Combined Biosynthetic Pathway For De Novo Production of UDP-Galactose: Catalysis with Multiple Enzymes Immobilized on Agarose Beads

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Regeneration of sugar nucleotides is a critical step in the biosynthetic pathway for the formation of oligosaccharides. To alleviate the difficulties in the production of sugar nucleotides, we have developed a method to produce uridine diphosphate galactose (UDP-galactose). The combined biosynthetic pathway, which involves seven enzymes, is composed of three parts: i) the main pathway to form UDP-galactose from galactose, with the enzymes galactokinase, galactose-1-phosphate uridyltransferase, UDP-glucose pyrophosphorylase, and inorganic pyrophosphatase, ii) the uridine triphosphate supply pathway catalyzed by uridine monophosphate (UMP) kinase and nucleotide diphosphate kinase, and iii) the adenosine triphosphate (ATP) regeneration pathway catalyzed by polyphosphate kinase with polyphosphate added as an energy resource. All of the enzymes were expressed individually and immobilized through their hexahistidine tags onto nickel agarose beads ("super beads"). The reaction requires a stoichiometric amount of UMP and galactose, and catalytic amounts of ATP and glucose 1-phosphate, all inexpensive starting materials. After continuous circulation of the reaction mixture through the super-bead column for 48 h, 50% of the UMP was converted into UDP-galactose. The results show that de novo production of UDP-galactose on the super-bead column is more efficient than in solution because of the stability of the immobilized enzymes.

KEYWORDS:

biosynthesis · enzymes · immobilization · polyphosphate · UDP-galactose

Introduction

Carbohydrates and glycoconjugates are gaining importance as a result of the discovery of their roles in molecular recognition, transmembrane signaling, and other physiological or pathological processes.[1] Much attention has been focused on biosynthetic pathways and preparative approaches to the synthesis of oligosaccharides, which are biologically distinct and pharmaceutically valuable. Leloir glycosyltransferase catalysis is one of the common ways to synthesize oligosaccharides, but is dependent on the availability of expensive sugar nucleotides.[2–5] In enzymatic glycosylation,[6] sugar nucleotides serve as transient intermediates that are regenerated continually in situ in catalytic amounts. The nucleotide portions are reused in the biosynthetic cycle. Previously, we have successfully synthesized a galactose-α1,3-galactose-terminated trisaccharide by using the enzymes involved in the regeneration of uridine diphospate galactose (UDP-Gal) and a truncated bovine α1,3-galactosyltransferase.[7, 8] Unlike the bovine enzyme, most important eukaryotic glycosyltransferases require post-translational modification that does not take place in bacteria.[9–12] Additionally, the optimal reaction conditions for the glycosyltransferases may be incompatible with those for the enzymes in the sugar nucleotide biosyntheses. Hence the separate preparation of sugar nucleotides in large quantities is necessary. The isolated sugar nucleotides can be conveniently used to synthesize any target oligosaccharides.

There are eight main sugar nucleotides that function as the donor substrates of glycosyltransferases in mammals: UDP-glucose (UDP-Glc), UDP-Gal, UDP-glucuronic acid, UDP-*N*-acetylglucosamine, UDP-*N*-acetylgalactosamine, cytosine monophosphate (CMP)-*N*-acetylneuraminic acid, guanine diphosphate (GDP)-mannose, and GDP-fucose.[13] Among these nucleotides, UDP-Gal and schemes for its regeneration are the most prevalent in the literature. In contrast to sugar nucleotide regeneration cycles, de novo production of sugar nucleotides requires a stoichiometric supply of the respective nucleotide and two high-energy equivalents for each product molecule formed. For instance, UDP-Gal has been enzymatically synthesized on the gram scale from uridine triphosphate (UTP; added stoichiometrically) and galactose 1-phosphate formed by the phosphorylation of galactose in a separate reaction.[14] UDP-Gal has also been produced from UDP-Glc with three enzymes coupled together to drive the reaction forward.[15] Recently, Kyowa Hakko Inc. (Japan) reported significant progress in the biosynthesis of oligosaccharides and sugar nucleotides, including UDP-Gal.[16–18] Desirable yields of oligosaccharides and sugar nucleotides were obtained based on an engineered strain of *Corynebacterium ammoniagenes* that produces UTP from orotic acid and was coupled with two recombinant *Escherischia coli* strains. Although accumulation of UDP-Gal was reported in their work, the major focus of researchers at Kyowa Hakko Inc. was on the final oligosaccharide product.

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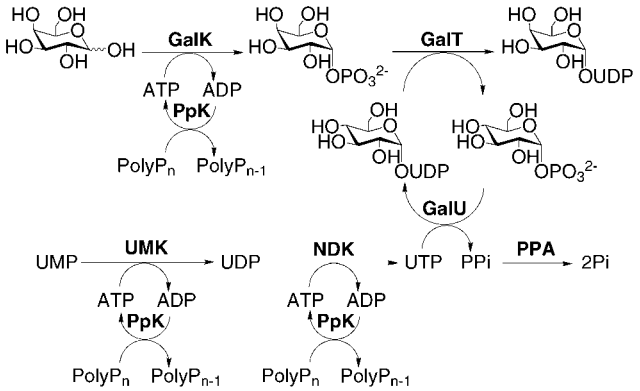
Herein, a combined approach to the de novo production of UDP-Gal is designed from previous knowledge and information on sugar nucleotide metabolism.[19] There are seven enzymes involved in this proposed biosynthetic pathway, which consists of three parts, the main UDP-Gal production pathway, the UTP supply pathway, and an adenosine triphosphate (ATP) regeneration pathway (Scheme 1). The main pathway starts with the



Scheme 1. *Biosynthetic pathway for UDP-Gal production. For abbreviations, see text.*

phosphorylation of galactose (Gal), catalyzed by galactokinase (GalK, EC 2.7.1.6).[20, 21] The galactose 1-phosphate (Gal-1-P) formed is then converted into UDP-Gal by galactose 1-phosphate uridyltransferase (GalT, EC 2.7.7.10),[22] which uses UDP-Glc as the nucleotide source and also produces glucose 1-phosphate (Glc-1-P). UDP-Glc is provided by UDP-glucose pyrophosphorylase (GalU, EC 2.7.7.9), which catalyzes the condensation of Glc-1-P with UTP.[23, 24] The reaction generates pyrophosphate (Ppi), which can be hydrolyzed by inorganic pyrophosphatase (PPA, EC 3.6.1.1) to form two phosphate (Pi) groups and thus drive the UDP-glucose production equilibrium forward.[25, 26] The necessary stoichiometric amount of UTP is made from uridine monophosphate (UMP), an inexpensive uridine source, by reactions catalyzed by UMP kinase (UMK, EC 2.7.4.14)[27, 28] and nucleotide diphosphate kinase (NDK, EC 2.7.4.6).[29, 30] ATP, which directly provides free energy, is regenerated from ADP and polyphosphate (polyP) by polyphosphate kinase (PpK, EC 2.7.4.1). Therefore, the inexpensive polyP serves as the ultimate energy source.[31–34]

Immobilization of the enzymes possesses many advantages, such as simplified isolation of the enzymes from the reaction mixture, stability and reuse of the enzymes, and possibly improved enzyme kinetics.[35–37] Immobilized metal affinity chromatography (IMAC) is a common approach to the purification of proteins with hexahistidine tags. The nickel agarose beads immobilized with the enzymes attached, can be used directly as the catalytic agent.[38] In our system (Figure 1), the reaction mixture is continually circulated through a column that contains the enzyme-charged beads. This system provides an efficient and practical method for the production of UDP-Gal from inexpensive starting materials.

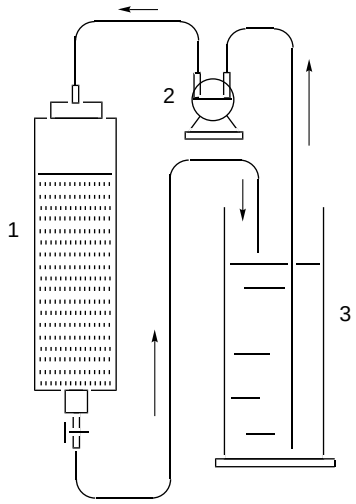


Figure 1. *Device for closed recirculating reaction. 1) column packed with bead-immobilized enzymes; 2) pump; 3) mixture reservoir.*

Results

Expression and characterization of enzymes

The recombinant *E. coli* strains were each cultured in the same volume (1 L). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) results showed the proteins to have molecular weights identical to the predicted values (GalK: 43.6 kD, GalT: 42.1 kDa, GalU: 35.5 kDa, PPA: 21.9 kDa, UMK: 28.3 kDa, NDK: 17.6 kDa, and PpK: 82.6 kDa; Figure 2). None of

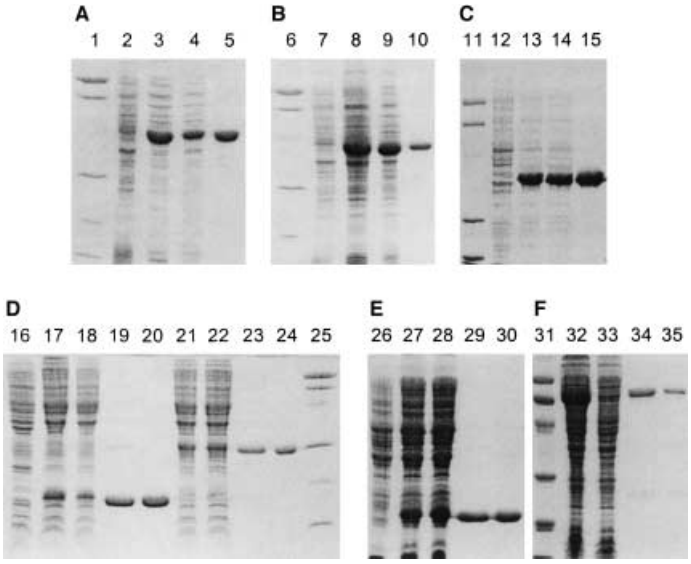


Figure 2. *SDS-PAGE for expression and purification of the enzymes. A, GalK; B, GalT; C, GalU; D, NDK and UMK; E, PPA; F, PpK. A, B, and D were performed in 15% gels, C, E, and F in 12.5% gels. Lanes 1, 6, 11, 25, and 31: low-range protein standards (from top to bottom: phosphorylase B, 97.4 kDa; serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa); lanes 2, 7, 12, and 16: BL21(DE3) host cells; lanes 3–5, 8–10, 13–15, 17–19, 21–23, 27–29, and 32—34: expressed cells, lysates, and proteins.*

the over-expressed proteins was toxic to the host, hence the cell populations were comparable to each other after growth. The protein expression levels were also comparable but the solubilities were different. It was estimated that GalK, UMK, and PPA are nearly 100% soluble; NDK, GalU, and GalT are approximately 80%, 70%, and 50% soluble, respectively; PpK is expressed mostly as inclusion bodies and is 30% soluble. The enzymes obtained after affinity chromatography were pure enough for enzymatic reactions. Gel filtration was carried out to identify the native conformations of the proteins in solution and further purified enzymes were used for the kinetic studies. The results suggested that GalK and PpK are monomers and GalT is a dimer, whereas GalU, PPA, UMK, and NDK are tetramers in solution (data not shown). The enzyme yields from the same culture volume were different because of the different solubilities. The activities of the enzymes varied considerably (Table 1). Therefore, the enzyme immobilization could be controlled by the volume of enzyme-containing lysates used, such that each enzyme had comparable overall activity within a test mixture. The mixture comprised the lysates from GalK (4 L), GalU and PpK (2 L each), GalT and NDK (1 L each), UMK (0.5 L), and PPA (0.25 L).

Table 1. *Purification of individual enzymes from 1 L bacterial culture.*

Enzyme	Amount	Purification step			
		homogenate	lysate	nickel affinity	gel filtration
GalK	protein[a]	1400	28	12	7.1
	activity[b]		40	30	20
GalT	protein[a]	1200	19	6.5	4.1
	activity[b]		500	350	260
GalU	protein[a]	1500	7	3.0	2.2
	activity[b]		300	200	160
PPA	protein[a]	1300	15	10	6.4
	activity[b]		2000	1400	1000
UMK	protein[a]	1300	8	3.0	2.1
	activity[b]		1500	1000	800
NDK	protein[a]	1500	10	3.3	2.0
	activity[b]		500	360	240
PpK	protein[a]	1400	10	3.2	2.0
	activity[b]		300	220	150

[a] Measured in mg. [b] Measured in Units (U).

Stepwise analysis of the biosynthetic pathway

The performance of each of the three parts of the proposed biosynthetic pathway was investigated by stepwise incorporation of more enzymes and simpler starting materials into the reaction. The results shown in Table 2 indicate that a 70% yield of UDP-Gal was generated based on UTP as the starting material (combination 1) in 24 h; 45% yield was obtained when the reaction started with UMP and ATP present (combination 2); 35% yield was obtained from UMP with the addition of polyP (combination 3). It was concluded that the proposed biosynthetic pathway to produce UDP-Gal is feasible in practice.

Kinetics of individual and multiple enzyme reactions

The kinetic behavior of every enzyme was examined prior to and following immobilization. The apparent Michaelis constant, K_m, of most enzymes for the major substrates increased only slightly upon immobilization but the GalU $K_m^{Glc\text{-}1\text{-}P}$ and PpK K_m^{ADP} values doubled after immobilization. The maximum reaction rate, V_{max}, for some of the enzymes was significantly changed upon immobilization. GalK and PpK had greater V_{max} values when immobilized rather than in solution. However, the V_{max} values of GalU and NDK decreased. The V_{max} values of GalT, PPA, and UMK were unchanged by the immobilization.

The kinetics of the multiple-enzyme reaction revealed that the apparent K_m values for the four main substrates were slightly affected by the immobilization. The K_m values for Gal, UMP, and ATP increased and that for Glc-1-P was decreased by immobilization. The V_{max} value for the whole reaction was 50% increased after immobilization, which suggests an improvement in the efficiency of UDP-Gal production when it is catalyzed by all the immobilized enzymes (Table 3), rather than produced in solution.

Table 3. *Kinetic parameters of individual or multiple-enzyme reactions.[a]*

Enzyme	Substrate	K_m [μM]		V_{max} [nmol min^{-1} mg^{-1}]		Product
		soluble	immobilized	soluble	immobilized	
GalK	Gal	200	250	2000	2800	Gal-1-P
	ATP	120	160			
GalT	Gal-1-P	450	500	55000	53000	UDP-Gal
	UDP-Glc	85	120			
GalU	Glc-1-P	200	400	7000	5000	UDP-Glc
	UTP	650	750			
PPA	PPi	0.20	0.22	150000	150000	Pi
UMK	UMP	0.09	0.12	200000	220000	UDP
	ATP	22	28			
NDK	UDP	30	35	15000	8000	UTP
	ATP	20	25			
PpK	ADP	150	300	2000	2500	ATP
Multi	Gal	300	400	2000	3000	UDP-Gal
	UMP	20	25			
	ATP	25	25			
	Glc-1-P	25	20			

[a] Each reaction (1 mL solution) was carried out with purified or resin-immobilized enzymes. The V_{max} value for more than one main substrate is given under saturating concentrations of the substrates. The results shown are representative of three independent experiments.

Table 2. *Stepwise production of UDP-Gal.*

Combination	Enzymes	Starting materials	UDP-Gal [%]
1	(GalK + GalT + GalU + PPA)	Gal + Glc-1-P(cat) + ATP + UTP	70
2	(GalK + GalT + GalU + PPA) + (UMK + NDK)	Gal + Glc-1-P(cat) + ATP + UMP	45
3	(GalK + GalT + GalU + PPA) + (UMK + NDK) + PpK	Gal + Glc-1-P(cat) + ATP(cat) + UMP + PolyP	35

Production of UDP-Gal

At first, small-scale reactions on mini Pasteur pipette columns were carried out to optimize the reaction conditions, then the gram-scale reaction was set up in the regular-sized column. In a reaction mixture (200 mL) with UMP (1.47 g, 4 mmol), galactose (0.72 g, 4 mmol), polyP (2.0 g, 1% (w/v)), ATP (0.11 g, 0.2 mmol), and Glc-1-P (0.06 g, 0.2 mmol), about 50% (gross yield) of the UMP was transformed into UDP-Gal (1.2 g, 2.0 mmol) in 48 h. Finally, 60% of the produced UDP-Gal (0.7 g, 1.2 mmol) remained after purification, which suggests an overall yield of 30% based on UMP. NMR spectroscopy and mass spectrometry (MS) were utilized to identify the produced UDP-Gal. Under the same reaction conditions (50 mL), incubating with the purified enzymes gave UDP-Gal in 35% gross yield based on UMP. UDP-Gal purification after the reaction was omitted in this case, but the overall yield after purification would clearly be lower than that of the on-column reaction. In summary, the space-time yield of the on-column reaction (1.80 g L^{-1} d^{-1}) was higher than that of the solution reaction (1.26 g L^{-1} d^{-1}), which suggests an improved efficiency of UDP-Gal production (Table 4).

Table 4. *UDP-Gal production in the solution or on-column reaction.*

	Solution[a]		On-column[b]	
	UMP	UDP-Gal	UMP	UDP-Gal
start [mmol]	1.00		4.00	
reacted [mmol]	0.40	0.35	1.00	2.00
purified [mmol]				1.20
gross Yield [%]	35	50		
overall Yield [%]		30		
space-time yield [g L^{-1} d^{-1}][c]	1.26	1.80		

[a] The reaction was carried out in solution (50 mL) with purified enzymes. [b] The reaction mixture (200 mL) was continually circulated on the column and the produced UDP-Gal was purified. [c] The data were calculated based on the presumably constant purification yields for both reactions.

The reaction time course suggested that the initial production rate in the on-column reaction is slightly lower than that in the solution reaction in the first 6 h. However, the on-column reaction reaches a plateau after 48 h as opposed to 24 h in the case of the solution reaction, therefore the on-column reaction results in higher product yields (Figure 3).

Reuse and productivity of enzymes

The column with the enzymes immobilized on beads was stored at 4 °C after washing out the reacted mixture. The column was reused for newly set up reactions, but only half the activity of the whole enzyme mixture remained after the first reaction. Lysates from PpK-expressed *E. coli* (1 L) were loaded onto the used column because PpK is even unstable in the purification and storage steps. The reaction yield was then 40%, which suggests that 80% of the whole activity was restored. When GalK lysates from the culture (2 L) were loaded as well as PpK lysates, the activity of the column could be almost completely restored if it was used within a one-month period. Except for PpK and GalK, which need to be refreshed for each

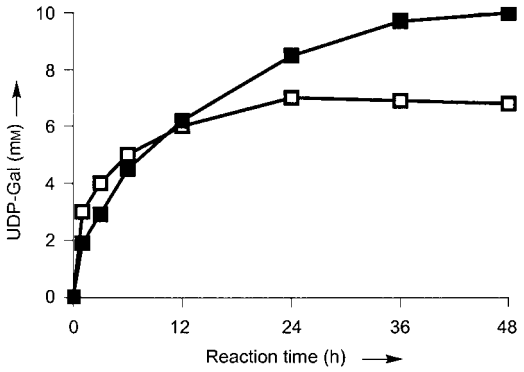


Figure 3. *Time course of UDP-Gal production. The reaction on the super-bead column (200 mL, filled squares) was performed with UMP and galactose (20 mM each), polyP (2% (w/v)), ATP and Glc-1-P (2 mM each). About 50% UMP was converted into UDP-Gal in 48 h. Reaction in the solution with the purified enzymes (50 mL, open squares) used the same reaction composition. About 35% UMP was converted in 24 h. The results shown are the mean values from three experiments. Standard error of the mean (S.E.M.) values (not shown) amounted to less than 5% of the corresponding means.*

new reaction, the enzymes on the column can be used at least four times in a month with only slight loss of their activity. After repeated syntheses, all the enzymes, deactivated or not, were removed from the nickel beads and the beads were recharged for further use. A limited amount of the enzymes can be used to catalyze a reaction with a large volume of substrate solution when the reaction mixture is continuously passed and recirculated through the column. In this way, a higher efficiency, beyond the capacity of these limited enzymes, can be realized. We think this recirculation would be another way to reuse the bead-immobilized enzymes.

The enzyme productivity of the on-column reaction has no advantage over that of the solution process in a single reaction. For example, the productivity of GalU in the on-column reaction (3.75 mg U^{-1}) is lower than that in the solution reaction (6.56 mg U^{-1}). With several reuses, the total productivity of most enzymes immobilized on the column can be significantly higher than that of those used once in solution.

Discussion

GalT catalyzes the conversion from UDP-Glc and Gal-1-P into UDP-Gal and Glc-1-P in the classical Leloir pathway. Phosphoglucomutase and Glc-6-P dehydrogenase are utilized to remove the byproduct Glc-1-P and shift the equilibrium in the UDP-Gal synthesis direction.[15, 19] However, these enzymes were not actually applied in the biosynthetic pathway engineered by others, for example Kyowa Hakko Inc., for the synthesis of oligosaccharides with UDP-Gal regeneration, in which galactosyltransferase could drive the whole reaction by consumption of the regenerated product, UDP-Gal.[16] Herein, UDP-Gal is the final product. Our designed biosynthetic pathway has two key steps: Gal-1-P supply and UDP-Glc regeneration. The phosphorylation of Gal by GalK is the critical step, because GalT catalysis requires a relatively high concentration of Gal-1-P so as to drive the reaction forward to UDP-Gal. The Glc-1-P byproduct is treated

with UTP to regenerate UDP-Glc at catalytic amounts in an interchangeable reaction catalyzed by GalU. PPA is used to consume PPi, the byproduct of UDP-Glc production, and drive the reaction in the forward direction.[25, 26]

The production of UDP-Gal was not affected by the presence of up to 20 mM ATP. Therefore, ATP can be added up to this concentration as an energy source. However, polyP is a much cheaper high-energy donor than ATP and, interestingly, not an inhibitor at any step of the biosynthetic pathway of sugar nucleotides.[33, 34] Hence, polyP was used as the ultimate energy source.

Only the GalU $K_m^{Gal-1-P}$ and PpK K_m^{ADP} values were significantly affected by immobilization, which suggests that the kinetic behavior of these two enzymes is much more sensitive to immobilization than that of the others. GalK and PpK were relatively unstable but their V_{max} values were significantly improved after immobilization. NDK has the smallest molecular weight among the seven enzymes and, as well as GalU, was observed to have a decreased V_{max} value after immobilization. Practically, K_m and/or V_{max} changes for the individual enzymes resulted in favorable effects on the overall reaction since the overall V_{max} value was increased by 50% after immobilization. However, the time course indicated that the initial rate of UDP-galactose production is slightly lower in the on-column reaction than for the solution reaction with the purified enzymes. If proximity leads to a higher reaction rate, as also discussed in our previous study,[39] immobilization may not enhance the production rate in a multiple-step reaction because of lower proximity of the enzymes than can be achieved in solution. Packing the immobilized enzymes onto a column should slow down the rate of the reaction. The immobilization resulted in increased overall yield of the reaction, albeit at the expense of increased reaction times. Stabilization of the enzymes would be the predominant effect of the immobilization and the reason for the yield improvement. Generally, the immobilized enzyme exhibits high stability against inactivation caused mainly by high temperature (room temperature or 37 °C) and long-term use (days). The stability is attributed both to the elimination of bacterial proteases and to the inherent stabilization of proteins that are associated with metal chelation.[40]

Following the protocol of Revers et al,[38] we removed the enzyme-charged resin slurry from the column into a beaker and set up a reaction under continuous stirring. We observed immobilized enzymes that were partially detached from the beads before the reaction ended. For this reason we abandoned this protocol and continued to use the enzymes in the column. The reactor allows for convenient product separation and avoids the centrifugation step necessary to remove the resin in the protocol of Revers et al. Even so, it was observed that about 10% of the enzymatic activity was lost after a 48 h reaction. In essence, these features render this approach highly practical, and may facilitate parallel development for other nucleotide sugars where progress is less advanced.

Affinity chromatography with nickel chelating resin is a common technique for purification of proteins tagged with oligohistidine at the N or C terminus. Other immobilization methods include the use of different tags such as glutathione *S*-transferase,[41] protein A,[42] and nonspecific covalent attachment to *N*-hydroxysuccinimide activated resin. These may be expected to have higher capacity and stronger protein binding ability. However, hexahistidine tags can be used for one-step purification of the recombinant proteins. This purification is a form of immobilization and has been shown previously by our group[8] and others[38] to be a successful method for obtaining active resin-bound enzymes for oligosaccharide biosynthesis.

UDP-Gal could be prepared at a low cost (< US$ 20 per g), mostly influenced by the price of UMP, with UMP and Gal (one equivalent each), polyP (1% (w/v)), and catalytic amounts of ATP and Glc-1-P (1/20th of one equivalent each) as the starting materials. It is clear that this is an extremely cost-efficient way to generate UDP-Gal as compared to the commercial prices of this compound (US$ 439.20 per 100 mg from Sigma).

Experimental Section

Materials: *E. coli* strain K-12 (MG 1655) was obtained from the American Tissue Culture Collection (ATCC); this served as a DNA template for gene cloning of the involved enzymes. Strain DH5α [*lacZ*Δ*M15 hsdR recA*], used for molecular cloning, was from Gibco. Expression vector pET15b and the host strain BL21(DE3) [F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm (DE3)*] were from Novagen. The restriction enzymes, T4 DNA ligase, and DNA ladder were purchased from Promega; the kits for purification of chromosomal DNA, plasmid DNA, PCR product, and DNA fragments were from Qiagen. Nickel-nitrilotriacetic acid (Ni – NTA) agarose was also a product of Qiagen. Low-range protein standards were purchased from Bio-Rad. Radioactive materials were purchased from Amersham Pharmacia Biotech. PolyP was from Sigma. Other chemical reagents were from Sigma or Fisher.

PCR and molecular cloning: The general procedure was as published.[43] The primers were synthesized by Gibco with incorporated appropriate restriction sites (Table 5). The DNA amplification was performed in a solution (50 μL) that contained template DNA (50 ng) and Taq polymerase (2.5 U) with an annealing temperature of 52 °C in a Thermolyne Amplitron I (Barnstead Thermolyne). PCR

Table 5. *Polymerase chain reaction (PCR) primers for cloning the genes from* E. coli *K-12 into* pET15b.

Primer name	Sequence[a]
galK-F	5′-GAT*CATATG*AGTCTGAAAGAAAAAACAC-3′/NdeI
galK-R	5′-CGC*GGATCC*TCAGCACTGTCCTGCTCCTTG-3′/BamHI
galT-F	5′-GGATC*CATATG*ACTAGTATGACGCAATTTAATCCC-3′/NdeI
galT-R	5′-AGC*GGATCC*TTACACTCCGGATTCGCG-3′/BamHI
galU-F	5′-GGATC*CTCGAG*ATGCCTGCCATTAATACG-3′/XhoI
galU-R	5′-CGC*GGATCC*ACTAGTTTACTTCTTAATGCCCATCTC-3′/BamHI
ppa-F	5′-GGAATTC*CATATG*AGCTTACTCAACGTCCCT-3′/NdeI
ppa-R	5′-CCG*CTCGAG*CGGTTATTTATTCTTTGCGCGCTC-3′/XhoI
umk-F	5′-GAAAGA*CATATG*GCTACCAATGCAAAA-3′/NdeI
umk-R	5′-CCATCA*CTCGAG*TTATTCCGTGATTAAAGT-3′/XhoI
ndk-F	5′-GAGGTA*CATATG*GCTATTGAACGTACT-3′/NdeI
ndk-R	5′-TTTACG*CTCGAG*TTAACGGGTGCGCGGGCA-3′/XhoI
ppk-F	5′-GGATC*CATATG*GGTCAGGAAAAGCTATAC-3′/NdeI
ppk-R	5′-CGC*GGATCC*GGTACCTTATTCAGGTTGTTCGAG-3′/BamHI

[a]The restriction site sequences are in italics.

products were purified by using a Qiaquick PCR purification kit, then cut by appropriate restriction enzymes and purified further on agarose gel with a Qiaex II gel extraction kit. DNA fragments were then ligated with plasmid pET15b which was double digested by suitable pairs of restriction enzymes. Ligates were used to transform DH5α, and the positive recombinants were identified by restriction mapping of the isolated plasmid.

Expression and purification of enzymes: Newly transformed BL21(DE3) carrying the above recombinant plasmids were cultured in Luria – Bertani (LB) medium (10 g L^{-1} peptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl; 50 mL) with ampicillin (150 μg mL^{-1}) overnight at 30 °C in an incubator – shaker (New Brunswick Scientific), then transferred into fresh LB medium(1 L) with ampicillin for another 2 h at 37 °C. When an optical density $A_{600} = 0.8 - 1.0$ was reached, the culture was induced with 400 μM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. The cells were harvested by centrifugation (4500 $\times$ *g*, 10 min, 4 °C) and the cell paste stored at -80 °C. The cell pastes were thawed and resuspended at 1/20th culture volume with lysis buffer (50 mM tris(hydroxymethyl)aminomethane (Tris)/HCl, (pH 8.5), 1% (v/v) Triton X-100, 10 mM 2-mercaptoethanol, 200 μg mL^{-1} lysozyme, 2 μg mL^{-1} DNase). The cells were broken by sonication (3 min) on ice by using a Branson Sonifier 450. The debris was removed by centrifugation (10 000 $\times$ *g*, 20 min, 4 °C). The supernatants were diluted with an equal volume of binding buffer (50 mM Tris/HCl (pH 7.9), 500 mM NaCl, 10 mM 2-mercaptoethanol), and applied at a flow rate of 2 mL min^{-1} to a Ni – NTA agarose column (25 $\times$ 40 mm = 20 mL) equilibrated in the same buffer at 4 °C. The column was washed with 5 column volumes (100 mL) of washing buffer (50 mM Tris/HCl (pH 7.9), 500 mM NaCl, 20 mM imidazole, 10 mM 2-mercaptoethanol). The proteins were eluted with eluting buffer (50 mM Tris/HCl (pH 7.9), 500 mM NaCl, 200 mM imidazole, 10 mM 2-mercaptoethanol). The fractions that contained the proteins were pooled and precipitated with 70% saturated ammonium sulfate. The pellets were dissolved in distilled water (2 mL), applied to a HiLoad 16/60 Superdex 200 column (Amersham Pharmacia Biotech), and eluted with a buffer (50 mM Tris/HCl, pH 7.9, 250 mM NaCl, 10% (v/v) glycerol, 10 mM 2-mercaptoethanol) at a flow rate of 0.4 mL min^{-1} on a ÄKTA*design* fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech). The chromatography was calibrated with standard proteins from a gel filtration calibration kit (Amersham Pharmacia Biotech). Purified proteins were stored at -20 °C after addition of glycerol to 30% of the final concentration. SDS-PAGE was done by the standard protocol of Laemmli[44] with a 12.5% or 15% uniform gel in a Mini Protean III cell gel electrophoresis unit (Bio-Rad), and stained with Brilliant Blue R-250 (Sigma). Protein concentration was measured by the method of Lowry et al.[45] Activity assays for the individual enzymes were carried out as described below.

Characterization of GalK: This enzyme catalyzes phosphorylation at the C1—OH of Gal. The activity assay for GalK was performed at room temperature for 30 min in a reaction solution (100 μL) that contained 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES; 100 mM; pH 7.4), [6-^{3}H]Gal (0.5 mM; 1000 cpm $nmole^{-1}$), ATP (50 mM), and a suitable amount of purified enzyme. The reaction was stopped by addition of cold ethylenediaminetetraacetate (EDTA; 100 mM; 100 μL) followed by Dowex 1 $\times$ 8 – 200 chloride anion exchange resin (0.8 mL) prepared in distilled water. After centrifugation, the supernatant (0.5 mL) was mixed with ScintiVerse BD (10 mL) and counted in a LS-3801 liquid scintillation counter (Beckmann).[20, 21] One unit of enzyme activity was defined as one μmol of product formed per minute at room temperature.

Characterization of GalT: This enzyme catalyzes the conversion from Gal-1-P and UDP-Glc into Glc-1-P and UDP-Gal. The activity was measured by observation of the UDP-Glc consumption coupled with the UDP-Glc dehydrogenase reaction. The first step was performed at room temperature for 15 min in a reaction solution (250 μL) that contained HEPES (100 mM, pH 7.4), Gal-1-P (1.6 mM), UDP-Glc (2.8 mM), and a suitable amount of purified enzyme. The reaction was stopped by addition of NaCl (0.15 M; 0.5 mL) and immediate transfer to a boiling water bath for 5 min. After the tube cooled down, the precipitate was spun down and the supernatant was subjected to the next step. The enzyme was assayed in a reaction solution (1 mL) that contained Tris/acetate (0.03 M, pH 8.7), nicotinamide adenine dinucleotide$^+$ (NAD^+; 1.36 mM), UDP-Glc dehydrogenase (3 mU), and the above supernatant (0.2 mL). The A_{340} change was recorded in an HP 845 $\times$ UV-Visible System (Hewlett Packard) and indicated the amount of UDP-Glc that remained.[22]

Characterization of GalU: This enzyme generates UDP-Glc from Glc-1-P and UTP in a reversible reaction. Two assay methods were used to assess the reaction in the forward direction.

Protocol I was a direct assay of the generated UDP-Glc by radioactive labeling of Glc-1-P, as described by Weissborn et al.[23] The reaction was performed at room temperature for 30 min in a reaction solution (0.1 mL) that contained Tris/HCl (50 mM, pH 7.8), KH_2PO_4 (2 mM), $MgCl_2$ (10 mM), [U-^{14}C]Glc-1-P (0.3 mM; 4000 cpm $nmol^{-1}$), UTP (0.3 mM), PPA (100 mU), and a suitable amount of purified enzyme. The reaction was stopped by the addition of ethanol (0.9 mL, 30% (v/v)). After centrifugation, the supernatant was loaded onto a mini diethylaminoethyl (DEAE)-cellulose acetate column (0.5 mL) packed in a Pasteur pipette. The column was washed with ethanol (30%, 1.1 mL) and acetic acid/ammonium acetate (0.05 M, pH 4.6) in ethanol (30%, 7 mL) to remove unutilized [U-^{14}C]Glc-1-P. The product, labeled UDP-Glc, was then eluted with acetic acid/ammonium acetate (0.15 mM, pH 4.6) in ethanol (30%, 4 mL). The radioactivity was counted in a liquid scintillation counter by mixing the eluate (1 mL) with ScintiVerse BD (10 mL).

Protocol II was an indirect assay that involved the generation of UDP-Glc coupled with the A_{340} increase caused by the formation of reduced nicotinamide adenine dinucleotide (NADH) from NAD^+ when UDP-Glc was oxidized by UDP-Glc dehydrogenase. The reaction was carried out at room temperature in a reaction solution (1 mL) that contained Tris/HCl (50 mM, pH 7.5), KH_2PO_4 (2 mM), $MgCl_2$ (10 mM), Glc-1-P (3 mM), UTP (3 mM), NAD (1.36 mM), UDP-Glc dehydrogenase (3 mU), and a suitable amount of purified enzyme. GalU activity was determined from the initial rate of the A_{340} increase.[24]

Characterization of PPA: This enzyme catalyzes the hydrolysis of PPi. A color reagent that consisted of malachite green (0.045%) base and ammonium molybdate (4.2%) in 4 M HCl (malachite green:ammonium molybdate = 3:1) was used to quantitatively determine the PPA activity. The samples that contained released phosphate developed a green color while negative controls remained yellow. The assay was carried out in a reaction solution (300 μL) that contained Tris/HCl (50 mM, pH 7.5), $MgCl_2$ (3 mM), sodium pyrophosphate (160 μM), and the purified PPA (30 μL). After 30 min at room temperature, the color reagent (600 μL) was added and the solution incubated for another 10 min. The A_{620} was measured.[25, 26]

Characterization of UMK: The activity was determined by using a coupled spectrophotometry assay. The reaction was carried out in a reaction solution (1 mL) that contained Tris/HCl (50 mM, pH 7.4), KCl (50 mM), $MgCl_2$ (2 mM), ATP (4 mM), UMP (2 mM), phosphoenolpyruvate (PEP; 1 mM), NADH (0.2 mM), pyruvate kinase (2 U), and lactate dehydrogenase (2 U). The reaction was started with the addition of a suitable amount of purified UMK. The A_{340} decrease was measured.[27, 28]

Characterization of NDK: A coupled-enzyme assay was used as described by Almaula et al.[29] The reaction mixture (1 mL) contained

Tris/HCl (50 mM, pH 7.4), KCl (200 mM), $MgCl_2$ (20 mM), ATP (0.9 mM), deoxythymidine diphosphate (dTDP; 1.8 mM), PEP (2 mM), NADH (0.2 mM), lactate dehydrogenase (5 U), and pyruvate kinase (5 U). The reaction was started by the addition of purified NDK and the A_{340} decrease was measured.[29, 30]

Characterization of PpK: This assay for PpK activity was established in this study. The enzyme catalyzes the formation of ATP from ADP, which could be coupled with the ATP-dependent phosphorylation of Glc.[32] The reaction was carried out in a mixture (1 mL) that contained Tris/HCl (50 mM, pH 7.4), $MgCl_2$ (10 mM), Glc (5 mM), ADP (5 mM), $NADP^+$ (0.5 mM), polyP (1 % (w/v), 75 mM as phosphate), glucokinase (1 U), Glc-6-P dehydrogenase (1.25 U), and a suitable amount of purified enzyme. The activity was determined at room temperature from the initial rate of the A_{340} increase.[33]

Immobilization of enzymes: The lysates of the expressed enzymes from *E. coli* were loaded individually onto a mini column (Pasteur pipette) with Ni – NTA agarose. To prepare the beads (immobilize them with the enzymes attached), a suitable amount of each of the individual enzymes was applied as determined by the specific activity. A mini column (1 mL) was used for a small-scale test and a regular-sized column (25 × 80 mm, 40 mL) was used for large-scale production. After loading of the individual or mixed enzyme(s), the columns were washed and equilibrated with the reaction buffer before use.

Kinetics: The enzymatic reaction was carried out in a buffer (1 mL) that consisted of Tris/HCl (50 mM, pH 7.4), KCl (10 mM), $MgCl_2$ (10 mM), Gal (20 mM), UMP (20 mM), ATP (2 mM), Glc-1-P (2 mM), polyP (1 % (w/v)), and the purified enzyme (1 U in each case). The reactions were performed at room temperature with rotation at 30 rpm. For this purpose, the beads immobilized with either individual enzymes or multiple enzymes were removed from the mini column and added into the enzymatic reaction as catalytic reagents. The amounts of immobilized proteins were also measured according to Lowry et al.[45] after their release from the beads by addition of EDTA (100 mM). The apparent kinetic parameters of the individual enzymes for their main substrates were determined. Additionally, the parameters for the entire pathway (7 enzymes) were determined for each of the four main substrates. Double-reciprocal plots of each enzymatic activity were constructed for the determination of K_m and V_{max} values for the respective main substrates.[28]

Reaction on the column: The reaction mixture was prepared in small scale (1 mL) or large scale (200 mL) and contained Tris/HCl (50 mM, pH 7.4), KCl (10 mM), $MgCl_2$ (10 mM), Gal (20 mM), UMP (20 mM), ATP (2 mM), Glc-1-P (2 mM), and polyP (1 % (w/v)). The mixtures were applied to the column in a continuous closed-circuit system at room temperature (for large scale, Figure 1).

HPLC analysis: To prepare a sample for HPLC, acetonitrile (0.5 mL) was added to an equal volume of the reaction mixture, the tube was vortexed, and the possible precipitate was spun down (14 000 × *g*, 5 min). Sugar nucleotides and other mixture components were measured by HPLC by a modified version of the method of Kittelmann et al.[46] The sample (20 µL) was injected into a Microsorb NH_2 100 Å column (4.6 × 250 mm) connected to the ProStar HPLC system (Varian). Elution was carried out with a linear gradient ratio of acetonitrile to potassium phosphate (20 mM, pH 6.2) from 70:30 to 0:100 in 40 min at a flow rate of 0.75 mL min^{-1} and was observed with a UV detector at 262 nm.

Purification of UDP-Gal: To isolate UDP-Gal, the eluant was collected from the column for the large-scale reaction. The diluted solution was applied to an ion-exchange column (22 × 120 mm, 45 mL) with DEAE-cellulose (Sigma) and was eluted with a gradient of NaCl from 0 to 0.5 M in 4 column volumes (180 mL) at a flow rate of 2 mL min^{-1}. The fractions that contained UDP-Gal were identified by HPLC. UDP-Gal was desalted with water in a Sephadex G-15 (Pharmacia) column (26 × 800 mm) under gravity flow and the purified product was lyophilized. NMR spectra were recorded in D_2O with a Varian VXR400 NMR spectrometer (data not shown). Electrospray ionisation (ESI)-MS spectrometry: found: 225.11 [$M+Na^+ + Rb^+ + H$]; 383.25 [$(M-H)+2Na^+ + K^+$]; consistent with the calculated values.

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