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Application of recombinant sucrose synthase-large scale synthesis of ADP-glucose

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Abstract

The synthesis of ADP-glucose with recombinant sucrose synthase from potato was combined with the synthesis of ADP from AMP and ATP catalysed by myokinase from rabbit muscle. By using the repetitive-batch-technique we were able to reach enzyme productivities (mg ADP-glucose/U enzyme) of 28 mg/U sucrose synthase and 140 mg/U myokinase yielding 2.8 g ADP-glucose (55% yield). After product isolation, 2.2 g ADP-glucose was obtained corresponding to 44% overall yield.

Keywords: ADP-glucose; Sucrose synthase; Nucleotide sugar

1. Introduction

The application of highly regio- and stereospecific Leloir glycosyltransferases in carbohydrate synthesis requires a facile access to nucleotide sugars as their donor substrates. We have demonstrated that the glycosyltransferase sucrose synthase (EC 2.4.1.13, SuSy) from rice grains is a versatile tool for the preparation of activated glucoses by exploitation of the enzymatic cleavage of sucrose with nucleoside diphosphates (NDP, N = U, dT, dU, A, C) [1–3]. The combination of SuSy with kinases and/or a dehydratase yielded nucleotide(deoxy)sugars from inexpensive nucleoside monophosphates and sucrose [4], and a central intermediate of the dTDP-deoxysugar pathways (dTDP-6-deoxy-4-ketoglucose) on a gram-scale [5]. In the present paper, we report on the enzymatic gram-scale synthesis of ADP-glucose utilizing the favourable substrate spectrum of recombinant SuSy from potato expressed in yeast [6] (Fig. 1). ADP-glucose is the donor substrate in plant starch and bacterial glycogen synthesis [7,8].

2. Experimental

2.1. Materials

Recombinant SuSy (from potato) was purified as described elsewhere [6]. Calf intestinal

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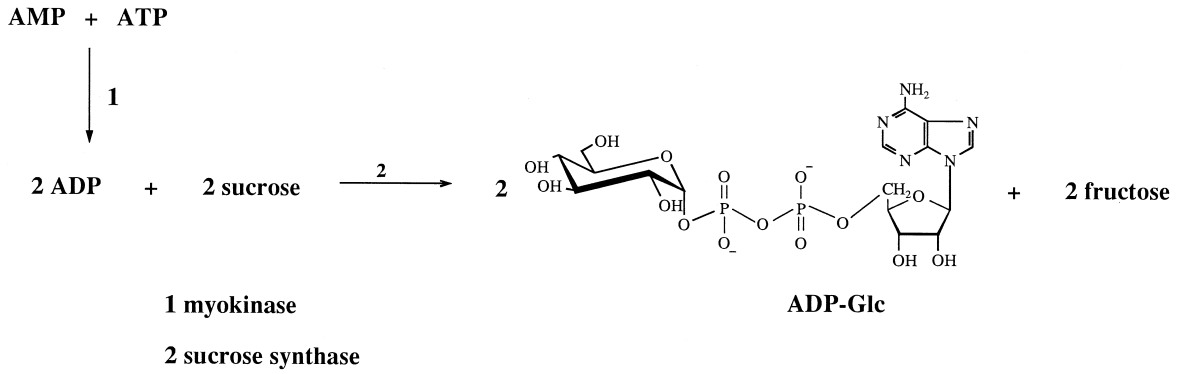

Fig. 1. Synthesis of ADP-glucose from AMP, ATP and sucrose with sucrose synthase and myokinase.

alkaline phosphatase (EC 3.1.3.1) was from Boehringer (Mannheim, Germany). Adenosine, AMP, ADP, ADP-glucose, myokinase (MK, EC 2.7.4.3) from rabbit muscle were supplied by Sigma (Deisenhofen, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany).

2.2. Analytical methods

Adenosine, AMP, ADP and ADP-glucose can be separated by ion-pair HPLC using a Hypersil ODS-column (5 μm particle size, 250×4.6 mm) from Chromatography Service (Langerwehe, Germany). The compounds were eluted with 100 mM potassium acetate pH 4.6, containing 0.013% (v/v) *n*-octylamine and 8% (v/v) methanol. The flow rate was 0.8 ml/min. The concentrations were read from calibration charts (0.2–3 mM) of the compounds vs. peak area (absorption at 254 nm).

2.3. Synthesis in presence of different Mg^{2+}-concentrations

Four millimolar AMP, 4 mM ATP, 1 U/ml SuSy and 0.25 U/ml MK were incubated in buffer A (200 mM Hepes–NaOH, pH 7.5 containing 500 mM sucrose, 3 mM DTT and 1 mg/ml bovine serum albumin) in the presence of 0.5 and 8 mM $MgCl_2$ at 30°C. The enzymatic synthesis was stopped by heating at 95°C for 5 min. The samples were analysed by HPLC.

2.4. Hydrolysis of ADP-glucose in the presence of Mg^{2+}

Two millimolar ADP-glucose was incubated for 24 h at 30°C in buffer (200 mM Hepes–NaOH, pH 7.5) in the presence of different $MgCl_2$-concentrations. The samples were analysed by HPLC.

2.5. Preparative synthesis of ADP-glucose

A batch synthesis was started by mixing 10 ml reaction solution of 40 mM AMP, 40 mM ATP, 10 U/ml SuSy, 1 U/ml MK, 10 mg/ml bovine serum albumin, 1.25 mM $MgCl_2$ in buffer A, respectively, and addition of 40 ml of buffer A (final volume 100 ml). After a sterile filtration, the solution was gently stirred overnight at 30°C. The product solution was separated from the enzymes by ultrafiltration. Therefore, the solution was concentrated to 10 ml in a stirred ultrafiltration cell (Amicon, Modell 8050, equipped with a membrane YM 10, cut-off 10.000 g/mol). The second and all subsequent batches was started by addition of 10 ml of solutions containing 40 mM AMP, 40 mM ATP, 1.25 mM $MgCl_2$, respectively, and 60 ml buffer A to the concentrate (final volume 100 ml).

The isolation of ADP-glucose was started by incubation of the product solution containing 4.45 mmol ADP-glucose with alkaline phosphatase (2 U/ml product solution) overnight at 30°C. The enzyme was removed by ultrafiltration. ADP-glucose was separated from the product solution by anion-exchange chromatography using Sepharose Q FF, Cl^--form (column: 5 × 23 cm). Prior to loading onto the column (flow rate: 8 ml/min), the pH of the product solution containing 1.3 g ADP-glucose was adjusted to 6.5. The column was subsequently washed with dest. water (adjusted to pH 2.0 with HCl) until the conductivity and the UV-absorption (254 nm) have dropped to a constant value. ADP-glucose was eluted with 0.4 M NaCl (pH 2.0 adjusted with HCl) (flow rate: 12 ml/min). The pH of the pool was adjusted to 7.0, and the solution was concentrated at 25–30°C and 20–25 mbar until NaCl precipitated. The precipitated salt was resolved in a minimal volume of water, and NaCl was partially precipitated by addition of 50% (v/v) ethanol. The solution was concentrated and aliquots containing 200 mg ADP-glucose were desalted on Sephadex G10 (column: 2.6 × 94 cm, flow rate: 1 ml/min, dest. water). 2.88 g product (containing 2.2 g ADP-glucose, NaCl and H_2O, HPLC: 100%,) were isolated after lyophilization. Overall yield: 3.48 mmol ADP-glucose (43.5% based on 4 mmol AMP and 4 mmol ATP). ^{1}H-NMR and ^{13}C-NMR-spectrum was essentially the same as that reported by Zervosen et al. [4].

3. Results and discussion

Recombinant SuSy from potato has a favourable substrate spectrum (UDP > ADP > dTDP > CDP > GDP) [6] in comparison to SuSy from rice (UDP > dUDP = dTDP > ADP > CDP > GDP) [1] which we used in our previous studies [4]. In contrast to our previously described method [4], we avoided the in situ regeneration of ATP with PEP and pyruvate kinase because of the low price of ATP and the problems to separate ADP-glucose from pyruvate.

Table 1
Hydrolysis of 2 mM ADP-glucose in the presence of different $MgCl_2$-concentrations

c ($MgCl_2$) (mM)	ADP-glucose (%)
0.125	92
0.5	95
4	85
8	72

ADP-glucose was incubated in buffer (Hepes–NaOH, pH 7.5) at 30°C for 24 h.
ADP-glucose (t = 0 h) was 100%.

Since Mg^{2+} is an important cofactor for myokinase, we investigated the stability of ADP-glucose in the presence of Mg^{2+}. Table 1 shows that ADP-glucose is hydrolysed in the presence of increasing $MgCl_2$-concentrations, implying that the Mg^{2+} concentration has to be minimized during synthesis. Fig. 2 demonstrates that a high concentration of ADP was already obtained after a short incubation time (5 min) either with a $MgCl_2$-concentration of 0.5 or 8 mM. We concluded that the reaction rate of myokinase is not affected by a low $MgCl_2$-concentration. Also, a low Mg^{2+}-concentration is favourable for SuSy, since a 4 × higher concentration of ADP-glucose has been obtained in the presence of 0.5 mM Mg^{2+} compared to 8 mM $MgCl_2$ (Fig. 2). Further experiments show that the $MgCl_2$-concentration could finally be minimized to 0.125 mM.

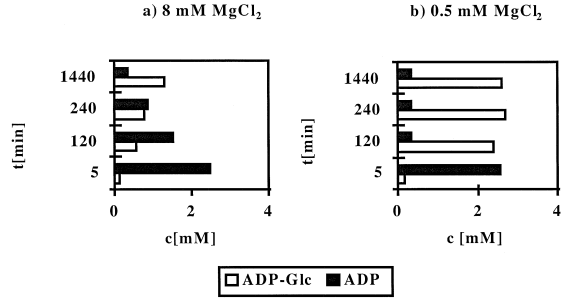

Fig. 2. Synthesis of ADP and ADP-glucose in the presence of different Mg^{2+}-concentrations.

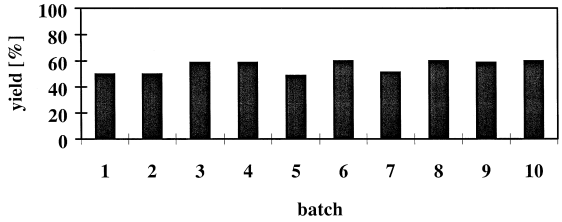

Fig. 3. Synthesis of ADP-glucose from AMP, ATP and sucrose using the repetitive-batch-technique (10 batches).

In order to obtain high enzyme productivities (mg ADP-glucose/U enzyme) we used the repetitive-batch-technique for the large-scale synthesis. Fig. 3 illustrates the high stability of all enzymes by a constant yield over 10 batches. With 20 U myokinase and 100 U SuSy, 2.8 g ADP-glucose was synthesized in 10 batches with an average yield of 55% referring to AMP and ATP.

In comparison to our previously published method [4], we have increased the enzymatic synthesis by 15% in the present study (Table 2). Several reasons account for the improved synthesis: (i) use of a recombinant SuSy from potato with a favourable acceptance of ADP, (ii) increase of the reaction rate of SuSy, as well as (iii) minimization of the hydrolysis of ADP-glucose by decreasing the Mg^{2+}-concentration, respectively.

Table 2
Comparison of ADP-glucose synthesis

Step	Ref. [4]	This paper
Yield synthesis	39%	55%
Yield product isolation	27%	79%
Overall yield	10%	44%
Isolated product	63 mg	2201 mg

Table 3
Isolation of ADP-glucose

Step	Yield (%)	Amount ADP-Glc (mg)
Synthesis	100	2804
Cleavage of nucleotides with alk. phosphatase	92.7	2599
Anion-exchange column	89.8	2517
Desalting	80.4	2254
Lyophilisation	78.5	2201

ADP-glucose was isolated in three steps yielding 2.2 g (79.5%) (Table 3). The yield of the product isolation was increased approx. 3 × (Table 2) because we have stabilized ADP-glucose during the anion-exchange chromatography by adjusting the pH of the eluting solutions to pH 2.0. Furthermore, we have found that the Na-salt of ADP-glucose is more stable during the concentration and lyophilization steps than the Li-salt. In conclusion, a 4-times higher overall yield was obtained compared to our previously described method (Table 2).

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