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PII: S0008-6215(15)00102-0

DOI: 10.1016/j.carres.2015.04.001

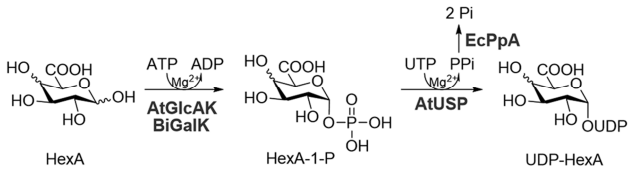
Reference: CAR 6973

To appear in: *Carbohydrate Research*

Received Date: 23 November 2014

Accepted Date: 3 March 2015

Please cite this article as: Guo Y, Fang J, Li T, Li X, Ma C, Wang X, Wang PG, Li L, Comparing substrate specificity of two UDP-sugar Pyrophosphorylases and Efficient One-pot Enzymatic Synthesis of UDP-GlcA and UDP-GalA, *Carbohydrate Research* (2015), doi: 10.1016/j.carres.2015.04.001.



Comparing substrate specificity of two UDP-sugar Pyrophosphorylases and Efficient One-pot Enzymatic Synthesis of UDP-GlcA and UDP-GalA

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Abstract

Uridine 5'-diphosphate-glucuronic acid (UDP-GlcA) and UDP-galacturonic acid (UDP-GalA), the unique carboxylic acid-formed sugar nucleotides, are key precursors involved in the biosynthesis of numerous cell components. Limited availability of those components has been hindering the development of efficient ways towards facile synthesis of bioactive glycans such as glycosaminoglycans. In current study, we biochemically characterized two UDP-sugar pyrophosphorylases from *Arabidopsis thaliana* (AtUSP) and *Bifidobacterium infantis* ATCC15697 (BiUSP), and compared their activities towards a panel of sugar-1-phosphates and derivatives. Both enzymes showed significant pyrophosphorylation activities towards GlcA-1-phosphate, and AtUSP also exhibited comparable activity towards GalA-1-phosphate. By combining with monosaccharide-1-phosphate kinases, we have developed an efficient and facile one-pot three-enzyme approach to quickly obtain hundreds milligrams of UDP-GlcA and UDP-GalA.

Key words: UDP-GalA, UDP-GlcA, UDP-sugar pyrophosphorylase, Synthesis

1. Introduction

Hexuronic acids (HexAs), a class of sugar acids with both carbonyl and carboxylic acid functional groups, are the oxidation products of the corresponding monosaccharides. Glucuronic acid (GlcA) is one of the most commonly found HexAs in the plant and animal kingdoms.[1] It is also prevalent in carbohydrate chains of proteoglycans.[2] Galacturonic acid (GalA) is a major sugar residue of plant pectic polysaccharides and a minor component of certain plant arabinogalactan proteins.[3] GalA is also found in various cell surface polysaccharides of different gram-negative bacteria,[4,5] as well as capsular polysaccharide of the gram-positive human pathogens, such as type I *Streptococcus pneumonae*.[6]

Because of the biological significance and structure complexity, glycans containing HexAs have attracted considerable attention in past decades.[7-9] One of the major bottlenecks in basic research and application of these components is the limited and cost-effective availability of uridine 5'-diphosphate glucuronic acid (UDP-GlcA) and uridine 5'-diphosphate galacturonic acid (UDP-GalA), the active forms of corresponding monosaccharides. UDP-GlcA and UDP-GalA are essential glycosyl donors for glycosyltransferases in the biosynthesis of various cell components including glycosaminoglycans, some N- and O-linked glycans, glycosphingolipids, pectins, etc.[10,11] Recently, several chemical or coupled enzymatic approaches have been developed for the synthesis of UDP-GlcA and UDP-GalA.[12,13] However, low yield, tedious steps and strict condition requirements have restricted the application of these approaches in large scale sugar nucleotide preparation.[14] Therefore, a simple, cost-effective and efficient approach for the preparation of UDP-GlcA and UDP-GalA is highly desirable for subsequent glycosyltransferase-catalyzed reactions.

Enzymatic synthesis, which mimics the biosynthesis pathway, is considered more attractive with advantages of high conversion rate and mild reaction conditions. As shown in **Figure 1**, the *de novo* biosynthesis of UDP-GlcA initiates from glucose-6-phosphate (Glc-6-P), an intermediate of the glycolytic pathway. Three enzymatic catalyzed steps were involved, including the equilibrium conversion of Glc-6-P and Glc-1-P, the pyrophosphorylation step to yield UDP-Glc, and the

oxidoreduction step catalyzed by UDP-Glc 6'-oxidoreductase. The complication of the pathway, the similarities between the final product and intermediates, as well as the consuming of expensive NAD^+ or $NADP^+$ have highly limited the application of such a pathway in large scale UDP-GlcA preparation. The *de novo* biosynthetic pathway of UDP-GalA is even more complicated (**Fig. 1**).

In contrast, salvage biosynthetic pathways of sugar nucleotides, usually involving only two reactions (sugar-1-phosphate kinase catalyzed formation of sugar-1-P from monosaccharide and ATP, and NDP-sugar pyrophosphorylase catalyzed formation of NDP-sugar from sugar -1-P and NTP) were found much more practical in large scale synthesis of sugar nucleotides. Using recombinant enzymes from salvage pathways, we have successfully developed efficient approaches for 100 mg scale synthesis of UDP-Gal, UDP-GlcNAc, UDP-GalNAc, GDP-Fuc, GDP-Man and derivatives.[15-22] Recently, a salvage biosynthetic pathway of UDP-GlcA had been discovered in *Arabidopsis thaliana* (**Fig. 1**).[23] In such a pathway, GlcA is first catalyzed by a glucuronokinase (AtGlcAK) to form GlcA-1-P in the presence of ATP and Mg^{2+}. Subsequently, a UDP-sugar pyrophosphorylase (AtUSP) catalyzes the conversion of GlcA-1-P into UDP-GlcA in the presence of UTP and Mg^{2+}.[24,25] A galacturonokinase (AtGalKA) was also found in *A. thaliana* [23] implies that a salvage biosynthetic way of UDP-GalA may also exist.

In this study, we cloned and characterized a UDP-sugar pyrophosphorylase from *Bifidobacterium infantis* ATCC15697 (BiUSP) which exhibited significant activity towards GlcA-1-P. In addition, we codon optimized the gene encoding AtUSP, and performed heterogeneous overexpression. AtUSP showed significant activities towards both GlcA-1-P and GalA-1-P. Furthermore, substrate specificity study exhibited that both USPs were promiscuous towards a panel of sugar-1-phosphates. Finally, by combing sugar-1-P kinases with USPs as well as pyrophosphatase, we developed an efficient one-pot three-enzyme approach to prepare hundreds of milligrams of UDP-GlcA and UDP-GalA.

2. Results and discussion

2.1 Gene cloning, expression, and purification of AtGlcAK, AtUSP, and BiUSP

Genes encoding AtGlcAK and AtUSP from *A. thaliana* were codon optimized (see DNA sequences in **supporting information**) and synthesized to achieve better heterologous expression in *E. coli*. The synthesized genes were cloned into pQE80L, a tightly controlled *E. coli* protein expression vector with a T5 promoter. Given that inductions were performed at a low temperature (16 °C) and with a low concentration of IPTG (0.2 mM), AtGlcAK and AtUSP were expressed almost entirely in soluble form. As shown in **Figure 2**, after one-step Ni-NTA affinity chromatography, both enzymes with N-terminal His_6-tag were purified to 90% with high yields (90 mg for AtGlcAK, 42 mg AtUSP per L of LB cultures). The apparent molecular weights of the enzymes on SDS-PAGE (**Fig. 2**, Lane 1 & 2) are consistent with theoretical values.

A bacterial UDP-sugar pyrophosphorylase gene, *Blon_1169*, was cloned from *B. infantis* ATCC 15697. The enzyme (BiUSP) encoded by this gene is highly identical (98%) to a previously reported UDP-sugar pyrophosphorylase, BLUSP.[26] BiUSP was expressed and purified with a C-terminal His_6-tag followed the abovementioned methods. The yield of pure BiUSP from 1 L of cultures is 43 mg. An approximate molecular weight of 60 KDa was observed on SDS-PAGE (**Fig. 2**, Lane 4), consistent with theoretical value (58 KDa).

2.2 Pyrophosphorylase activities of AtUSP and BiUSP

Capillary Electrophoresis (CE) was used to analyze the formation of UDP-sugars. Retention times of 21.7, 18.2, 16.6 min were observed on CE profiles for UTP, UDP-GlcA and UDP-GalA standards (**Fig 3A**, **B** & **C**), respectively. The results shows that BiUSP could catalyze the formation of UDP-GlcA from GlcA-1-P and UTP efficiently (**Fig**. **3D**), whereas the formation of UDP-GalA was not clearly observed (CE profile not shown), indicating that the C-4 hydroxyl group may be necessary for BiUSP recognition. This is opposite with results yielded form activity assays towards Glc-1-P and Gal-

1-P, where both substrate can be well accepted (**Table 2**). One possible explanation could be the bulky carboxylic acid group on C-6 position altered the configuration of C-4 hydroxyl group, makes GalA not tolerable by BiUSP.

On the other hand, AtUSP could efficiently (68% and 65% towards GlcA-1-P and GalA-1-P) catalyze the formation of both UDP-GlcA and UDP-GalA (**Fig**. **3E** & **F**). The activity of BiUSP and AtUSP towards GlcA-1-P are comparable (**Fig**. **3D** & **E**). It is worth to note that AtUSP can be stored for up to 6 months at -20 °C without significant activity loss, whereas BiUSP showed dramatically activity decrease after 6 months storage under the same condition.

Freshly prepared AtUSP or BiUSP was applied in time course experiment and kinetics studies. As shown in **Figure 4**, time course results implies that for AtUSP, the formation rate of UDP-GalA is slightly lower than that of UDP-GlcA. In kinetics studies, to ensure initial reaction rates, enzyme dilutions were selected so that less than 15% of substrate conversion was achieved within 5 min for each reaction. Kinetic parameters were calculated according to Lineweaver–Burk equation (**Table 1**). The K_m value of AtUSP towards GlcA-1-P and GalA-1-P are 2.36×10^{-3} μM and 5.50×10^{-3} μM, indicates that AtUSP has a higher affinity for GlcA-1-P that GalA-1-P. The k_{cat}/K_m values of AtUSP towards GlcA-1-P is 1.8 times as that of GalA-1-P, implies that AtUSP is more efficient for the pyrophosphorylation of GlcA-1-P. The k_{cat}/K_m values of AtUSP (4.66×10^3 s^{-1} μM^{-1}) and BiUSP (4.72×10^3 s^{-1} μM^{-1}) towards GlcA-1-P are similar, indicating they are equally efficient in the synthesis of UDP-GlcA. The kinetics of reverse reactions (hydrolysis of UDP-GlcA and UDP-GalA) (**Table 1**) revealed that AtUSP and BiUSP are equally active towards UDP-GlcA, whereas only AtUSP can catalyze the hydrolysis of UDP-GalA. Such results are consistent with that of forward reactions (formation of UPD-GlcA and UDP-GalA).

2.3 Substrate specificity study of AtUSP and BiUSP towards sugar-1-phosphates

It was previously reported that AtUSP possessed relaxed substrate specificity towards sugar-1-phosphate. For example, it could well accept Gal-1-P, Glc-1-P, 4-deoxy-4-azido-Gal-1-P, D-Fuc-1-P, L-Ara-1-P, and Xyl-1-P.[21,24,25] To further investigate the substrate specificities of AtUSP and BiUSP, the specific activities towards a panel of sugar-1-phosphates were measured. Not surprisingly, BiUSP exhibited similar substrate specificity as BLUSP [26] towards sugar-1-phosphates (**Table 2**). In addition, both AtUSP and BiUSP showed broad substrate specificities, but towards partially different sugar-1-phosphates. For example, AtUSP are highly active towards GlcA-1-P, GalA-1-P, Glc-1-P, Gal-1-P, D-Fuc-1-P, 2-deoxy-Glc-1-P, 2-deoxy-Gal-1-P, whereas BiUSP are highly active towards GlcA-1-P, Glc-1-P, Gal-1-P, Man-1-P, 2-deoxy-Glc-1-P, and 2-deoxy-2-amino-Glc-1-P. Such information are of great importance in applying the USPs in large scale synthesis of UDP-sugar and derivatives.

2.4 Preparation and characterization of UDP-GlcA and UDP-GalA

Given the stability and efficiency, AtUSP was chosen for hundreds milligrams scale synthesis of UDP-HexA. As shown in **Figure 5**, a one-pot three enzyme approach was employed. In the case of UDP-GlcA preparation, the monosaccharide GlcA was first converted into GlcA-1-P catalyzed by AtGlcAK, the glucuronokinase from *A. thaliana*.[23] The formation of UDP-GlcA was then achieved by AtUSP catalyzed reaction in the presence of UTP, and Mg^{2+}. Since UDP-sugar pyrophosphorylases catalyzed relations are usually reversible,[3] a pyrophosphatase from *E. coli* (EcPpA) was added to digest the diphosphate byproduct. The same as previously proved,[20] addition of EcPpA greatly improved the final yield (207 mg pure UDP-GlcA in a yield of 89%) by driving the reaction forward. The synthesis of UDP-GalA was performed following the same route, except that AtGlcAK was substituted by BiGalK, a promiscuous galactokinase exhibiting phosphorylation activity towards GalA.[27] The total yield of UDP-GalA after P2 purification was 70% (162 mg).

2.4.1 Spectroscopic data of Uridine 5'-diphosphate glucuronic acid

HRMS (ESI): Calcd 580.0343 for $C_{15}H_{22}N_2O_{18}P_2$; found: 579. 0256 $[M\text{-}H^+]^-$;

^{1}H NMR (400 MHz, D_2O): δ 3.51 (t, *J* = 9.6 Hz, 1 H), 3.57-3.60 (m,1 H), 3.74 (s,1 H), 3.79 (t, *J* = 9.6 Hz, 1H), 4.14 (d, *J* = 10.4 Hz, 1H), 4.20-4.29 (m,2 H), 4.37-4.38 (m,2 H), 5.62 (dd, *J* = 2.8 Hz, *J* = 7.2 Hz,1 H), 5.97-5.99 (m,2 H), 7.95 (d, *J* = 8.0 Hz, 1 H); ^{13}C NMR (100 MHz, D_2O): δ 58.33, 63.95 (J_{C-P}= 20.4 Hz), 68.65, 70.37 (J_{C-P}= 33.6 Hz), 70.85, 71.58, 72.02, 72.77, 82.27 (J_{C-P}= 35.6 Hz), 87.27, 94.36 (J_{C-P}= 26.0 Hz), 101.68, 140.56, 150.84, 165.23; ^{31}P NMR (162 MHz, D_2O): δ -12.06, -10.40;

2.4.2 Spectroscopic data of Uridine 5'-diphosphate galacturonic acid

HRMS (ESI): Calcd 580.0343 for $C_{15}H_{22}N_2O_{18}P_2$; found: 579. 0258 [M-H^+]$^-$;

^{1}H NMR (400 MHz, D_2O): δ 3.83 (d, *J* = 10.4 Hz, 1 H), 3.98 (dd, *J* = 3.2 Hz, *J* = 10.4 Hz,1 H), 4.17-4.22 (m,2 H), 4.29-4.32 (m,2 H), 4.36-4.37 (m,2 H), 4.53 (s,1 H), 5.68-5.69 (m,1 H), 5.97-5.99 (m,2 H), 7.96 (d, *J* = 8.0 Hz, 1 H); ^{13}C NMR (100 MHz, D_2O): δ 58.31, 63.91 (J_{C-P}= 16.0 Hz), 67.50 (J_{C-P}= 29.2 Hz), 68.32, 68.63, 69.57, 71.70, 72.78, 82.28 (J_{C-P}= 35.6 Hz), 87.25, 94.71 (J_{C-P}= 20.4 Hz), 101.67, 140.57, 150.84, 165.23; ^{31}P NMR (162 MHz, D_2O): δ -11.90, -10.35; HRMS (ESI):

3. Conclusion

In summary, we have biochemically characterized two highly efficient UDP-sugar pyrophosphorylases (AtUSP and BiUSP) for the purpose of large scale UDP-HexA preparation. Genes or expression conditions were optimized to obtain high yields of enzymes (40-90 mg per L culture). Both enzymes showed comparable activities towards GlcA-1-P, whereas only AtUSP exhibited activity towards GalA-1-P. Specific activity study revealed that both USPs possessed broad substrate specificity, but towards partially different sugar-1-phosphates. In addition, we have developed a one-pot three-enzyme approach for efficient and rapid preparation of UDP-GlcA and UDP-GalA starting from inexpensive monosaccharides and nucleotides. Using such an approach, 207 and 162 mgs of UDP-GlcA and UDP-GalA were prepared within one week.

4. Experimental section

4.1 Materials

All chemical reagents were purchased from Sigma unless otherwise noted. Ni-NTA agarose was purchased from Qiagen. Talose-1-P,[20] D-Fucose-1-P,[27] 2-deoxy-Gal-1-P,[27] 2-deoxy-Glc-1-P [20] were prepared as previously described. The preparation of 6-deoxy-6-azido-Gal-1-P will be reported subsequently. Codon optimized gene of AtGlcAK, AtUSP were synthesized by Genscript (Piscataway, NJ). Genomic DNA of *B. infantis* ATCC 15697 was purchase from ATCC. *E. coli* DH5α and BL21 (DE3), as well as vector pET22b were purchased from EMD Millipore (Bilerica, MA). Vector pQE80Lwas purchased from Qiagen (Germantown, MD). BiGalK expression *E. coli* strain was kept by our group.

4.2 Molecular Cloning, Protein Expression and Purification

The glucuronokinase gene (*AtGlcAK*) and UDP-sugar pyrophosphorylase gene (*AtUSP*) from *A. thaliana* was codon optimized and synthesized with restriction sites (*Sac*I and *Hind*III) (see supporting information for optimized DNA sequences). The genes were then cleaved by *Sal*I and *Hind*III, and inserted into pQE80L pretreated with corresponding restriction enzymes. The UDP-sugar pyrophosphorylase gene (*BiUSP, Blon_1169*) from *B. infantis* was amplified by PCR using *B. infantis* ATCC15697 genomic DNA as templates. Primers used are, Blon_1169F: GAGCATATGACCGAAATAAACGATAAGGCC and Blon_1169R: TATCTCGAGCACCCAATCGTCCGGTTCGAT. Yielded PCR fragments were digested with *Nde*I and *Hind*III and subsequently inserted into pET22b pretreated with corresponding enzymes. The recombinant plasmids were verified by DNA sequencing and transformed into *E. coli* BL21 (DE3) for protein expression.

E. coli BL21(DE3) cells harboring the above recombinant plasmids were grown in LB medium at 37 °C with shaking (20 rpm) till OD_{600} reached 0.6-0.8, followed by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM. After 20 h induction at 16 °C the

cells were harvested by brief centrifugation, and stored at -80 °C until used. Protein purification was performed utilizing a Ni-NTA resin (Qiagen) column according to the manufacture instructions. The purified proteins were desalted by PD-10 column (GE life science, USA) against 50 mM Tris-HCl (pH 8.0) and 20% of glycerol. Enzymes were kept at -20 °C until used.

4.3 Activity assays of AtUSP and BiUSP towards GlcA-1-P and GalA-1-P

A 50 μL reaction system in 20 mM Tris-HCl (pH 8.0) consisted of 0.8 mM GlcA-1-P or GalA-1-P, 1 mM UTP, 2 mM $MgCl_2$, and varying amount of purified AtUSP or BiUSP. Reactions were allowed to proceed at 37 °C for 20 min, and quenched by the addition of equal volumes of cold ethanol. The formation of UDP-HexA and consumption of UTP was analyzed by Capillary Electrophoresis (Beckman Coulter) using the following conditions: 75 μm i.d. capillary (Beckman Coulter), 25 KV/140 μA, 5 s vacuum injection, 50 mM sodium tetraborate running buffer, pH 9.4., monitored at 254 nm.

4.4 Determination of kinetic parameters

Kinetic parameters of both AtUSP and BiUSP towards Glc-1-P and Gal-1-P were measured in 50 μL reactions in 20 mM Tris-HCl (pH 8.0). The reaction mixtures contain varying concentrations of GlcA-1-P or GalA-1-P (0.05, 0.1, 0.2, 0.4, 0.6, 0.8 mM), 1 mM UTP, 2 mM $MgCl_2$. Reactions were initiated by the addition of 40 ng of AtUSP or BiUSP, performed at 37 °C for 5 min, and quenched by addition of equal volumes of cold ethanol. The sugar-1-P substrate concentrations used in the time course studies were 0.05 and 0.8 mM. The reactions were quenched at 0, 2, 5, 10, 15, 30, and 60 min. Analysis were performed by CE as described above, and the average value of triplicate assays was employed to generate Line-weaver-Burk plot, and K_m, V_{max}, k_{cat} values were calculated accordingly. Kinetic parameters of AtUSP and BiUSP towards UDP-GlcA and UDP-GalA were measured similarly,

by replacing GlcA-1-P or GalA-1-P into UDP-GlcA or UDP-GalA, and replacing UTP into pyrophosphate.

4.5 Substrate specificity study of AtUSP and BiUSP towards various sugar-1-phosphates

The reaction mixtures (50 μL total volume) contain 0.8 mM of different sugar-1-phosphates (**Table 2**), 1 mM UTP and 2 mM $MgCl_2$. Reactions were initiated by the addition of varying amounts (0.4-40 μg, to make sure the reactions convert no more than 25%) of AtUSP or BiUSP, performed at 37 °C for 10 min, and quenched by the addition of equal volumes of cold ethanol. Analysis were performed by CE as described above, and the average value of triplicate assays was employed to generate specific activities towards each sugar-1-phosphate.

4.6 One-pot three-enzyme synthesis of UDP-GlcA and UDP-GalA

Preparative-scale synthesis of UDP-GlcA and UDP-GalA were performed in a 20 mL reaction system containing 50 mM pH 8.0 Tris-HCl buffer, 20 mM GlcA or GalA, 22 mM ATP, 22 mM UTP, 20 mM $MgCl_2$, and 0.1 to 2 mg of different enzymes. For the synthesis of UDP-GlcA, 0.6 mg of AtGlcAK, 0.4 mg of AtUSP, and 0.1 mg of EcPpA[20] were employed; for the synthesis of UDP-GalA, 2 mg of BiGalK, 0.4 mg of AtUSP, and 0.1 mg of EcPpA were employed. The reaction mixtures were incubated at 37 °C for 4 to 12 hours with gentle shaking. The generation of UDP-sugars were monitored by TLC (Isopropanol: NH_4OH: H_2O = 7: 3: 2). After 90% of monosaccharides converted into UDP-sugar, reactions were quenched by boiling for 5 min, centrifuged to remove precipitants, and concentrated for separation. The separation of UDP-sugars from the reaction mixtures were performed by gel filtration (Bio-Gel P2, Bio-Rad), fractions containing products were pooled and lyophilized. Digestion of nucleotides by alkaline phosphatase and further P2 purification were applied to achieve minimum 95% purity. The final products were lyophilized and characterized by HR-MS and NMR.

Acknowledgement

This work was supported by National Institutes of Health (Grant No.: R01 GM085267 & R01 AI083754 to P.G.W).

Supplementary materials

Supplementary materials associated with this article can be found in the online version.

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Figure Legends:

Figure 1. *De novo* and salvage biosynthetic pathway of UDP-GlcA and UDP-GalA. A), Glucuronokinase (GlcAK); B) UDP-sugar pyrophosphorylase (USP); C) UDP-glucose 6-dehydrogenase; D) Phosphoglucomutase; E) UTP: glucose-1-phosphate uridylyltransferase (GalU); F) UDP-Glc 4'-epimerase (GalE); G) UDP-galactose 6-dehydrogenase; H) galacturonokinase (GalAK); I) UDP- galacturonate pyrophosphorylase.

Figure 2. SDS-PAGE analysis of purified enzymes. Lanes: 1, AtGlcAK; 2, AtUSP; 3, BiGalK; 4, BiUSP.

Figure 3. CE Profiles of compound standards, and BiUSP/AtUSP catalyzed reactions. (A) 0.5 mM UTP; (B) 0.5 mM UDP-GlcA; (C) 0.5 mM of UDP-GalA; (D) BiUSP catalyzed generation of UDP-GlcA; (E) AtUSP catalyzed generation of UDP-GlcA; (F) AtUSP catalyzed generation of UDP-GalA. For each reaction, 0.8 mM sugar-1-P, 1 mM UTP, and 1 μg enzyme was used in a total 50 μL system, reactions were proceeded at 37 °C for 20 min. UTP has a retention time of 21.7 ± 0.1 min, UDP-GlcA has a retention time of 18.2 ± 0.1 min, and UDP-GalA has a retention time of 16.6 ± 0.1 min.

Figure 4. Time course for UDP-GlcA and UDP-GalA reactions. Reactions contains 1 mM UTP, 40 ng of At USP or BiUSP, and: 1, AtUSP and 0.05 mM GlcA-1-P; 2, AtUSP and 0.8 mM GlcA-1-P; 3, AtUSP and 0.05 mM GalA-1-P; 4, AtUSP and 0.8 mM GalA-1-P; 5, BiUSP and 0.05 mM GlcA-1-P; 6, BiUSP and 0.8 mM GlcA-1-P.

Figure 5. One-pot three-enzyme synthesis of UDP-GlcA and UDP-GalA. Enzymes used: AtGlcAK: Glucuronokinase from *A. thaliana*; BiGalK, Galactokinase from *B. infantis* ATCC15697; AtUSP, UDP-sugar pyrophosphorylase from *A. thaliana*; EcPpA, Pyrophosphatase from *E. coli*.

Table 1

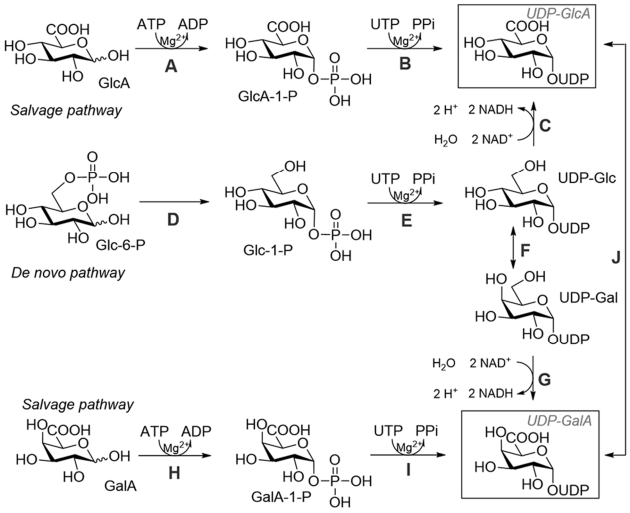
Kinetic parameters of AtUSP and BiUSP towards GlcA-1-P, GalA-1-P, UDP-GlcA and UDP-GalA.

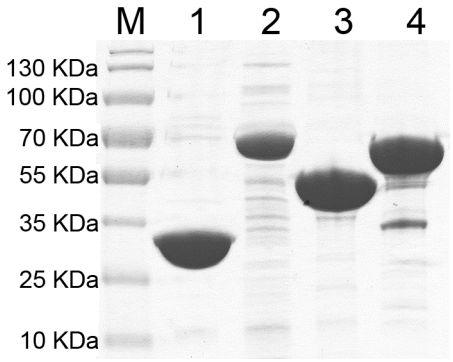
	K_m (μM)	V_{max} ($\mu M\, mg^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)
AtUSP				
GlcA-1-P	2.36×10^{-3}	3.63×10^{-4}	11.0	4.66×10^{3}
GalA-1-P	5.50×10^{-3}	4.45×10^{-4}	13.5	2.45×10^{3}
UDP-GlcA	2.65×10^{-3}	4.06×10^{-4}	12.3	4.64×10^{3}
UDP-GalA	4.96×10^{-3}	4.59×10^{-4}	13.9	2.80×10^{3}
BiUSP				
GlcA-1-P	1.76×10^{-3}	3.38×10^{-4}	8.30	4.72×10^{3}
GalA-1-P	-	-	-	-
UDP-GlcA	2.02×10^{-3}	3.71×10^{-4}	9.12	4.51×10^{3}
UDP-GalA	-	-	-	-

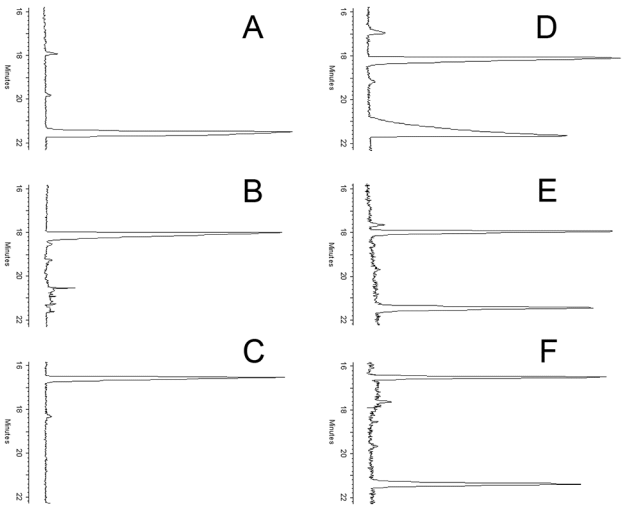
Table 2

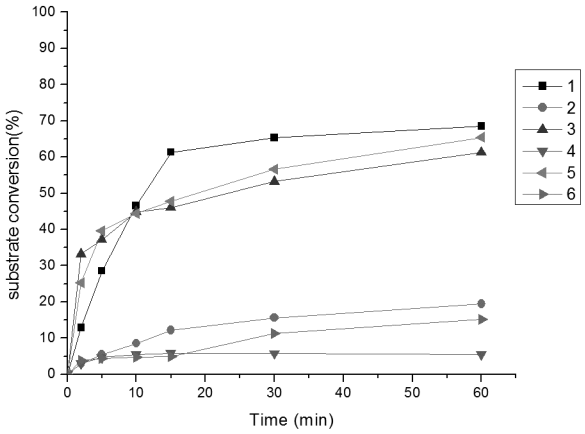
Substrate specificity of AtUSP and BiUSP towards sugar-1-phosphates. Specific activity of AtUSP towards GlcA-1-P (12.4 μmol min^{-1} mg^{-1}) was set as 100. ND, not detected.

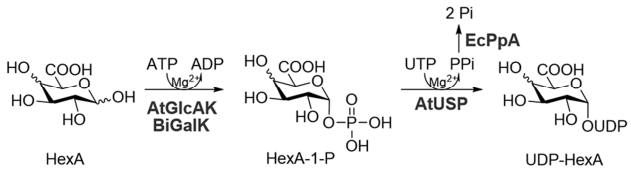
Sugar-1-phosphate	Relative activities	
	AtUSP	BiUSP
GlcA-1-P	100	91
GalA-1-P	75.7	ND
Glc-1-P	130	141
Gal-1-P	123	136
GlcNAc-1-P	ND	ND
GalNAc-1-P	ND	ND
Man-1-P	ND	37.2
Talose-1-P	ND	9.65
D-Fuc-1-P	61.7	15.4
2-deoxy-Glc-1-P	73.8	82.0
2-deoxy-Gal-1-P	53.2	ND
6-deoxy-6-azido-Gal-1-P	11.6	ND
2-deoxy-2-amino-Glc-1-P	7.50	41.8
2-deoxy-2-amino-Gal-1-P	ND	ND











UDP-sugar pyrophosphorylase (AtUSP) from *Arabidopsis thaliana* showed significant activity towards both GlcA-1-P and GalA-1-P

UDP-sugar pyrophosphorylase (BiUSP) from *Bifidobacterium infantis* showed significant activity towards GlcA-1-P

Both USPs showed broad substrate specificity towards various sugar-1-phosphates.

Both AtUSP an BiUSP were expressed and purified in high yields (over 40 mg per L);

A one-pot three-enzyme approach was applied efficiently for hundreds mgs scale of UDP-GlcA and UDP-GalA.

Comparing substrate specificity of two UDP-sugar Pyrophosphorylases and Efficient One-pot Enzymatic Synthesis of UDP-GlcA and UDP-GalA

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Supporting Information

Codon optimized *AtGlcAK* gene:

ATGGATCCGAACAGCACCGTTTCTGGCGATGGTCAGGCTACCGCAGCGATTGAACAC

CGCTCCTTTGCCCGCATTGGCTTTCTGGGTAACCCGTCAGATGTTTATTTTGGCCGCA

CCATTTCGCTGACGATCGGTAATTTCTGGGCGAGCGTCAAACTGGAACCGTCTGAAC

ATCTGGTGATTAAACCGCATCCGTTTCACGATCTGGTGCAGTTCACCAGCCTGGACC

ACCTGCTGAACCGTCTGCAAAATGAAGGCTATTACGGCGGTGTTCGCCTGCTGATGG

CGATCTGCAAAGTCTTCCGTAACTACTGTAAAGAAAACGATATCCAGCTGCATCAAG

CCAACTTCTCACTGTCGTACGACACCAATATTCCGCGCCAGACGGGCCTGAGTGGTA

GCTCTGCAATCGTCTCCGCGGCCCTGAACTGCCTGCTGGATTTTTATAATGTGCGCCA

TCTGATTAAAGTGCAAGTTCGTCCGAACATCGTGCTGAGTGCTGAAAAAGAACTGGG

CATTGTTGCAGGTCTGCAGGATCGTGTTGCTCAAGTCTACGGCGGTCTGGTTCACATG

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Codon optimized *AtUSP* gene:

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GATGGCGATTAAAGGCCGCAATGTGTTCATCAAAGATCTGAGTCTGGACGGTGCGCT

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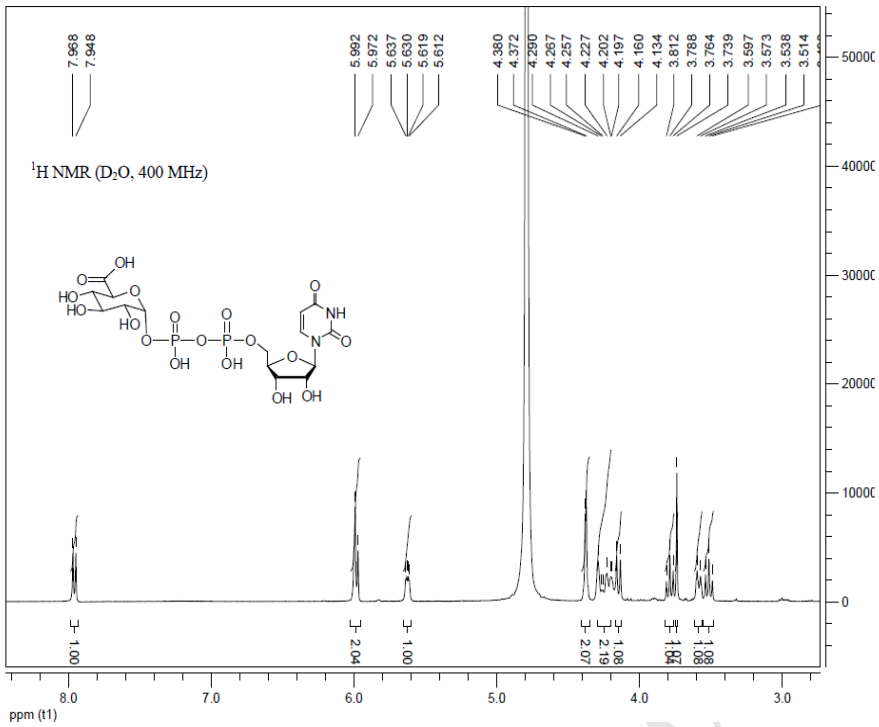
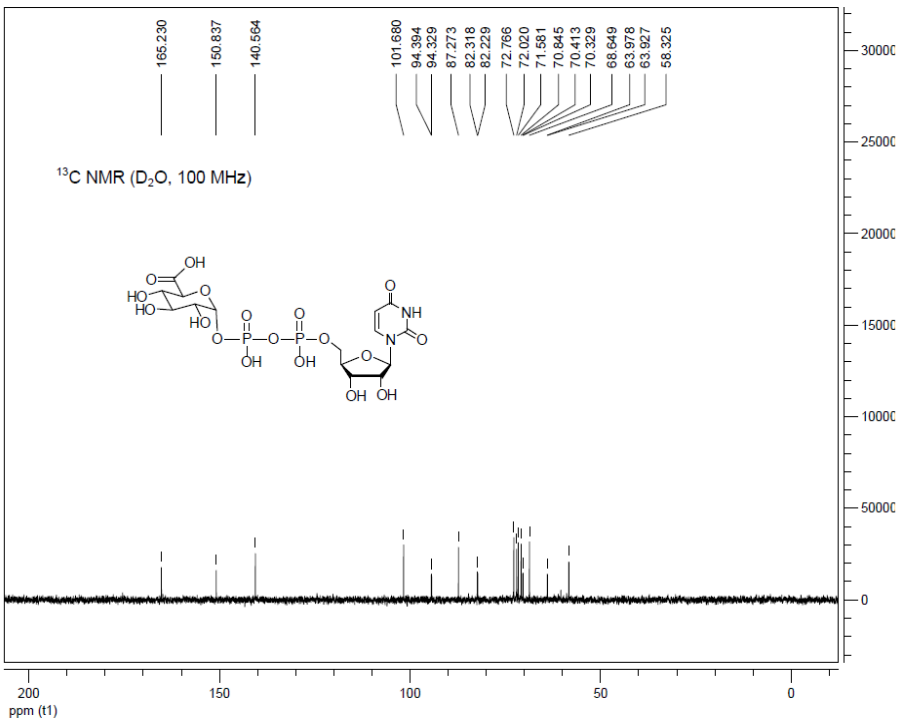
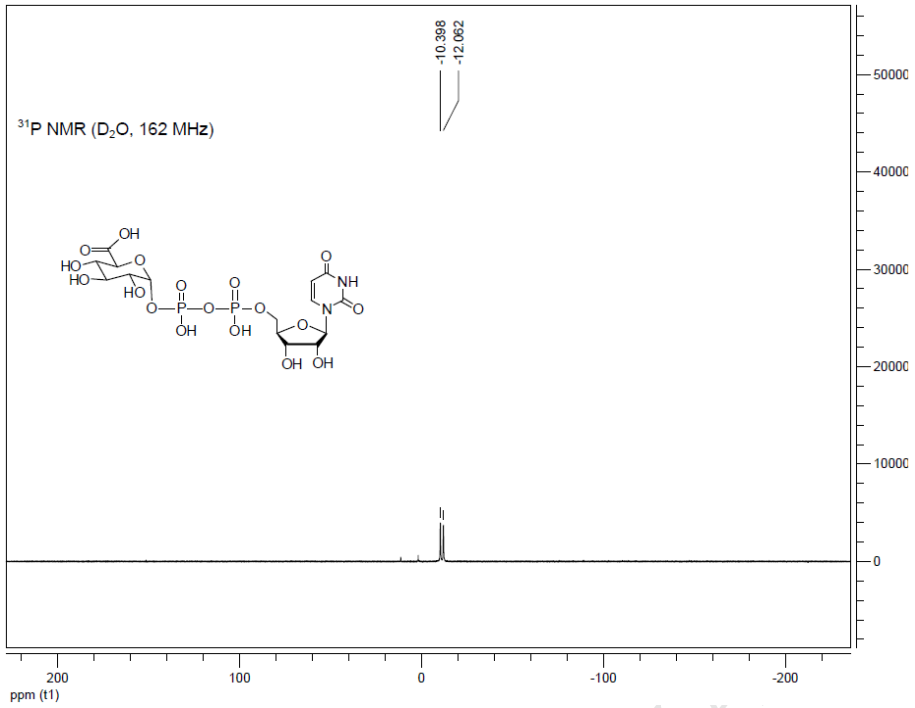
^{1}H NMR Spectrum of UDP-GlcA



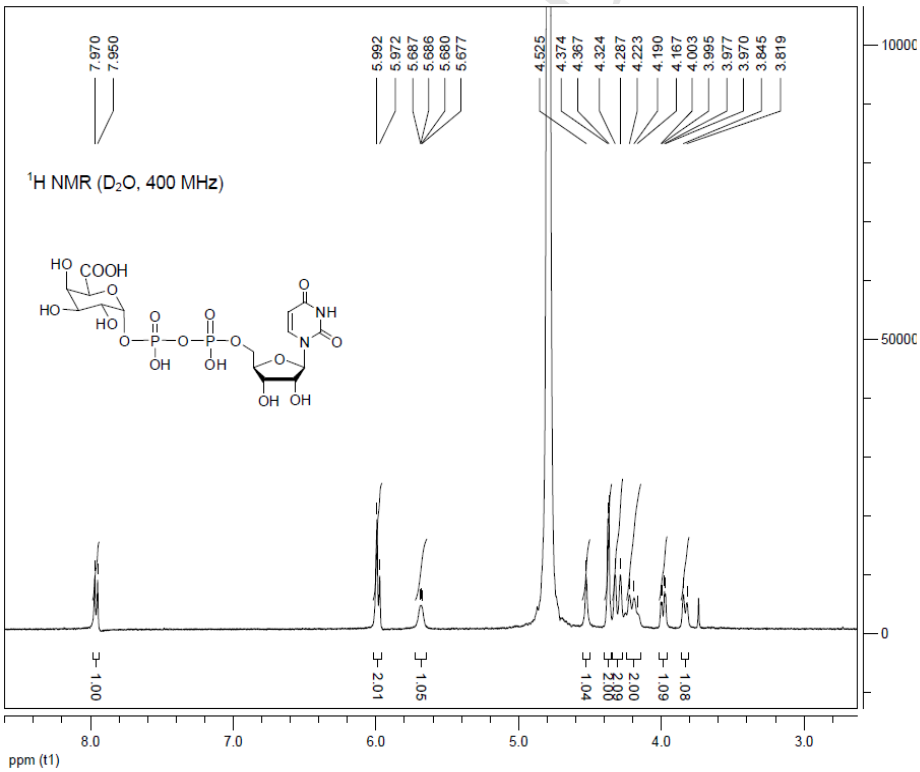
^{13}C NMR Spectrum of UDP-GlcA



^{31}P NMR Spectrum of UDP-GlcA



^{1}H NMR Spectrum of UDP-GalA



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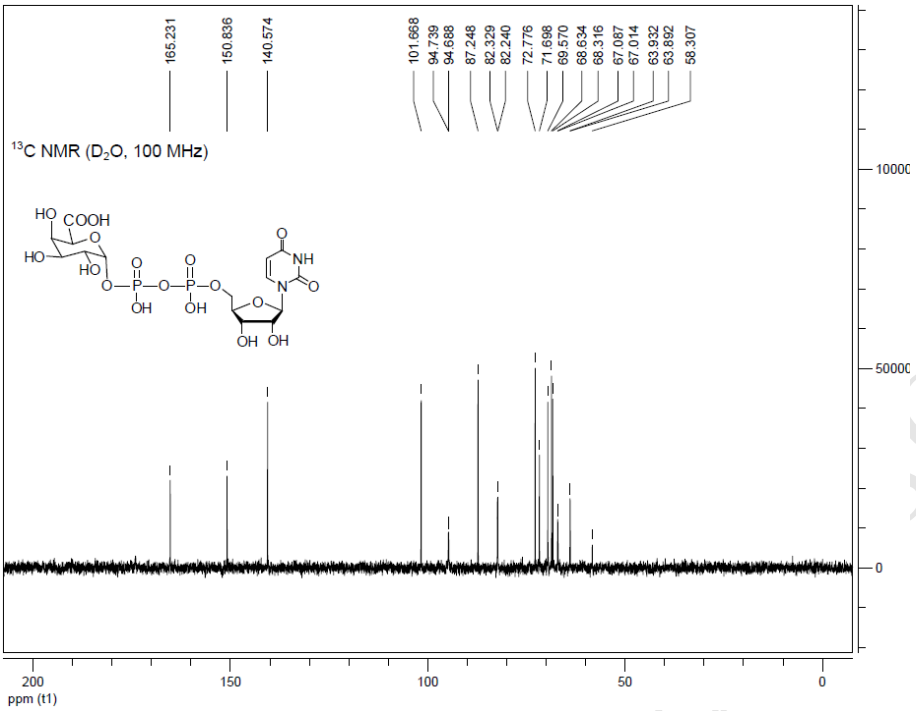

^{31}P NMR Spectrum of UDP-GlcA