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Enzymatic synthesis of UDP-galactose on a gram scale

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Abstract

Uridine 5′-diphospho-α-D-galactose (UDP-Gal) was synthesized on a gram scale from uridine 5′-diphospho-α-D-glucose and α-D-galactose 1-phosphate using the enzymes galactose-1-phosphate uridyltransferase (EC 2.7.7.12), phosphoglucomutase (EC 2.7.5.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.27). The synthesis was performed in a repetitive batch mode in which the enzymes, some of which are expensive, were used in 16 subsequent batches without any loss of enzyme activity. The space time yield of the synthesis was 7.1 g/l d. The overall yield of the synthesis amounted to 40% and 1.1 gram of pure UDP-Gal was obtained.

Keywords: Enzymatic synthesis; UDP-galactose; Nucleotide sugars; Repetitive batch

1. Introduction

Uridine 5′-diphospho-α-D-galactose (UDP-Gal) is one of the nucleotide sugars that are most relevant for glycoconjugate biosynthesis in mammals. These activated sugars are the substrates of the Leloir glycosyltransferases, that are used in the enzymatic syntheses of oligosaccharides and glycoconjugates [1]. For these synthetic applications, large amounts of activated sugars are needed. UDP-Gal was synthesized previously on a small scale [2]. This synthesis yielded a mixture of uridine 5′-diphospho-α-D-glucose (UDP-Glc) (16%) and UDP-Gal. Therefore, UDP-Glc had to be converted to uridine 5′-diphospho-α-D-glucuronic acid in an additional enzymatic reaction. Furthermore, the purification procedure was not suitable for large scale production of UDP-Gal. Heidlas et al. [3] synthesized UDP-Gal on a gram scale yielding a product that contained 10% of uridine 5′-monophosphate after purification. UDP-Gal was also synthesized with permeabilized cells as bioreactors [4]. We here report on a synthesis suitable for large scale that yields a high amount of pure UDP-Gal.

2. Experimental

2.1. Materials

Phosphoglucomutase, glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* and α-D-glucose 1,6-bisphosphate were purchased from Boehringer (Mannheim, Ger-

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many). D,L-dithiothreitol, UDP-Gal (sodium salt), UDP-Glc (sodium salt), α-D-galactose 1-phosphate (sodium salt) and galactose-1-phosphate uridyltransferase from yeast were from Sigma (Deisenhofen, Germany). All other chemicals were from Merck (Darmstadt, Germany).

2.2. Analytical methods

The synthesis of UDP-Gal was followed by high-performance liquid chromatography (HPLC) [5]. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AM-400 spectrometer.

2.3. Synthesis of UDP-Gal

The enzymatic synthesis of UDP-Gal was performed with the repetitive batch technique. A buffer solution of 100 ml 50 mM Tris–acetate (pH 7.7) containing 2.1 mM UDP-Glc (a), 2.8 mM α-D-galactose 1-phosphate (b), 2.5 mM nicotinamide adenine dinucleotide (NAD) (c), 500 mM sucrose (d), 0.002 mM α-D-glucose 1,6-bisphosphate (e), 2 mM D,L-dithiothreitol (f), 2 mM $MgSO_4$ (g), 100 mg BSA, 10 U galactose-1-phosphate uridyltransferase, 40 U phosphoglucomutase, 40 U glucose-6-phosphate dehydrogenase was prepared and stirred at an incubation temperature of 30°C. After an incubation time of 2 h, the conversion was complete. The reaction mixture was transferred into a stirred ultrafiltration cell (model 8050, equipped with a YM 10 membrane, Amicon (Witten, Germany)). The filtrate was collected and stored at −20°C. The 10 ml of retentate, containing the enzymes and BSA were used for the next batch. Substrates (a)–(g) were added in the corresponding volume of buffer to readjust the initial concentrations and volume. The resulting solution was subsequently treated like the first batch. In summary, 16 batches yielded 1600 ml of combined filtrates containing 3.36 mmol UDP-Gal. The product solution was applied to a column of an anion exchange resin, (Dowex 1*2 in Cl^--form, 100–200 mesh, ∅ 5 cm, length: 21 cm Serva (Heidelberg, Germany)) at a flow rate of 5 ml/min, which had been equilibrated with 3 l of 10 mM Tris–acetate, pH 7.0 (buffer A). The column was washed with buffer A until the conductivity of the eluate reached the value measured before the application of the sample. The product was eluted with a linear NaCl-gradient (0–1 M, volume: 4 l) at a NaCl-concentration of 0.25 M and a flow rate of 10 ml/min. The fractions containing UDP-Gal were combined and concentrated at reduced pressure to a volume of 50 ml. The concentration of NaCl in the product solution was reduced by the addition of one volume ethanol. The sample was further desalted by passing through a gel filtration column (Sephadex G 10, ∅ 2.6 cm, length: 93 cm, Pharmacia (Uppsala, Sweden)) using water as eluent at a flow rate of 1 ml/min. Lyophilisation yielded UDP-Gal (disodium salt, 1.1 g, 40% overall yield, HPLC-purity: 99%), indistinguishable from authentic material by ^{1}H and ^{13}C NMR spectroscopy.

3. Results and discussion

We used a straightforward synthesis that was established by Anderson et al. [2] (Fig. 1). Galactose-1-phosphate uridyltransferase catalyzed the transfer of uridine 5′-monophosphate from UDP-Glc to α-D-galactose 1-phosphate (Gal-1-*P*). The yield of the reaction is limited by its unfavourable equilibrium constant (K_{eq} = 1.1) and an additional inhibition of galactose-1-phosphate uridyltransferase by the side product α-D-glucose 1-phosphate (Glc-1-*P*) [6,7]. Therefore, Glc-1-*P* was converted to D-gluconate 6-phosphate by the enzymes phosphoglucomutase and glucose-6-phosphate dehydrogenase. By means of these additional reactions and a slight excess of Gal-1-*P*, the equilibrium of the galactose-1-phosphate uridyltransferase-catalyzed reaction was completely shifted to the product side. The key enzyme galactose-1-phosphate

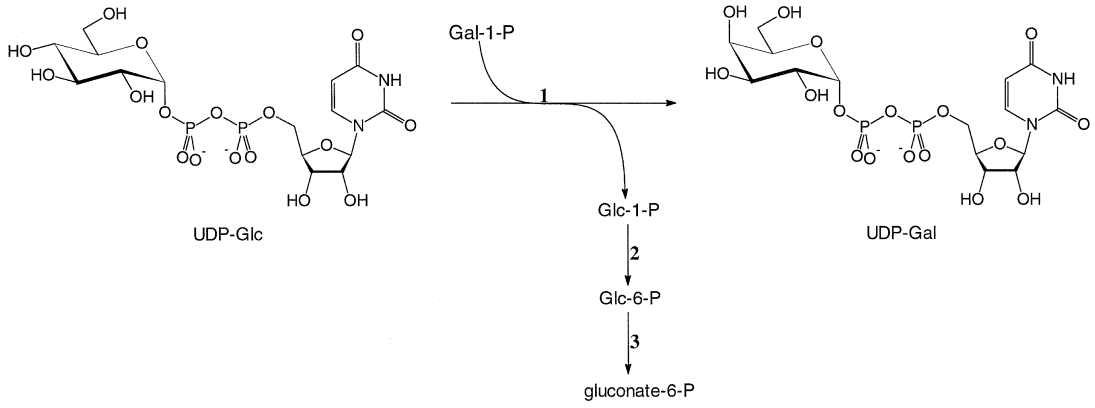

Fig. 1. Synthesis of UDP-Gal from UDP-Glc and Gal-1-*P*. **1**: Galactose-1-phosphate uridyltransferase (EC 2.7.7.12), **2**: phosphoglucomutase (EC 2.7.5.1), **3**: glucose-6-phosphate dehydrogenase (EC 1.1.1.49).

uridyltransferase is an expensive catalyst representing the main cost factor of the batch synthesis. We made use of the repetitive batch technique [8] to maximize the enzyme productivity. At the end of each batch, the product was separated from the enzymes by ultrafiltration. The next batch was started by the addition of new substrate solution. In this way, 16 batches were performed in 37 h (Fig. 2). Sucrose was added to the reaction mixture in order to stabilize the enzymes during incubation [9,10]. The yield of the synthesis was 75% referring to Gal-1-*P* (Fig. 2). The 16 batches yielded 3.36 mmol UDP-Gal. The conversion of UDP-Glc to UDP-Gal was quantitative in all batches after 2 h of incubation, indicating no loss in enzyme activity. The space time yield of the synthesis was 7.1 g/l d. The product solution was purified by anion exchange chromatography, ethanol precipitation of NaCl and gel filtration (Table 1). The overall yield of the synthesis was 40% and 1.1 gram of pure UDP-Gal were obtained. The space time yield of the synthesis was more than four times higher than in the gram scale synthesis published previously (1.5 g/l d) [3]. The enzyme productivity reached 111 mg/U (galactose-1-phosphate uridyltransferase) and this value could be increased easily by running more repetitive batches. We demonstrated previously that the synthesis of nucleotide sugars with purified enzymes is an effective alternative compared to biotransformation using whole cells

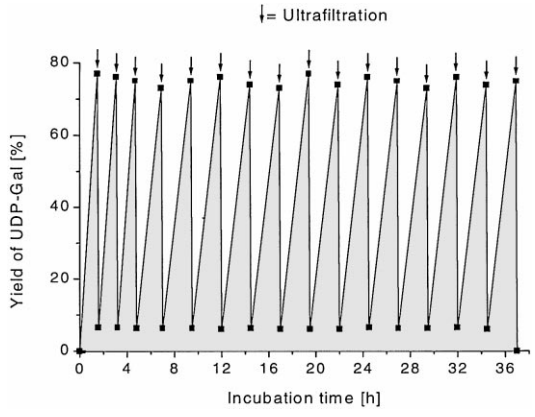

Fig. 2. Time-course of the repetitive batch synthesis. Sixteen batches were performed in 37 h. Yield refers to the concentration of Gal-1-*P*.

Table 1
Yields of synthesis and isolation of UDP-Gal

Step	Amount (g)	Purity (HPLC) (%)	Yield (%)
Synthesis	2.05	72	75
Anion exchange chromatography	1.6	89	59
Ethanol precipitation	1.4	89	52
Gel filtration	1.1	99	40

[10,11]. The repetitive use of all enzymes, the stabilizing conditions and the ease of product isolation contribute to the reduction of production costs [12]. With whole cells, higher space time yields are possible (46 g/l d) [4]. However, the efficient isolation of UDP-Gal out of the cell broth still remains to be demonstrated and compared to the isolation out of a defined product solution, as demonstrated herein.

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