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An enzyme-coupled continuous spectrophotometric assay for glycogen synthases

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Abstract The metabolic pathways leading to the synthesis of bacterial glycogen involve the action of several enzymes, among which glycogen synthase (GS) catalyzes the elongation of the α-1,4-glucan. GS from *Agrobacterium tumefaciens* uses preferentially ADPGlc, although UDPGlc can also be used as glycosyl donor with less efficiency. We present here a continuous spectrophotometric assay for the determination of GS activity using ADP- or UDPGlc. When ADPGlc was used as the substrate, the production of ADP is coupled to NADH oxidation via pyruvate kinase (PK) and lactate dehydrogenase (LDH). With UDPGlc as substrate, UDP was converted to ADP via adenylate kinase and subsequent coupling to PK and LDH reactions. Using this assay, we determined the kinetic parameters of GS and compared them with those obtained with the classical radiochemical method. For this purpose, we improved the expression procedure of *A. tumefaciens* GS using *Escherichia coli* BL21(DE3)-RIL cells. This assay allows the continuous monitoring of glycosyltransferase activity using ADPGlc or UDPGlc as sugar-nucleotide donors.

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Introduction

Glycosyltransferases are a group of enzymes that catalyze the transfer of a monosaccharide from an activated glycosyl donor onto an oligosaccharide acceptor [1]. This family of enzymes is highly specific for their donor and acceptor and with few exceptions, each type of glycosidic linkage requires a unique glycosyltransferase [1, 2].

Glycogen and starch are the major carbohydrate storage molecules in most living organisms ranging from archaea, bacteria, yeast, animals and plants [3, 4]. The synthesis of bacterial glycogen and plant starch involves the action of three enzymes: ADP-glucose pyrophosphorylase (ADPGlc PPase, EC 2.7.7.27), glycogen (starch) synthase (GS or SS, EC 2.4.1.21), and branching enzyme (BE, EC 2.4.1.18) [4–6]. The regulatory step in both biosynthetic routes takes place at the level of the sugar-nucleotide production, a reaction catalyzed by ADPGlc PPase; whereas GSs or SSs catalyze the elongation of the α-1,4 glucans by adding glucose units from the sugar-nucleotide to the non-reducing end of the growing chain [4–6].

Bacterial and plant glycosyltransferases belong to the GT5 family (http://afmb.cnrsmrs.fr/CAZY/) [1, 7]. This group of enzymes prefer ADPGlc as sugar donor and appear to be unregulated proteins [4]. However, some of these enzymes (i.e. archaeal GSs or some SS isoforms) could use UDPGlc with similar or less efficiency [8–11].

Different methods to assay fucosyl-, sialyl-, galactosyl-, mannosyl-, *N*-acetylglucosaminyl- and *N*-acetyllactosaminyltransferase activity have been described, including radiochemical, spectrophotometric, immunological, chromatographic,

and solid-phase determinations [12–17]. In this article we report a coupled continuous method for the determination of the activity of glycogen synthase from *Agrobacterium tumefaciens* utilizing ADPGlc or UDPGlc. The production of the ADP or UDP nucleotides were coupled to NADH oxidation via pyruvate kinase (PK) and lactate dehydrogenase (LDH) (for ADP) or PK, nucleotide kinase (PNK) and LDH (for UDP). The method was compared with the discontinuous gold standard radiochemical assay previously described [18].

Materials and methods

Strains and culture media

Escherichia coli XL1Blue: *endA*1, *gyrA*46, *hsdR*17, *lac*$^-$, *recA*1, *relA*1, *supE*44, *thi*-1, F′ [*proAB*$^+$, *lacI*^{q} *lacZ*ΔM15, Tn*10*(tet^r)] and *E. coli* BL21-CodonPlus(DE3)-RIL strain: *E. coli* B F-*ompT hsdS*(rB- mB-) *dcm* + Tetr *gal* (DE3) *endA* Hte [*argU ileY leuW* Cam^r] were used in this study (Stratagene, La Jolla, CA, USA). *E. coli* strains were grown at 37°C in LB medium containing the appropriate antibiotics.

Cloning, expression and purification of glycogen synthase

cDNA of GS from *A. tumefaciens* cloned in pBG19 plasmid [19] was PCR amplified using the following primers: GSfw: AAA*CATATG*AATGTCCTTTCGGTTTC (NdeI site italicized), GSrv: AAA*CTCGAG*ATGGCCTTTCGAAAT AAGCTG (*Xho*I site italicized). The PCR product was digested using restriction endonucleases and cloned into pET32c vector (Novagen, Madison, WI, USA). Positive clones were verified by DNA sequencing. Recombinant plasmid named pHNAL32 (containing a C-terminal His-tag sequence) were used to transform *E. coli* BL21 (DE3)-RIL cells for expression purposes.

After growing the cells at 37°C for 4 h (until $OD_{600} = 0.6$), 1 mM isopropyl-β-D-thiogalactoside (IPTG) was added to the culture medium and incubated at 28°C for at least 4 h. Cells were harvested by centrifugation at 4,000×*g* for 10 min at 4°C. The pellet was washed and suspended in buffer containing 20 mM Tris–HCl, pH 8 (1 ml/g of wet cells) and 1 mM PMSF (phenylmethylsulphonyl fluoride). Cells were disrupted using an ultrasonicator (VCX130, Sonics and Materials Inc., Connecticut, USA) and centrifuged at 12,000×*g* for 15 min at 4°C.

GS was purified using one step Ni-chelating chromatography as described previously [19]. Briefly, the homogenates were filtered through a 0.2 μm cellulose acetate membrane filter and loaded onto a HiTrap^{TM} Chelating HP columns (GE Healthcare) equilibrated with binding buffer 20 mM potassium-phosphate, pH 7.4, 0.3 M NaCl, 1 mM β-mercaptoethanol; 20 mM imidazole. The column was washed at least with 10–15 volumes of binding buffer, and each protein was eluted using a linear gradient of binding buffer and elution buffer (20 mM potassium-phosphate, pH 7.4, 0.3 M NaCl, 1 mM β-mercaptoethanol; 20–500 mM imidazole). Recombinant GS eluted in the fraction between 200 and 300 mM imidazole. The active fractions were pooled and concentrated to >1 mg/ml. The concentrated proteins were desalted and used to determine enzyme activity or stored at −20°C until use.

Determination of glycogen synthase activity

The activity of GS was determined using two methods, a spectrophotometric method and a radiochemical method previously described with some modifications [18, 19].

The spectrophotometric determination of GS activity using ADPGlc was performed as follows: The reaction medium contained 50 mM Bicine, pH 8.0, 80 mM $MnCl_2$, 0.4 mM NADH, 1 mM phosphoenolpyruvate (PEP), 1 U lactate dehydrogenase (LDH) (Sigma-Aldrich, St. Louis, USA), 1 U pyruvate kinase (PK) (Sigma-Aldrich), 10 mg of rabbit muscle glycogen and 3.5 mM ADPGlc.

When UDPGlc was used as glycosyl donor, the medium contained 50 mM Bicine, pH 8.0, 80 mM $MnCl_2$, 0.4 mM NADH, 1 mM phosphoenolpyruvate (PEP), 1 U lactate dehydrogenase (LDH), 1 U pyruvate kinase (PK), 1 U adenylate kinase (AK, Sigma-Aldrich), 5 mM ATP, 10 mg of rabbit muscle glycogen and 20 mM UDPGlc. In both cases, the final volume of each reaction was 400 μl. GS activity was measured by following the oxidation of NADH at 340 nm and 30°C. One unit (U) is defined as the amount of enzyme that catalyzes the consumption of 1 μmol of NADH per min under the specified assay conditions.

The radiochemical determination of GS activity was performed as follows: assays were initiated by the addition of enzyme and also were performed at different enzyme concentrations to ensure steady-state conditions. The assay medium contained 100 mM MOPS, pH 7.5, 0.25 mM KCl, 10 mg/ml of rabbit muscle glycogen, and 3.5 mM ADP-[^{14}C]Glc (1.3 μCi/nmol) or 20 mM of UDP-[^{14}C]Glc (1.1 μCi/nmol) as glucosyl donor. The final volume of each reaction was 100 μl. After 20 min at 30°C, reactions were stopped by the addition of 500 μl of 75% (v/v) methanol and 1% (w/v) KCl, centrifuged and pellets washed twice with the same solution. Radioactivity incorporated in the final pellet suspended in 200 μl of distilled water was determined in a liquid scintillation counter. One unit of activity is defined as the amount of enzyme catalyzing the

incorporation into glycogen of 1 μmol of [^{14}C]Glc (from ADPGlc or UDPGlc) per minute at 30°C.

Kinetic studies and protein measurements

The concentrations giving 50% maximal velocity (K_m), Hill coefficients (n_H), and k_{cat} were calculated according to Brooks [20]. All kinetic parameters are means of at least three determinations and the average values ± SE are reported.

Total protein was determined using the Bradford method as described [21].

Gel electrophoresis and immunological studies

SDS-PAGE was performed with the Bio-Rad Mini Protean system using 10% polyacrylamide/bisacrylamide gels as described [22]. Gels were revealed by staining with Coomassie brilliant blue (R250, Sigma-Aldrich) or electroblotted onto nitrocellulose membranes (Trans-Blot Transfer Medium, Nitrocellulose Membrane, Bio-Rad). After electroblotting, membranes were treated with penta-His antibody (anti-His antibody selector kit, Quiagen). The antigen–antibody complex was visualized with alkaline phosphatase linked anti-mouse IgG, followed by staining with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) [23].

Results and discussion

Determination of kinetic parameters for GS using ADPGlc

First, we tested the expression of recombinant *A. tumefaciens* GS in two different hosts, *E. coli* BL21(DE3) and *E. coli* BL21(DE3)-RIL cells. The isolation procedure of His-tagged GS is highly reproducible in both *E. coli* hosts. Using BL21(DE3) cells we obtained about 1.2 mg of purified protein per gram of cells, whereas about 2.3 mg of protein per gram were obtained from the BL21(DE3)-RIL host. Therefore, the BL21(DE3)-RIL strain was used for further experiments. The purification yield of GS was about 10- and 1,100-fold higher than other previously reported values from *Pasteurella pseudotuberculosis* and *E. coli* GSs, respectively [24, 25]; and also about threefold higher than the yield obtained from its expression using the pBG19 plasmid [19]. The different expression level could be due to the presence of 5 rare codons in *A. tumefaciens* GS (1 AGA, 1 AUA, 1 AGG and 2 CUA). The *E. coli* BL21(DE3)-RIL strain contains extra copies of the *argU*, *ileY* and *leuW* tRNA genes that recognize the AGA/AGG (arginine), AUA (isoleucine), and CUA (leucine) codons. It has been described that synonymous codons (those encoding the same aminoacid) are not equally used [26]. In addition, the presence of rare codons could influence the level of gene expression, affecting the amount and quality of the proteins produced in *E. coli* hosts [27–30]. We evaluated by SDS-PAGE analysis the purity of the recombinant GS used in this study. A single protein band of about 52 kDa was eluted from the column in the range between 200 and 300 mM imidazole (Fig. 1a). We confirmed the presence of the recombinant GS by immunoblot using penta-His antibodies that react against the His_6C-terminal tag of the protein (Fig. 1b). The absence of endogenous GS activity by Western blot and by measuring enzymatic activity was investigated as described previously [10]. We determined that *E. coli* GS elutes from the Ni^{2+} chelating column during the washing step. Thus, this procedure completely separates the small amount of endogenous GS from recombinant GS (not shown).

Purified recombinant GS from *A. tumefaciens* was used to perform the enzymatic assays. Table 1 shows the kinetic parameters of GS for ADPGlc and glycogen using the radiochemical technique. Using glycogen as saturating substrate, GS display Michaelis kinetics, indicating a non-cooperative reaction. The enzyme exhibited a K_m value for ADPGlc of 0.37 ± 0.04 mM. Indeed, GS showed a k_{cat} of 288 ± 25 s^{-1} and a catalytic efficiency (k_{cat}/K_m) of 778 ± 149 mM^{-1} s^{-1}. Table 1 also shows the kinetic parameters of GS for the polysaccharide substrate. The K_m value for glycogen was 1.3 ± 0.2 mg/ml and the k_{cat} obtained was 293 ± 36 s^{-1}. The K_m value reported for

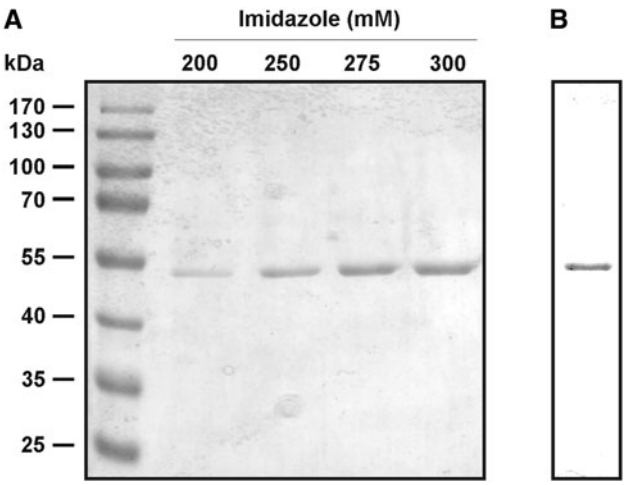

Fig. 1 **a** SDS-PAGE analysis of recombinant GS from *A. tumefaciens*. Imidazole concentrations used for the elution of each chromatographic fraction are indicated above the respective lane. The amount of protein loaded ranged from 0.2 (200 mM imidazole fraction) to 1.5 μg (300 mM imidazole fraction). *Numerals* indicate the molecular masses of markers (Fermentas Page Ruler Prestained Protein Ladder). **b** Western blot detection of purified recombinant GS shown in panel A using penta-His antibodies

Table 1 Kinetic parameters of *A. tumefaciens* GS for ADPGlc and glycogen using the radiochemical or spectrophotometric method

Substrate	K_m^a	n_H	k_{cat} (s^{-1})	k_{cat}/K_m^b
Radiochemical method				
ADPGlc	0.37 ± 0.04	1.3 ± 0.4	288 ± 25	778 ± 149
Glycogen	1.3 ± 0.2	1.1 ± 0.2	293 ± 36	225 ± 62
Spectrophotometric method				
ADPGlc	0.41 ± 0.07	1.2 ± 0.2	264 ± 14	644 ± 144
Glycogen	1.1 ± 0.3	1.0 ± 0.3	272 ± 10	247 ± 77

[a] K_m values are expressed in mM for ADPGlc or mg/ml for glycogen

[b] k_{cat}/K_m values are expressed in s^{-1} mM^{-1} for ADPGlc or s^{-1} $(mg/ml)^{-1}$ for glycogen

ADPGlc is within the range of 0.035 to 4.1 mM values, reported for other GSs or SSs (0.035 mM for *E. coli* GS [31]; 0.13 mM for *Zea mays* SSII [32]; 0.2 mM for *Spinacea oleracea* SSI [33]; 0.29 mM for *Pisum sativum* SSI [34]; 0.4 mM for *P. pseudotuberculosis* GS [24]; 1.3 mM for *Solanum tuberosum* GBSSI [35] and 4.1 mM for *A. thaliana* SSIII [10, 11]).

The kinetic parameters of GS for ADPGlc and glycogen were also determined using the spectrophotometric technique. First, we determined that the coupled assay has a linear dependence to the amount of GS and the coupled enzymes present in the mixture (see supplementary Fig. 1). The K_m for ADPGlc (0.41 ± 0.07 mM) showed no significant differences to that obtained with the radiochemical technique. Indeed, we also obtained similar k_{cat} and catalytic efficiency value for ADPGlc when compared to the radiochemical method. Under variable glycogen concentrations the K_m value was 1.1 ± 0.3 mg/ml and the k_{cat} 272 ± 10 s^{-1}. In addition, similar results of GS catalytic efficiency were obtained for the polysaccharide substrate using both techniques (Table 1). The saturation plots for glycogen and ADPGlc are shown in Fig. 2a, b. Thus, the method presented here produces identical results to that described previously using radiochemistry. Moreover, the specific activity value obtained for GS (about 165 U/mg) is at least tenfold higher than the specific activity values of the SSs enzymes from plants (ranging from 0.02 to 15.8 U/mg [33, 34]), however, it is threefold lower respect to the value reported for *E. coli* GS (about 500 U/mg) [25].

Determination of kinetic parameters for GS using UDPGlc

As described previously, most of GSs (and SSs) use preferentially ADPGlc [6]. However, some GSs including the archaeal enzymes use both, ADP- or UDPGlc with the same efficiency [9]. Indeed, some SS isoforms such as SSIII can also use UDPGlc as substrate [10]. The binding

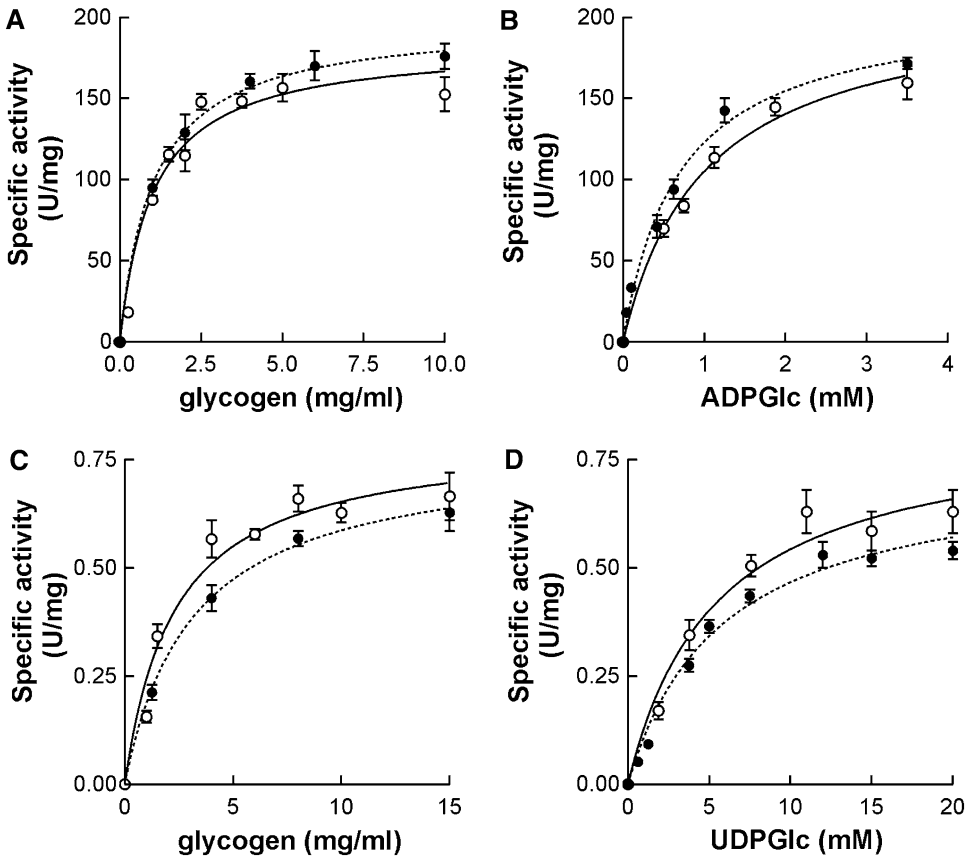

Fig. 2 Comparison of the substrate kinetics for GS by the radiochemical method (*filled circles*, *discontinuous curve*) and the spectrophotometric coupling assay method (*open circles*, *continuous curve*). Glycogen saturation plots for GS determined in the presence of 3.5 mM ADPGlc (**a**) or 20 mM UDPGlc (**c**). ADPGlc (**b**) or UDPGlc (**d**) saturation plots in the presence of 10 mg/ml glycogen

site for the sugar nucleotide in *A. tumefaciens* GS is located in the C-terminal region of the enzyme [36]. The specificity for the sugar nucleotide is determined by two weak hydrogen bonds between nitrogens N1 and N6 of the heterocycle with the protein backbone (the carbonyl and amide groups of Gly353 and Asn355, respectively) and stacking interactions with Tyr354 [36]. As mentioned above, Horcajada et al. [9] reported the GS form *Pyrococcus abyssi* can use ADPGlc or UDPGlc with the same efficiency. The reported 3D-models of *P. abyssi* GS with UDP or ADP in the donor binding pocket predicts that the nucleotide binding site of this protein allows the binding of both, ADPGlc or UDPGlc. In addition, the flexibility of a methionine residue (Met316) side chain would facilitate the stacking either with adenine or uracil [9]. We found that GS from *A. tumefaciens* use ADPGlc as the major sugar-nucleotide donor, but UDPGlc can also be used with less efficiency. In *A. tumefaciens* GS the Lys291 and Met316 residues are not conserved and are replaced by Ala340 and Tyr354, respectively. This could explain the less sensitivity of *Agrobacterium* GS for UDPGlc. It has been reported that other GSs such as that from *E. coli*, can use ADPGlc or UDPGlc (also with less efficiency) in the absence of added polysaccharide primer [37].

Table 2 shows the kinetic data of GS for UDPGlc and glycogen using the radiochemical and spectrophotometric methods. When UDPGlc was used as the donor substrate, GS follows Michaelis kinetics (n_H values close to 1), also indicating a non-cooperative reaction. At varying UDPGlc levels and saturating concentrations of glycogen, the K_m value for the sugar-nucleotide was 6.3 ± 0.5 mM and the k_{cat} measured was 0.89 ± 0.07 s^{-1} (Table 2). When glycogen was the variable substrate, the K_m value was 2.3 ± 0.2 mg/ml and the k_{cat} 1.05 ± 0.07 s^{-1} (Table 2).

When GS activity was determined in the presence of ADPGlc using the spectrophotometric method, the production of ADP was coupled to NADH oxidation via PK and LDH enzymes. PK is an allosteric protein and a substrate promiscuous enzyme, however, the most used PK to perform in vitro enzyme reactions is obtained from rabbit muscle. It has been described that this enzyme is more specific on ADP, and less specific for the other NDPs [38].

Thus, when UDPGlc was used as glycosyl donor substrate, the activity of GS was measured in the presence of PK, adenylate kinase (AK, nucleoside 5′-diphosphate kinase) and LDH. The AK transfers one phosphate group from many NTP to NDP. Thus, the UDP produced was coupled to NADH oxidation via the following reactions Eq. 1–4:

$$\text{UDPGlc} + \text{glycogen} \xrightarrow{\text{GS}} (\text{glycogen})n + 1 + \text{UDP} \quad (1)$$

$$\text{ATP} + \text{UDP} \xrightarrow{\text{AK}} \text{ADP} + \text{UTP} \quad (2)$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{pyruvate} + \text{ATP} \quad (3)$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^{+} \quad (4)$$

First, we compared the specific activity of GS in the absence or presence of AK. The k_{cat} value obtained in the absence of AK was about tenfold lower (near 0.05 s^{-1}) compared to that obtained with the addition of AK to the reaction mixture. This data is in agreement with the low specificity of PK for UDP. Taking into account the results described above, the kinetic parameters of GS for UDP and glycogen were determined using the spectrophotometric method in the presence of AK in the coupling assay (see Materials and methods section).

The results revealed that, in all cases, GS displays Michaelis kinetics. The K_m for UDPGlc was 6.0 ± 0.4 mM and the k_{cat} was 1.15 ± 0.12 s^{-1}, similar to those values obtained with the radiochemical technique (Table 2). Under saturating concentrations of UDPGlc, the K_m value for glycogen was 2.1 ± 0.3 and the k_{cat} 1.05 ± 0.05 s^{-1} (Table 2). The saturation plots for glycogen and UDPGlc are shown in Fig. 2c, d. The kinetic parameters obtained with the coupling assay are in also in agreement with the values reported using the radiochemical method. Indeed, the catalytic efficiency values obtained for UDPGlc and glycogen were similar regardless of the technique used (see Table 2).

In conclusion, in this work we have reported a continuous spectrophotometric method for the determination of GS glycosyltransferase activity using ADPGlc or UDPGlc as glycosyl donor substrates. For this purpose, we also performed the cloning and purification of *A. tumefaciens* GS and improved the expression protocol using BL21(DE3)-RIL hosts. We demonstrate that both methods, the radiochemical and the enzymatic coupling assay, render similar kinetic parameters for the sugar-nucleotides and the polysaccharide substrate. Indeed, we reported that the addition

Table 2 Kinetic parameters of *A. tumefaciens* GS for UDPGlc and glycogen using the radiochemical or spectrophotometric method

Substrate	K_m^a	n_H	k_{cat} (s^{-1})	k_{cat}/K_m^b
Radiochemical method				
UDPGlc	6.3 ± 0.5	1.3 ± 0.2	0.89 ± 0.07	0.14 ± 0.02
Glycogen	2.3 ± 0.2	0.9 ± 0.1	1.05 ± 0.07	0.46 ± 0.07
Spectrophotometric method				
UDPGlc	6.0 ± 0.4	0.8 ± 0.3	1.15 ± 0.12	0.19 ± 0.03
Glycogen	2.1 ± 0.3	1.4 ± 0.2	1.05 ± 0.05	0.50 ± 0.09

[a] K_m values are expressed in mM for ADPGlc or mg/ml for glycogen

[b] k_{cat}/K_m values are expressed in s^{-1} mM^{-1} for ADPGlc or s^{-1} $(mg/ml)^{-1}$ for glycogen

of AK to the reaction media is critical when GS activity was measured using UDPGlc as the glysosyl donor. It has been reported that glycosyltransferases are low abundant within the cells, therefore, their limited availability precluded a spectrophotometric assay due to the lack of specificity and sensibility, specially in preparations that use partially purified enzymes [13]. However, the cloning and high level expression of many glycosyltransferases allows the choice of this continuous spectrophotometric technique. The method described has the advantages of being simple to use, rapid, precise and non-radioactive. In addition, the technique allows the continuous monitoring of the enzyme activity and therefore, the detailed kinetic studies to characterize the glycosyltransferase reaction. Moreover, this assay can be used for the determination of the activity of other glycosyltransferases, such as those grouped in the GT3 family (for example animal and fungal GSs) which use UDPGlc as the sugar-nucleotide substrate.

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