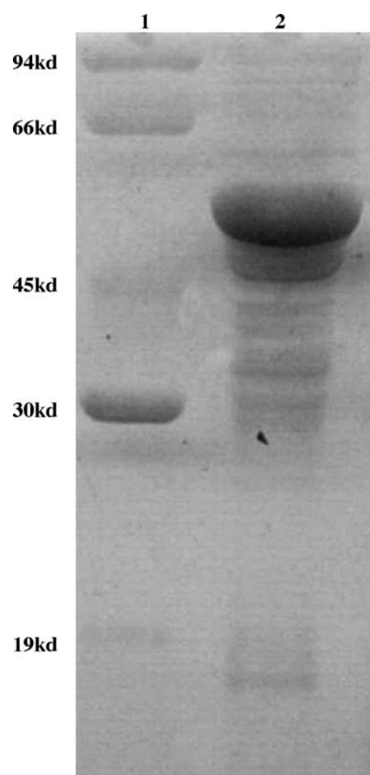

Fig. 1. SDS-PAGE of ManC. Lanes: (1) protein standards; (2) purified ManC enzyme.

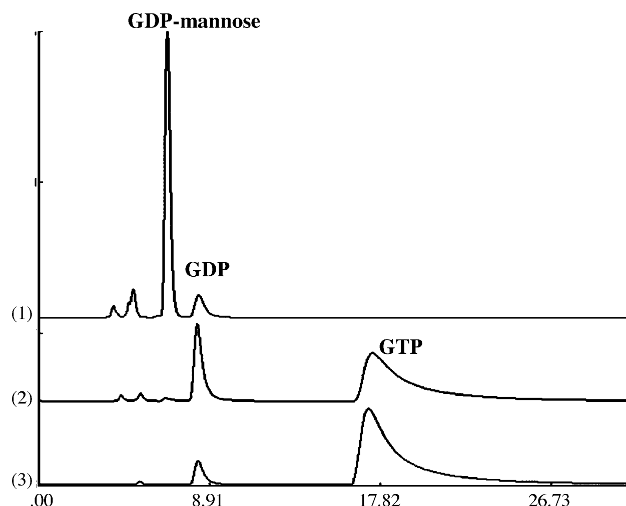

Fig. 2. Analysis of the reaction mixture by HPLC: (1) at $t=2$ h with ManC; (2) at $t=2$ h with *E. coli* cell extract; (3) at $t=0$. Retention times at the condition were as follows: GMP (5.3 min), GDP-mannose (6.9 min), GDP (8.5 min), and GTP (16.7 min).

phate (pH 3.5) and acetonitrile (9:1 volume ratio) were used.

Mass analysis (ESI-MS) was performed with LCQ Deca XP ion trap mass spectrometer (Thermo Electron Corp, USA) with standard Thermo ESI source in negative ion mode at the spray voltage of 5 kV. Samples were infused by syringe pump at a rate of 5 μl min^{-1} and nebulized with dry nitrogen gas. The heated capillary was maintained at a temperature of 270 °C. The maximum ion collection time was set to 50 ms and five microscans were averaged per scan.

3. Results and discussion

3.1. ManC expression and its characterization

ManC gene from *E. coli* O157 was cloned into pET24ma under the control of T7 promoter and expressed in *E. coli* BL21. The production yield of the enzyme from the crude cell extract was about 21%, calculated by ImageMaster 2D platinum (Amersham Biosciences). The analysis of purified protein by 12% SDS-PAGE confirmed 54 kD molecular weight (MW) of ManC (Fig. 1), and the enzyme activity from each purification step was summarized in Table 1. Then, optimal pH, Mg^{2+} ion concentration, and the ratio of mannose-1-phosphate to GTP for ManC activity were pH 8.5, 5 mM Mg^{2+} concentration and above eight, respectively. ManC activity was measured by the ratio of GMP and GDP to GDP-mannose peaks [13]. Under high and low metal ion concentrations, large GMP and GDP peaks were found, respectively, suggesting that metal ion concentration is closely related with GDP-mannose hydrolysis (data not shown). While the molar ratio of mannose-1-phosphate to GTP was increasing up to eight, the amount of GDP-mannose synthesized was monotonously increased, simply explaining that the excess of mannose-1-phosphate to GTP would shift the equilibrium of the reaction toward the synthesis GDP-mannose and function properly.

3.2. Synthesis of NDP-mannose, GDP-glucose and GDP-2-deoxy-glucose

When other NTP nucleotides, i.e. ATP, dTTP, UTP, and CTP were used as the substrate, relative reactivities for synthesizing corresponding nucleotide-mannoses [20] were relatively high. 20 mM of mannose-1-phosphate and 5 mM of GTP resulted almost full conversion (100%) within 2 h (Fig. 2), where as the same concentrations of ATP, UTP, CTP, and dTTP yielded 24, 20, 5, and 0% conversion, respectively.

Table 1
Purification of ManC over-expressed by *E. coli* BL21

Purification step	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell-free extract	1100	1.8	100	1
Q-sepharose FF	166.9	7.0	59	3.9
Butyl-sepharose FF	140.7	8.3	42	4.6

Table 2
Conversion of NTP and sugar-1-phosphate by ManC

NTP	Sugar					
	Man-1-pi	Glc-1-pi	2DGlc-1-pi	Gal-1-pi	GlcN-1-pi	NGlcN-1-pi
GTP	100%	13%	29%	0%	P	NP
ATP	24%	P	P	NP	P	NP
CTP	5%	P	P	NP	P	NP
UTP	20%	P	P	NP	4%	0%
dTTP	0%	NP	NP	NP	NP	NP

Man-1-pi: mannose-1-phosphate, Glc-1-pi: glucose-1-phosphate, 2DGlc-1-pi: 2-deoxyglucose-1-phosphate, Gal-1-pi: galactose-1-phosphate, GlcN-1-pi: glucosamine-1-phosphate, NGlcN-1-pi: *N*-acetylglucosamine-1-phosphate, P: possible considered from these experiments, and NP: not possible.

This result shows a broad substrate specificity of ManC for the nucleotides other than GTP. As ATP has a similar structure to GTP, it is no wonder for ATP to show similar reactivity to make ADP-mannose. However, the relatively high reactivities forUTP and CTP were unusual, considering structural differences between purine and pyrimidine nucleotides. No activity for dTTP which has no OH group at C-2 position of the ribose, indicates that the broad substrate specificity of ManC for various nucleotide originates from the recognition of the ribose moiety rather than the base changes in the nucleotides, because dTTP has only deoxyribose unit among NTPs. To explain these results, more studies on the enzyme structure and function are needed in the future.

Substrate specificities of ManC for other sugar-phosphates were also examined. Between glucose-1-phosphate and galactose-1-phosphate, ManC showed an activity only for glucose-1-phoshate, i.e. 13% of that of mannose-1-phosphate, suggesting that OH-group epimer at C-2 of the mannose is tolerated, but the epimer at C-4 position, i.e. galatose-1-phosphate, is not acceptable to ManC. This result suggests that OH group at C-4 carbon becomes a key determinant for the sugar-phosphate substrates of ManC. Based upon the above result, we attempted to use 2-deoxy-glucose-1-phosphate for ManC reaction. To make 2-deoxy-glucose-1-phosphate, the phosphate group of 2-deoxy-glucose-6-phosphate was moved to C-1 position using

Table 3
m/z of product and nucleotide diphosphate

Product [M]	Structure	Expected *m/z* of [M-H]$^{-}$ [a]	Detected *m/z* of [M-H]$^{-}$	Expected *m/z* of [NDP-H]$^{-}$	Detected *m/z* of [NDP-H]$^{-}$
GDP-mannose		604.3	604.2	442.2	442.2
ADP-mannose		588.3[b]	588.2	426.2	426.0
CDP-mannose		564.3[b]	564.1	402.1	402.1
UDP-mannose		565.3[b]	565.2	403.1	403.2
GDP-2-deoxyglucose		588	588.1	442.2	442.8
GDP-glucose		604.3[c]	604.8	442.2	442.0

[a] All the expected mass were from http://www.chemfinder.com.
[b] Masses of ADP-, CDP-, and UDP-mannose were not available, all the masses in the table were from mass of ADP-, CDP-, and UDP-glucose.
[c] Calculated.

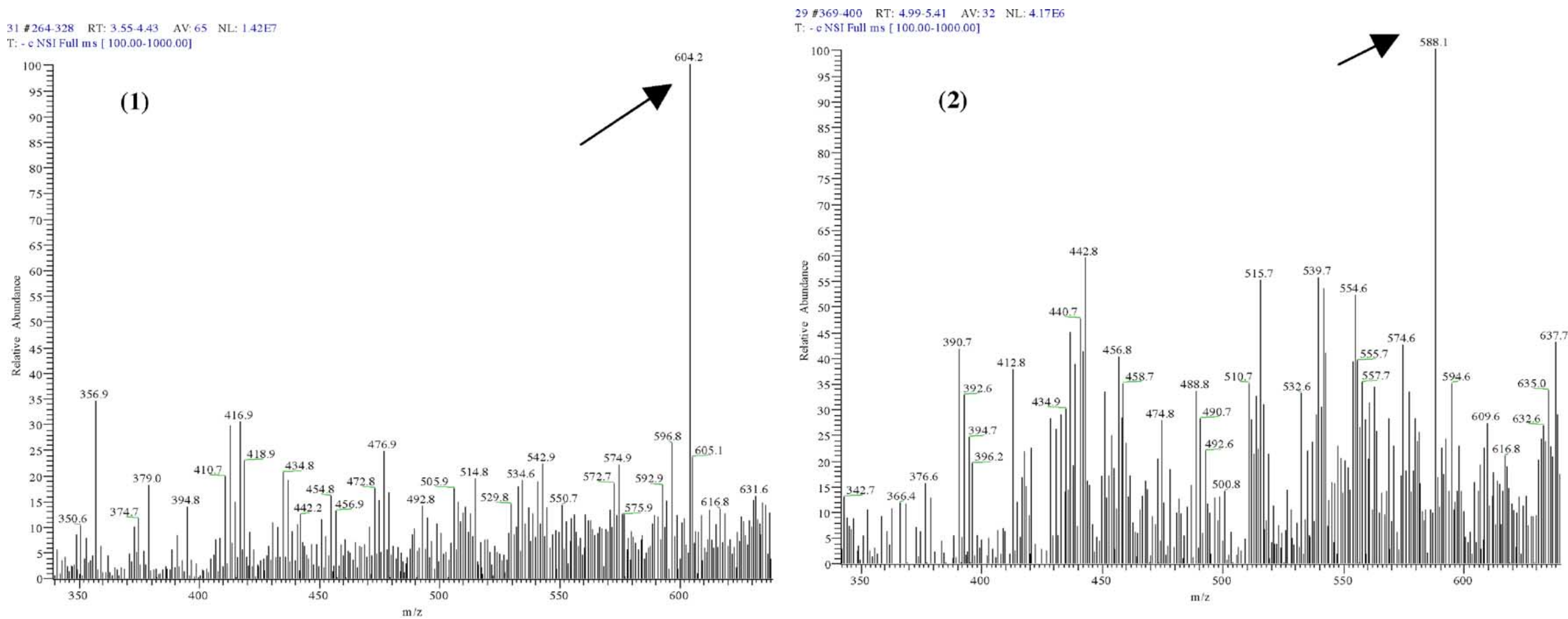

Fig. 3. The negative-ion ESI mass spectrum of m/z for each product: (1) GDP-mannose; (2) GDP-2-deoxy-glucose; (3) ADP-mannose; (4) GDP-glucose; (5) CDP-mannose; (6) UDP-mannose.

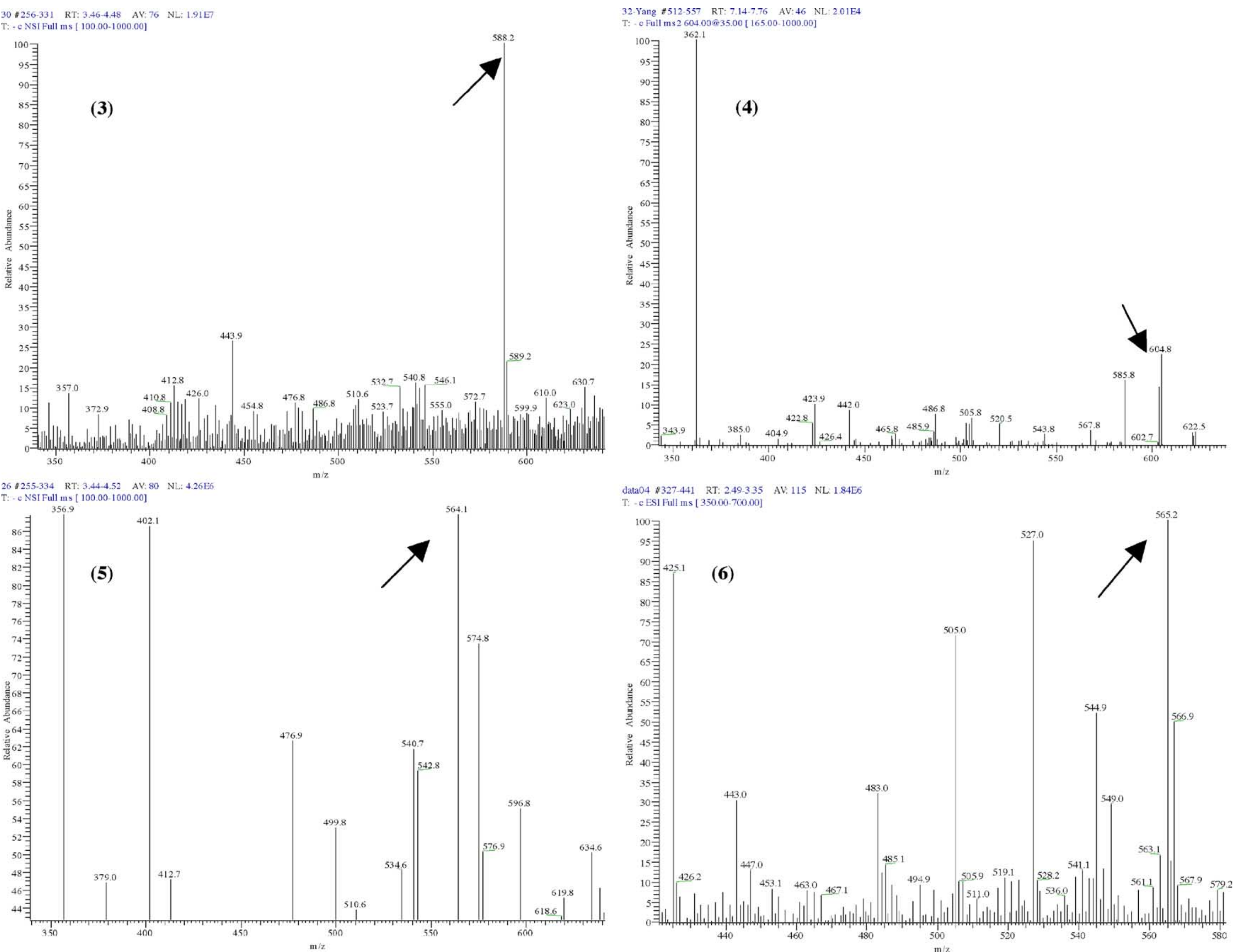

Fig. 3. (*Continued*).

phosphomannomutase (1 U). As expected, ManC activity for 2-deoxy-glucose-1-phosphate also displayed 29% of that of mannose-1-phosphate, resulting a good yield of GDP-2-deoxy-glucose. In result, we could confirm that ManC shows no preference in the recognition of the OH group epimers at C-2 of mannose (Table 2).

Although the reaction products were analyzed by HPLC, only the masses of the products were confirmed by ESI-MS, since we did not have their authentic samples. GDP-mannose peak was found at *m*/*z* 604 in the negative mode, and further confirmation was done with ESI-MS/MS (data not shown). Likewise all the products, i.e. ADP-mannose, CDP-mannose, UDP-mannose, GDP-glucose, and GDP-2-deoxy-glucose were confirmed by their *m*/*z* values of 588, 564, 565, 604, and 588, respectively (Table 3 and Fig. 3). As expected from HPLC data, we could not find any peaks for GDP-galactose, dTDP-mannose by ESI-MS.

To further examine the broad sugar-phosphate and nucleotide specificities of ManC, D-glucosamine-1-phosphate and *N*-acetyl-D-glucosamine-1-phosphate [21] having bulky groups at C-2 of glucose were used with UTP. Surprisingly, still the peak corresponding to UDP-glucosamine has been observed (Table 2), and its molecular mass was compared with those of the authentic sample and confirmed by ESI-MS (data not shown).

To roughly explain this broad substrate specificity of ManC, the crystal structure of RmlA (glucose-1-phophate thymidylyltransferase) complexed with its dTDP-rhamnose (1G1L) from *Pseudomonas aeruginosa* [22] was investigated. The RmlA has some conserved residues with ManC in N-terminal amino acid sequences for NDP binding sites, and Gly146, Glu161 and Tyr176 for sugar binding sites. According to the RmlA structure, interestingly we could identify a vacant space in the substrate binding site for various hexose sugars at C-2 position, so that no special functional groups from the vicinity amino acid residues in the substrate binding site are exerting to make specific hydrogen bondings with the OH group epimers at C-2, once again supporting our findings (data not shown). To explain the broad substrate specificities of ManC to the levels of detailed bondings, further researches on the structure of ManC are necessary.

4. Conclusions

GDP-mannose pyrophosphorylase, i.e. ManC, was characterized, and its broad substrate specificities on the nucleotide moieties as well as the epimers at C-2 of mannose were found out. In fact, the substrate specificity on various sugar-1-phosphate of sugar nucleotidyl transferase such as dTTP/UTP pyrophosphorylase has been studied from various sources [23,24], but little is known about GDP-mannose pyrophosphorylase on substrate specificity. Using the broad substrate specificities of ManC, various NDP-sugars can be easily synthesized using only ManC, which are confirmed by LC/ESI-MS. As the property of ManC is very interesting and remarkable, it is not surprising to see that an efficient route to synthesize NDP-rare sugars can be developed using it, and further study is remained to be seen.

Acknowledgements

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