Carbohydrate Research 345 (2010) 1622–1626

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Note

Efficient biosynthesis of uridine diphosphate glucose from maltodextrin by multiple enzymes immobilized on magnetic nanoparticles

Qing Dong, Li-Ming Ouyang, Hui-Lei Yu, Jian-He Xu *

Laboratory of Biocatalysis and Bioprocessing, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, 200237 Shanghai, PR China

ARTICLE INFO

Article history:
Received 10 March 2010
Received in revised form 25 April 2010
Accepted 27 April 2010
Available online 4 June 2010

Keywords:
UDP-Glc
Biocatalytic synthesis
One-pot
Magnetic nanoparticles
Immobilized biocatalyst

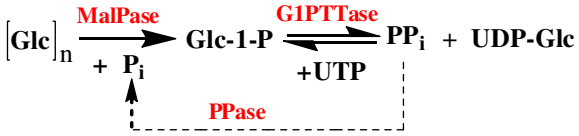
ABSTRACT

Uridine diphosphate glucose (UDP-Glc) serves as a glucosyl donor in many enzymatic glycosylation processes. This paper describes a multiple enzyme, one-pot, biocatalytic system for the synthesis of UDP-Glc from low cost raw materials: maltodextrin and uridine triphosphate. Three enzymes needed for the synthesis of UDP-Glc (maltodextrin phosphorylase, glucose-1-phosphate thymidylytransferase, and pyrophosphatase) were expressed in *Escherichia coli* and then immobilized individually on amino-functionalized magnetic nanoparticles. The conditions for biocatalysis were optimized and the immobilized multiple-enzyme biocatalyst could be easily recovered and reused up to five times in repeated syntheses of UDP-Glc. After a simple purification, approximately 630 mg of crystallized UDP-Glc was obtained from 1 l of reaction mixture, for a moderate yield of around 50% (UTP conversion) at very low cost.

Glycosylation is an important reaction in biology. Drug research now indicates that the actions of many drugs with principal roles in molecular recognition, transmembrane signaling, and other physiological or pathological processes involve glycosylation reactions.[1] Glycosylation is catalyzed almost exclusively by glycosyltransferases that utilize sugar nucleotides in the uridine form.[2,3] In biological systems, uridine diphosphoglucose (UDP-Glc) is the most commonly encountered sugar nucleotide and is widely used as a glucosyl donor in glucosyltransferase reactions. Although sugar nucleotides such as UDP-Glc can be synthesized by chemical methods, glycosylation using the enzymes that widely exist in nature is more attractive due to the strong regioselectivity, the high conversion rate, and the mild reaction conditions of enzymatic reactions. For example, biosynthesis of UDP-Glc has been catalyzed using glucose-1-phosphate thymidylytransferase, with UTP and glucose-1-phosphate (Glc-1-P) as substrates.[4] However, this reaction is not economically feasible for industrial scale production of UDP-Glc because of the high cost of Glc-1-P. Leloir glycosyltransferase catalysis is also one of the common ways to synthesize oligosaccharides,[5,6] but it also depends on the availability of expensive sugar phosphates.

In this paper, inexpensive raw materials—maltodextrin, UTP, and phosphate—were utilized for UDP-Glc synthesis with a newly designed multiple-enzyme system for in vitro synthesis of UDP-Glc (Scheme 1). Three enzymes, maltodextrin phosphorylase (MalPase, EC 2.4.1.1), glucose-1-phosphate thymidylytransferase (G1PTTase, EC 2.7.7.24), and inorganic pyrophosphatase (PPase, EC 3.6.1.1), were cloned and expressed in *Escherichia coli* and then immobilized on amino-functionalized magnetic nanoparticles. In this system, maltodextrin is first phosphorolyzed to glucose monophosphate by MalPase. The Glc-1-P is then converted to UDP-Glc by G1PTTase, generating pyrophosphate (PPi) as a byproduct. PPi is further hydrolyzed to phosphoric acid (Pi) by PPase, which serves both to drive the reaction scheme in the UDP-Glc direction and also to regenerate one starting product for the MalPase reaction.

Although this reaction sequence will very effectively allow the synthesis of UDP-Glc, all of the reaction components, that is, the enzymes, their substrates, and their products, are highly water soluble, which makes the purification of UDP-Glc from the reaction mixture very complicated. Immobilization of enzymes can be helpful for isolating the protein biocatalysts from the reaction mixture. As another benefit, immobilization can also improve the stability and reusability of the enzymes, thus further reducing the cost of this type of production process. In the present paper, we exploit amino-functionalized magnetic nanoparticles for this purpose.



Scheme 1. Biosynthesis of UDP-Glc by one-pot reaction with multiple enzymes.

* Corresponding author. Fax: +86 21 6425 2250.
E-mail address: jianhexu@ecust.edu.cn (J.-H. Xu).

0008-6215/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2010.04.025

Amino-functionalized magnetic nanoparticles are a relatively new material for enzyme immobilization, with many advantages that are now attracting much attention.[7–9] The immobilization of enzymes onto aldehyde-functionalized magnetic nanoparticles is a rapid process and these nanoparticles act as very effective supports for immobilized enzymes. More importantly, the nanoparticle-bound enzymes can easily be separated from the reaction mixture and recycled simply by using a magnetic field. The low cost of fabrication of amino-functionalized magnetic nanoparticles also makes this form of immobilization promising for industrial scale enzyme processes. Therefore, we used amino-functionalized magnetic nanoparticles in the present study to co-immobilize MalPase, GlPTTase, and PPase for the efficient synthesis of UDP-Glc from maltodextrin and UTP.

1. Materials and methods

1.1. Materials

Taq DNA polymerase, agarose gel DNA Purification Kit Ver.2.0, and T4 DNA ligase were purchased from TaKaRa (Dalian, China). The plasmid vectors of pET-11a (+) and pET-24a (+) were from our laboratory. Primers were synthesized by Invitrogen (Shanghai, China). UTP was purchased from Meiyapharm (Hangzhou, China). Amino-functionalized magnetic particles[7] were from our laboratory.

1.2. Cloning, expression, and preparation of enzymes

Genes coding for MalPase,[10,11] GlPTTase[3], and PPase[12–14] were amplified from *E. coli* K12 genomic DNA by PCR. The plasmids and strains used are listed in Table 1. All of the experimental procedures used were general methods.[15] *E. coli* BL21 (DE3) containing exogenous recombinant plasmids were grown in LB medium at 37 °C, with shaking at 200 rpm. When OD_{600} of the culture broth reached about 0.7, IPTG (isopropyl-1-thio-β-D-galactopyranoside) was added and the cultivation was continued at a suitable temperature to induce the expression of each target enzyme protein. Induction and expression conditions for different recombinant strains are listed in Table 2. The cells were then harvested by centrifugation at 12,000*g* for 10 min at 4 °C, followed by disruption by ultrasonication (400 W). The whole process of ultrasonication lasted for 100 cycles. Each cycle included 4 s of sonication and 6 s of pause. The supernatant was collected and lyophilized to obtain a crude enzyme powder.

1.3. Activity assays

(i) *MalPase*: The activity of MalPase was defined as the release of UDP-Glc, which can be measured using a coupled assay containing MalPase proteins, G1PTTase, and PPase. The reaction mixture contained 100 mM phosphate (pH 7.5), 5% maltodextrin, 5 mM $MgCl_2$, 5 mM UTP, 0.25 U G1PTTase, 1 U PPase, and 1 mg MalPase protein in a total volume of 1 ml. The reaction mixture was incubated at 30 °C for 30 min. One unit of MalPase was defined as the amount of enzyme producing 1 μmol of UDP-Glc within 1 min under the described conditions.

(ii) *G1PTTase*: The activity of G1PTTase was defined as the release of UDP-Glc. The reaction is as follows: the reaction mixture contained 50 mM phosphate (pH 7.5), 5 mM $MgCl_2$, 5 mM glucose-1-phosphate, 2 mM UTP, and 1 mg G1PTTase protein in a total volume of 1 ml. The reaction mixture was incubated at 30 °C for 1 min. One unit of G1PTTase was defined as the activity to produce 1 μmol of UDP-Glc in 1 min under the described conditions.[16,17]

(iii) *PPase*: The assay mixture contained 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM $Na_4P_2O_7$, and 1 mg PPase crude enzyme powder in a total volume of 1 ml. The reaction mixture lacking the enzyme was used as a control. The reaction mixture was incubated at 30 °C for 1 min. After the reaction, 20 μl of reaction mixture was withdrawn and transferred to the system which contained 500 μl fresh phosphorus-determining reagent solution (17% H_2SO_4 : 2.5% Na_2MoO_4–$(NH_4)_2SO_4$: 10% L-ascorbic acid: H_2O = 1:1:1:2) and 480 μl H_2O. Then the reaction was incubated at 45 °C for 10 min. The absorbance at 660 nm was recorded. One unit of PPase was defined as the activity to produce 1 μmol of inorganic phosphate in 1 min under the described conditions.[18,19]

Table 2
Induction and expression conditions for recombinant strains

Enzyme	IPTG concn (mM)	Induction temperature (°C)	Induction time (h)
MalPase	0.05	15	20
G1PTase	1	30	4
PPase	1	30	4

1.4. HPLC analysis of UDP-Glc

The chromatography was performed on a Shimadzu system (LC-10ADVP) equipped with an Agela SAX NH_2 column (150 μm, 4.6 × 150 mm) using an isocratic elution with 67 mM Na_2HPO_4–KH_2PO_4 (pH 5.3) at 30 °C. The flow rate was 1.5 ml/min and UV detection was performed at 260 nm.

1.5. Immobilization of enzyme

Amino-functionalized magnetic particles (10 mg/ml) were suspended in phosphate buffer or Tris–HCl buffer (50 mM, pH 7.5) and then ultrasonicated for 20 min. Thereafter, 25% glutaraldehyde (20 μl/ml) was added and shaken for 12 h at 30 °C, 1100 rpm. The nanoparticles were then separated by magnetic decantation and washed five times with phosphate buffer or Tris–HCl buffer (50 mM, pH 7.5) to eliminate the excess unreacted glutaraldehyde. Each enzyme solution was mixed separately with aldehyde-functionalized magnetic nanoparticles and shaken at 30 °C for 30 min, 1100 rpm. After this reaction period, each immobilized enzyme was removed by magnetic decantation and washed five times with phosphate buffer or Tris–HCl buffer (50 mM, pH 7.5) to remove any unbound enzyme.

Table 1
Endonucleases, vectors, and hosts for different genes

Gene	Endonucleases	Vector	Host	Primers (5′→3′)
malpase	*BamHI/NotI*	pET24a (+)	*E. coli* BL21 (DE3)	MP1: ctggatccatgtcacaacctatttttaacg MP2: cttgcggccgcttagcgttttgcctgccag
g1pttase	*BamHI/NdeI*	pET11a (+)	*E. coli* BL21 (DE3)	GP1: ctgggaattccatatgatgaaaatgcgtaaag GP2: cttgcggatccttaaacttaggaagc
ppase	*BamHI/NdeI*	pET11a (+)	*E. coli* BL21 (DE3)	PP1: ctgggaattccatatgatgagcttactcaacg PP2: cttgcggatccttatttattctttgcgcgctcg

1.6. Reuse of immobilized enzymes in repeated synthesis of UDP-Glc

A reaction mixture (200 ml in a 500 ml narrow neck flask), maltodextrin 5% (m/v), UTP 2 mM, and $MgCl_2$ 10 mM, in 100 mM sodium phosphate buffer (pH 7.5) was combined with immobilized enzymes for the one-pot synthesis. The product concentration was analyzed periodically by HPLC. When the UDP-Glc concentration showed no further increases, the reaction was terminated and the immobilized enzymes were recovered by magnetic separation. The recovered immobilized enzymes were washed three times with phosphate buffer (100 mM, pH 7.5) and then employed again in the next batch of fresh reaction mixture.

1.7. Purification of UDP-Glc

To isolate UDP-Glc, the immobilized enzymes were removed with a magnet and an equal volume of methanol was added to the remaining reaction mixture containing the UDP-Glc product. This mixture was cooled at 4 °C for 15 min, then centrifuged at 12,000*g* for 5 min at 4 °C. The supernatant was concentrated by rotary evaporation at 50 °C and the concentrated solution was applied to SAX (strong anion exchange) ion-exchanger column (40 μm, 25 × 300 mm) and eluted with 67 mM Na_2HPO_4–KH_2PO_4 (pH 5.3) at a flow rate of 2 ml/min. The fractions containing UDP-Glc were identified by HPLC, pooled, and desalted on an ODS (octadecylsilyl) column (40 μm, 25 × 300 mm) using isocratic elution with 70% methanol (aq) at a flow rate of 2 ml/min. The purified product was lyophilized and stored at 4 °C.

2. Results and discussion

2.1. Expression of enzymes

On SDS–PAGE gels, the three enzymes expressed in *E. coli* had molecular weights identical to the predicted values (MalPase, 88 kDa; G1PTTase, 32 kDa; PPase, 19 kDa; Fig. 1). G1PTTase and PPase were mainly expressed as soluble forms, while Malpase was mainly expressed as an insoluble form.

2.2. Immobilized enzymes

The enzymes were immobilized on amino-functionalized magnetic nanoparticles by the method represented in Section 1.5. The protein loading and activity recovery are shown in Table 3. After immobilization, more than 70% of the original activity of the recombinant enzymes was retained. The specific activities of the immobilized enzymes were MalPase 5.66 U/g, G1PTTase 102.49 U/g, and PPase 506.23 U/g.

Table 3
Protein loading and activity recovery for different enzymes

	MalPase	G1PTase	PPase
Protein loading[a] (mg/g support)	47.5	65	68.4
Activity recovery (%)	85.8	71	93.2

[a] The amount of protein was determined by the Bradford method using BSA as a standard.

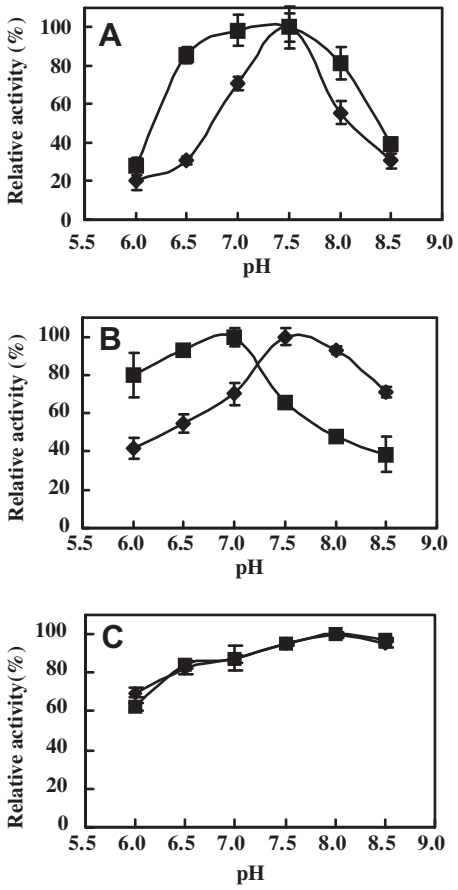


Figure 2. Effect of initial pH on activities of free enzymes (♦) and immobilized enzymes (■). (A) MalPase; (B) GlPTTase; (C) PPase.

2.3. Properties of the free and immobilized enzymes

Figure 2 shows a comparison of reactions of the free and the immobilized enzymes in response to external pH. The overall trends of activity were similar for both the free and the immobilized

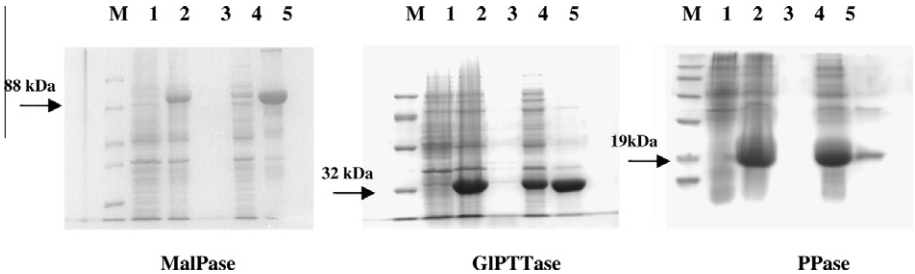


Figure 1. SDS–PAGE analysis of the recombinant enzymes expressed in *E. coli* which were used for one-pot biosynthesis of UDP-Glc. For each enzyme, lane M was the low-range molecular weight marker proteins; lane 1 was for the total protein of BL21 (DE3) host cells (control); lane 2 was the total protein of recombinant cells; lane 3 was the supernatant of recombinant cell culture; lane 4 was the soluble fraction of intracellular proteins of the recombinant cells; lane 5 was the insoluble fraction (pellet) of the recombinant cell lysate.

Table 4
Kinetic parameters of individual enzyme

Enzyme	Substrate	K_m (mM)		V_{max} (μmol L^{-1} min^{-1})	
		Free	Immobilized	Free	Immobilized
MalPase	Maltodextrin	39.5	59.5	7.2	23.0
	Sodium phosphate	23.2	40.0	9.0	30.9
G1PTase	Glu-1-P	2.72	1.69	420	410
	UTP	0.27	0.28	420	580
PPase	PPi	7.47	8.35	28,200	33,600

enzymes, with the exception of G1PTTase. Immobilized MalPase appeared to be more stable to pH changes than the free MalPase. This might be correlated with a prevention of hydrogen ion distribution between the surface and the solution by the amino groups on the nanoparticles.

The kinetic behavior of every enzyme was examined prior to and following immobilization. The apparent Michaelis constant (K_m) and the maximal reaction rate (V_{max}) are presented in Table 4. After immobilization, K_m value and V_{max} values of every enzyme for the major substrates increased. For example, the K_m value of MalPase toward maltodextrin increased 1.5 times and that toward phosphate increased 1.7 times as compared with the free enzyme, indicating some resistance of the carrier against the interaction between the substrate and the enzyme. The V_{max} values of the immobilized MalPase were almost 3.2-fold (toward maltodextrin) and 3.4-fold (toward phosphate) higher than those of the free enzyme. It is well known that the immobilization of enzyme may cause the variation of enzymes kinetic behavior. This suggests an improvement in the efficiency of UDP-Glc production when it is catalyzed by the immobilized enzymes, rather than by free enzyme in homogeneous solution.

2.4. Optimization of reaction conditions

The substrate concentration was optimized in 1.5 ml tubes (Eppendorf SafeLock). Figure 3A shows that increasing the

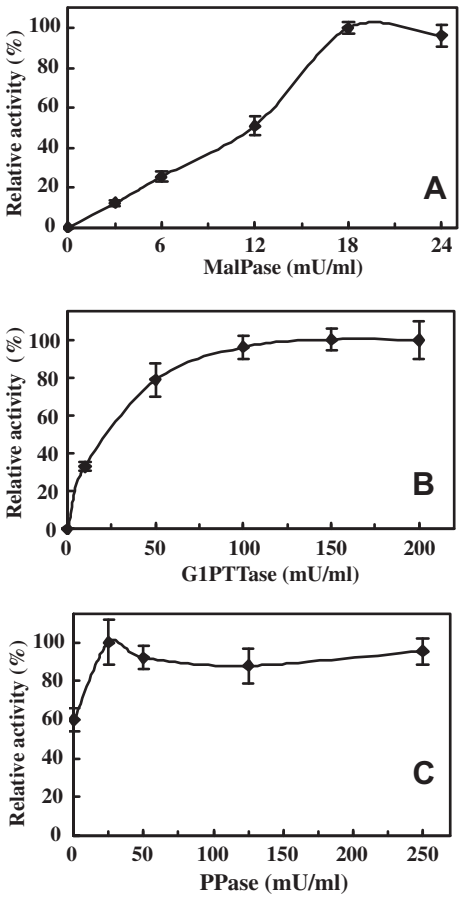


Figure 4. Optimization of immobilized enzymes load for the UDP-Glc production. Reaction conditions: maltodextrin, 5% (m/v); UTP, 2.5 mM; $MgCl_2$, 10 mM; buffer, sodium phosphate, pH 7.5, 100 mM; 30 °C, 4 h. (A) MalPase, 0–24 mU/ml; GlPTTase, 50 mU/ml; PPase, 50 mU/ml; (B) MalPase, 18 mU/ml; GlPTTase, 0–200 mU /ml; PPase, 50 mU/ml; (C) MalPase, 18 mU/ml; GlPTTase, 100 mU/ml; PPase, 0–250 mU/ml.

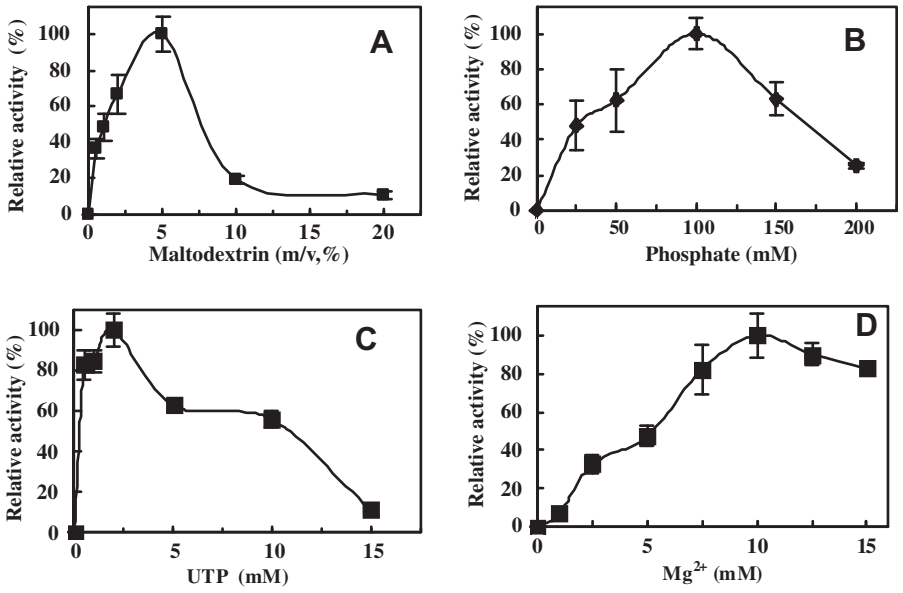


Figure 3. Effect of substrate concentration on the UDP-Glc production. Common reaction conditions: MalPase, 6 mU/ml; GlPTTase, 50 mU/ml; PPase, 50 mU/ml; 30 °C, 4 h. Other conditions: (A) maltodextrin, 0–20% (m/v); UTP, 2 mM; $MgCl_2$, 2 mM; buffer, sodium phosphate, pH 7.5, 100 mM; (B) maltodextrin, 5% (m/v); UTP, 2 mM; $MgCl_2$, 2 mM; buffer, sodium phosphate, pH 7.5, 0–200 mM; (C) maltodextrin, 5% (m/v); UTP, 0–15 mM; $MgCl_2$, 2 mM; buffer, sodium phosphate, pH 7.5, 100 mM; (D) maltodextrin, 5% (m/v); UTP, 2.5 mM; $MgCl_2$, 0–15 mM; buffer, sodium phosphate, pH 7.5, 100 mM. *The relative activity is defined as: $R(\%) = \frac{C_M - C_0}{CM} \times 100$ where R is the relative activity, C_M is the yield of optimal condition for UDP-Glc; C_0 is the yield of certain conditions for UDP-Glc.

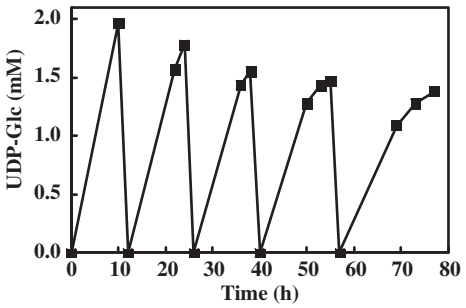


Figure 5. Time course of the repeated UDP-Glc production using three enzymes (MalPase, GlPTTase, and PPase) immobilized on amino-functionalized magnetic nanoparticles. Reaction conditions: MalPase, 18 mU/ml; GlPTTase, 100 mU/ml; PPase, 25 mU/ml; maltodextrin, 5% (m/v); UTP, 2 mM; $MgCl_2$, 10 mM; buffer, sodium phosphate, pH 7.5, 100 mM; 30 °C; total volume, 200 ml.

concentration of maltodextrin did not increase the rate of production of UDP-Glc. This was because a high concentration of maltodextrin made the reaction system too viscous and caused caking of the immobilized enzymes. Suitable concentrations of phosphate and UTP in this system were 100 mM and 2.5 mM, respectively (Fig. 3B and C). A too high salt concentration of salt may inactivate the enzymes by destroying their hydration layer. The Mg^{2+} concentration required to activate G1PTTase and PPase also modulated the synthesis of UDP-Glc. A fivefold increase in UDP-Glc production resulted from an increase in Mg^{2+} from 2 mM to 10 mM, but there were no further increases beyond 10 mM (Fig. 3D). A 1:5 ratio of UTP and Mg^{2+} was the most suitable for UDP-Glc synthesis.

Figure 4A and B shows the effects of manipulation of immobilized enzyme concentrations. Optimal UDP-Glc production was obtained with 18 mU/ml of MalPase and 100 mU/ml of G1PTTase. If the load of immobilized enzymes was increased further, the product yield became saturated. We speculate that too great an addition of amino-functionalized magnetic nanoparticles in the system may hamper the free movement of enzymes.

PPase played an auxiliary role in the synthesis of UDP-Glc and UDP-Glc could be synthesized even in the absence of added immobilized PPase (Fig. 4C). The addition of PPase to a certain level improved the yield of UDP-Glc, indicating a pivotal role for PPase in maximizing the reaction, but additions beyond this level had no further effect.

The time course of UDP-Glc production also was studied. We found that 2 mM UTP was completely transformed by the immobilized enzymes in about 10 h.

The optimal reaction conditions for UDP-Glc synthesis by multiple immobilized enzymes were: MalPase, 18 mU/ml; G1PTTase, 100 mU/ml; PPase, 25 mU/ml; maltodextrin, 5% (m/v); UTP, 2 mM; and $MgCl_2$, 10 mM; in 100 mM sodium phosphate buffer, pH 7.5; at 30 °C for 10 h.

2.5. Preparation and characterization of UDP-Glc

After the optimization of reaction conditions, a gram-scale reaction was performed in 200 ml. The same immobilized enzymes were retrieved with a magnet and reused five times. As shown in Figure 5, the reaction rate and the UDP-Glc yield decreased as the number of reuses increased, so the reaction time for each batch was extended to obtain a higher yield. After five consecutive reactions (about 80 h totally), the immobilized enzymes still retained some activity. This suggested that the covalently immobilized enzymes were stable on the magnetic nanoparticles.

The supernatants of all five batch reactions were combined and concentrated and the concentrate was purified by successive SAX column and ODS column, as described earlier. A white floccule of about 0.63 g was obtained after lyophilization. The isolated yield of product was 53% (UTP conversion). The product was characterized by HPLC and MS and confirmed to be UDP-Glc. MS results were: 605.2 [$M+K^+$]; 634.2 [$M+2K^+$]; 665.3 [$M+2K^++Na^+$]; 681.2 [$M+3K^+$]; 682.2 [$M+3K^++H^+$] which were consistent with calculated values.

3. Conclusions

We have established a one-pot system to synthesize the UDP-Glc from cheap substrates, maltodextrin, UTP, and phosphate, thus greatly reducing the cost compared to reactions that depend on expensive glucose-1-P. Three enzymes were cloned and expressed in *E. coli* and individually immobilized onto amino-functionalized magnetic nanoparticles. These immobilized enzymes were operationally stable and reusable and retained efficient catalytic activity. A gram-scale synthesis of UDP-Glc by reuse of the immobilized enzymes in repeated batch reactions was achieved. The reaction system described here has great potential for production of NDP-nucleotide sugars.

References

1. Gabius, H. J.. *Naturwissenschaften* **2000**, *87*, 108–121.
2. Jiang, J. Q.; Biggins, J. B.; Thorson, J. S. *J. Am. Chem. Soc.* **2000**, *122*, 6803–6804.
3. Barton, W. A.; Biggins, J. B.; Jiang, J. Q.; Thorson, J. S.; Nikolov, D. B. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 13397–13402.
4. Jiang, J. Q.; Biggins, J. B.; Thorson, J. S. *Angew. Chem., Int. Ed.* **2001**, *40*, 1502–1505.
5. Wymer, N.; Toone, E. J. *Curr. Opin. Chem. Biol.* **2000**, *4*, 110–119.
6. Palcic, M. M. *Curr. Opin. Biotechnol.* **1999**, *10*, 616–624.
7. Hu, B.; Pan, J.; Yu, H. L.; Liu, J. W.; Xu, J. H. *Proc. Biochem.* **2009**, *44*, 1019–1024.
8. Yong, Y.; Bai, Y. X.; Li, Y. F.; Lin, L.; Cui, Y. J.; Xia, C. G. *Proc. Biochem.* **2008**, *43*, 1179–1185.
9. Li, Y. G.; Gao, H. S.; Li, W. L.; Xing, J. M.; Liu, H. Z. *Bioresour. Technol.* **2009**, *21*, 5092–5096.
10. Chen, S.; Liu, J.; Pei, H.; Li, J.; Zhou, J.; Xiang, H. *Enzyme Microb. Technol.* **2007**, *41*, 390–396.
11. Bae, J.; Lee, D. H.; Kim, D.; Cho, S. J.; Park, J. E.; Koh, S.; Kim, J.; Park, B. H.; Choi, Y.; Shin, H. J.; Hong, S. I.; Lee, D. S. *Proc. Biochem.* **2005**, *40*, 3707–3713.
12. Jardin, P. D.; Bellran, J. R.; Gebhardt, C.; Brasseur, R. *Plant Physiol.* **1995**, *109*, 853–860.
13. Farre, E. M.; Bachmann, A.; Willmitzer, L.; Trethwey, R. N. *Nat. Biotechnol.* **2001**, *19*, 268–272.
14. Hajirezaei, M.; Sonnewald, U. *Potato Res.* **1999**, *42*, 353–372.
15. Sambrook, J.; Russell, D. *Molecular Cloning 3*; Cold Spring Harbor Laboratory Press, 2001.
16. Marolda, C. L.; Valvano, M. A. *Am. Soc. Microbiol.* **1995**, *78*, 5539–5546.
17. Barton, W. A.; Lesniak, J.; Biggins, J. B.; Jeffrey, P. D.; Jiang, J. Q.; Rajashankar, K. R.; Thorson, J. S.; Nikolov, D. B. *Nat. Struct. Biol.* **2001**, *93*, 545–551.
18. Kunitz, M. J. *Gen. Physiol.* **1952**, *35*, 423–449.
19. Lahti, R.; Pitkaranta, T.; Valve, E.; Ira, I.; Kalske, E. K.; Heinonen, J. *Bacteriology* **1988**, *170*, 5901–5907.