THE FORMATION OF UDP-L-RHAMNOSE FROM UDP-D-GLUCOSE BY AN ENZYME PREPARATION OF RED CAMPION (*SILENE DIOICA* (L) CLAIRV) LEAVES

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1. Introduction

In microorganisms at least three enzymes are involved in the conversion of TDP-D-glucose to TDP-L-rhamnose [1,2]. The formation of TDP-4-keto-6-deoxy-D-glucose, the first demonstrable intermediate, is catalyzed by TDP-D-glucose-4,6-hydrolyase (EC 4.2.1.43). This enzyme [3,4], which consists of two subunits firmly bound by one molecule NAD^+, initially attacks TDP-D-glucose at C-4 to yield TDP-4-keto-D-glucose and enzyme-NADH. This TDP-4-keto-D-glucose then rearranges by β-elimination of water between C-5 and C-6 to form an unsaturated glucoseen, which serves as hydrogen acceptor for the enzyme-NADH, and leads to the intermediate TDP-4-keto-6-deoxy-D-glucose.

Possibly via the ene-diol form, TDP-4-keto-6-deoxy-D-glucose; 3,5-epimerase catalyzes the epimerizations at C-3 and C-5. The epimerizations are followed by a stereospecific reduction by NADPH: TDP-6-deoxy-L-lyxo-4-hexulose; 4-reductase. The 3,5-epimerase and 4-reductase are sometimes considered to be one single enzyme: TDP-L-rhamnose synthetase (see also fig.1) [5].

In plants the conversion of TDP-D-glucose to TDP-L-rhamnose proceeds with a very low efficiency [6,7]. With UDP-D-glucose, however, the formation of UDP-L-rhamnose takes place at an appreciable higher rate [7]. It is not known whether in plants the synthesis proceeds according to the same reaction mechanism as in microorganisms.

We here want to describe the conversion of UDP-D-glucose to UDP-L-rhamnose in *Silene dioica* and demonstrate that UDP-4-keto-6-deoxy-D-glucose is formed in the interconversion of the sugar nucleotide, suggesting that in plants the same pathway is operating as in microorganisms, but that uridine instead of thymidine is used as nucleoside.

2. Experimental

2.1. *Enzyme purification*

All operations were performed in the cold (4°C). Leaves 100 g were homogenized with an Ultra-turrax homogenizer in 200 ml of 50 mM β-mercaptoethanol, 0.1% Triton X-100, 5% PVP, 150 mM Tris–HCl buffer (pH 8.0). The homogenate was squeezed through two layers of cheese-cloth, centrifuged at 38 000 × *g* for 20 min. The supernatant solution was treated with 0.05 vol. 1 M $MnCl_2$, stirred for 15 min, and the precipitate removed by centrifugation. An equal volume of saturated ammonium sulfate solution (pH 7.5) was added slowly with stirring to the supernatant. After centrifugation the precipitate was suspended in 5 ml 10 mM β-mercaptoethanol, 25 mM Tris–HCl buffer, pH 8.0. The protein solution was passed through a column of Sephadex G-25/PVP (two equal layers, 2.5 × 15 cm). The protein eluate was precipitated with an equal volume of saturated ammonium sulfate solution, and the precipitate formed after centrifugation was dissolved in 1 ml same buffer, and dialyzed overnight against 2 litre buffer. Unless otherwise stated, this preparation, with a protein content of 60–120 mg/ml was used in the studies here described.

2.2. *UDP-D-glucose-4,6-hydrolyase assay*

The enzyme was assayed by measuring the increase in A_{314}, a characteristic A_{max} for UDP-4-keto-6-deoxy-D-glucose at alkaline pH (ϵ_{314} = 4800). The assay mixture contained in 50 μl: 80 nmoles β-mercaptoethanol, 5 μmol Tris–HCl (pH 8.0), 125 nmol UDP-D-glucose and 40 μl enzyme. The reaction was stopped after 15 min incubation at 38°C by the addition of 600 μl 0.1 N NaOH. The mixture was kept at 38°C for 20 min according to [8]. Blancs were obtained by addition of sodium hydroxide at zero time. The blancs were kept in ice and placed at 38°C before measuring the absorbance. Protein was determined according to [9].

2.3. *Synthesis of UDP-4-keto-6-deoxy-D-[U-^{14}C]-glucose*

Incubation mixtures contained in total vol. 200 μl: 0.1 μmol β-mercaptoethanol, 0.6 μmol Tris–HCl (pH 8.0), 0.1 μmol UDP-D-[U-^{14}C]glucose (about 1 Ci/mol) and 190 μl enzyme. After incubation for 4 h at 38°C the reaction was terminated by the addition of 600 μl ethanol. Ten individual incubations were combined and the pH adjusted to pH 4.0–5.0. The protein was removed by centrifugation and a 5% suspension of Darco (grade K-B) charcoal in H_2O added to the supernatant (0.1 ml/0.25 μmol nucleotide) [10]. After standing for 5–10 min, the charcoal was removed by centrifugation, and washed once with 0.001 N HCl. Then the nucleotide was eluted with 50% ethanol–water containing 0.015 N NH_4OH. Two elutions of 20–30 min each were employed. The eluate was concentrated and purified by paper chromatography on Whatman I paper in the solvent system isobutyric acid–1 N ammonia (6:4, v/v). The portion of the chromatogram bearing the UDP-4-keto-6-deoxy-D-glucose was washed with acetone to remove the isobutyric acid and subsequently the product was eluted with water.

2.4. *UDP-L-rhamnose synthetase assay*

The reaction mixture contained in total vol. 50 μl: 80 nmol β-mercaptoethanol, 5 μmol Tris–HCl (pH 8.0), 1 nmol UDP-4-keto-6-deoxy-D-[U-^{14}C]-glucose, 10 nmol NADPH and 40 μl enzyme. After 30 min incubation at 38°C the reaction was stopped by the addition of equal vol. trichloroacetic acid in methanol. The rhamnose was liberated from the nucleotide by hydrolysis in 0.5 N HCl at 100°C for 20 min. The hydrolyzate together with carrier rhamnose was applied as a spot on Whatman III paper and developed two-dimensionally in the solvents *n*-butanol–acetic acid–water (4:1:5, v/v/v) and *n*-propanol–ethylacetate–water (7:1:2, v/v/v). The rhamnose was made visible by spraying with a solution of 2.58 g aniline hydrogenphtalate in 100 ml *n*-butanol saturated with water and heated for 5 min at 110°C [11]. The portion of the chromatogram bearing the rhamnose was cut out, and counted in a Packard Tricarb scintillation spectrophotometer. For the determination of the zero time control, trichloroacetic acid was added to the reaction mixture before incubation. The radioactivity found at the rhamnose spot in this case was about 30 cpm.

2.5. *Assay for the overall formation of UDP-L-rhamnose from UDP-D-glucose*

The assay mixture contained in 40 μl: 50 nmol β-mercaptoethanol, 0.3 μmol Tris–HCl (pH 8.0), 25 nmol UDP-D-[U-^{14}C]glucose (1 Ci/mol), 50 nmol NADPH and 25 μl leaf protein extract. The mixture was incubated for 45 min at 38°C. The reaction was stopped with 5% trichloroacetic acid in methanol. The mixture was treated as above.

3. Results and discussion

Petals of *S. dioica* plants, with a dominant allele of gene *N*, contain cyanidin 3-rhamnosylglucoside. Incubation of an anthocyanin free protein preparation of petals of these plants, with cyanidin 3-glucoside, UDP-D-[U-^{14}C]glucose and NADPH lead to the formation of cyanidin 3-rhamnosylglucoside. Hydrolysis and isolation of the product formed, revealed that the label was only present in the rhamnosyl moiety. In a protein preparation of petals of homozygous recessive *n/n* plants, which contain cyanidin 3-glucoside, this reaction could not be demonstrated. These findings suggest that gene *N* either is involved in the formation of UDP-L-rhamnose from UDP-D-glucose or that this reaction takes place in both genotypes but that the transfer of rhamnose from UDP-L-rhamnose to cyanidin 3-glucoside is governed by gene *N*. To explain this discrepancy in properties between *N/N* and *n/n* protein preparations we studied

the formation of UDP-L-rhamnose in these genotypes.

Both in *N/·* and *n/n* petal- or leaf-protein extract the formation of UDP-4-keto-6-deoxy-D-glucose could be followed by measuring the increase in A_{314} in alkali after incubation [8]. The enzyme UDP-D-glucose-4,6-hydrolyase (UDP-D-glucose oxidoreductase) which catalyzes this formation was rather stable and had an optimum pH 8.1, with half maximum velocities at pH 6.2 and pH 9.8. The amount of UDP-4-keto-6-deoxy-D-glucose formed from UDP-D-glucose was found to be proportional to time for periods up to 30 minutes and to added enzyme. The K_m for UDP-D-glucose was 3.5×10^{-2} mM. NAD or NADH did not stimulate the reaction rate. *p*-Chloromercuribenzoate (2 mM) inhibited the reaction completely.

However, when NADPH was added to the assay system, the preparation converts UDP-D-glucose to UDP-L-rhamnose. This overall reaction was followed by using UDP-D-[U-^{14}C]glucose as substrate and counting the radioactivity in the rhamnose formed after hydrolysis of the reaction mixture and purification of the labelled rhamnose liberated.

The conversion of UDP-D-glucose to UDP-L-rhamnose was (after a lag period of 9 min) proportional to time for periods up to 40 min and to added enzyme. There was no activity in the absence of NADPH. Neither ATP, NADH, EDTA nor divalent metal ions did stimulate the reaction rate.

From UDP-4-keto-6-deoxy-D-glucose the reaction took place without a lag period, was also NADPH dependant, and was not influenced by NADH. This shows that UDP-4-keto-6-deoxy-D-glucose is an

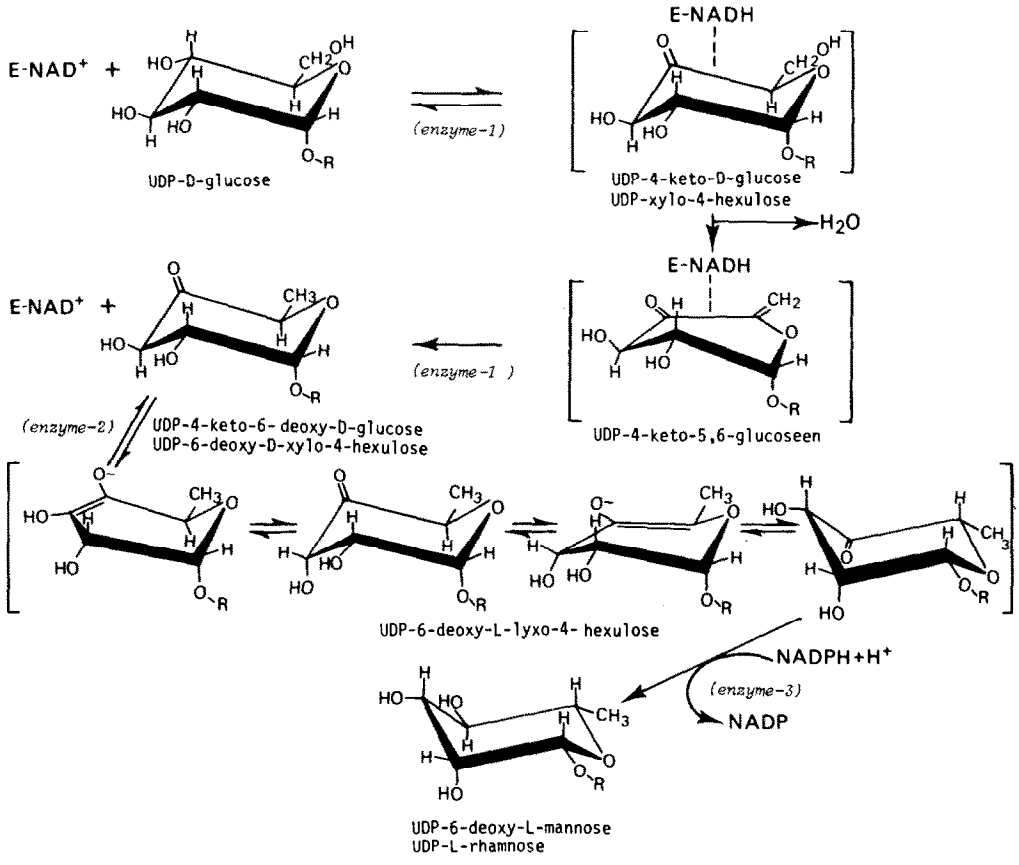

Fig.1. Hypothetical pathway for the biosynthesis of UDP-L-rhamnose from UDP-D-glucose. (*enzyme - 1* = UDP-D-glucose: 4,6-hydrolyase, *2* = UDP-4-keto-6-deoxy-D-glucose; 3,5-epimerase, *3* = NADPH: UDP-6-deoxy-L-lyxo-4-hexulose, 4-reductase.)

intermediate in this conversion. The K_m values for UDP-L-rhamnose synthetase are, respectively, for UDP-4-keto-6-deoxy-D-glucose, 0.01 mM and for NADPH 0.07 mM. We therefore propose that the synthesis of UDP-L-rhamnose in *S. dioica* proceeds analogous to the synthesis of TDP-L-rhamnose in microorganisms (fig.1).

The conversion of UDP-D-glucose to UDP-L-rhamnose via the intermediate, UDP-4-keto-6-deoxy-D-glucose proceeds both in *N/·* and *n/n S. dioica* plants. Therefore gene *N* is not involved in the synthesis of UDP-L-rhamnose, but probably governs the production of the enzyme, UDP-L-rhamnose: anthocyanidin 3-*O*-glucoside, 6″-*O*-rhamnosyltransferase, which catalyzes the transfer of rhamnose from UDP-L-rhamnose to the 3-*O*-bound glucose of cyanidine 3-*O*-glucoside.

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