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UDP-GALACTOSE 4′-EPIMERASE FROM *VICIA FABA* SEEDS

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Key Word Index—*Vicia faba*; Leguminosae; broad bean; galactose metabolism; UDP-galactose 4′-epimerase.

Abstract—UDP-Galactose 4′-epimerase was purified *ca* 800-fold through a multi-step procedure which included affinity chromatography using NAD^+-Agarose. Three forms of the enzyme were separated by gel-filtration but only the major form was purified. The pH optimum of the enzyme was 9.5. Exogenous NAD^+ was not required for enzymic activity but its removal caused inactivation. The enzyme was unstable below pH 7.0 but stable at pH 8.0 in the presence of glycerol and at −20° for two months. The equilibrium constant for the enzyme-catalysed reaction was 3.2 ± 0.15. The K_m for UDP-galactose and UDP-glucose were 0.12 mM and 0.25 mM, respectively. The inhibition by NADH was competitive, with a K_i of 5 μM. The MW of the enzyme was 78 000; the two minor forms showed the values of 158 000 and 39 000, respectively.

INTRODUCTION

UDP-Galactose 4′-epimerase (EC 5.1.3.2) is one of the important enzymes of galactose metabolism catalysing the interconversion of UDP-galactose and UDP-glucose. Since the discovery of this enzyme by Leloir [1] from D-galactose grown *Saccharomyces fragilis*, it has been isolated from a number of microbial, plant and animal sources [2–4]. The microbial enzymes have been most extensively studied [5–12]. Detailed description of the mechanism of action has been presented in recent reviews [3, 4].

The presence of this enzyme may be predicted in most plant organs that involve galactose metabolism, especially in seeds where galactose-containing oligosaccharides (raffinose-family) or polysaccharides (galactomannans) serve as storage carbohydrates [13, 14]. These carbohydrates are mobilized during seed germination; however, free galactose is rarely detected [14, 15]. It is believed that galactose is rapidly metabolized [14, 16]. Although it may be realised that UDP-galactose 4′-epimerase plays a key role in galactose metabolism, only few enzymes from plant sources have been thus far studied [17–23]. As from seeds, the only example of isolation and purification is that from fenugreek [23]. A potential pathway of metabolism of galactose involving UDP-galactose 4′-epimerase has been studied in cucumber plant [24, 25]. The members of the raffinose family of oligosaccharides constitute important translocates in this plant [26]. The generation of UDP-glucose in plants probably plays an important role as a 'branch-point' metabolite, for example, forming glucose-1-phosphate for energy needs, in the formation of transportable sucrose and in cell-wall synthesis in developing seedlings. As a prelude to understanding more fully the metabolism of galactose-containing oligosaccharides in *V. faba* [15] and determining the roles of various controlling enzymes (e.g. galactokinase [16]), we have now for the first time isolated and studied UDP-galactose 4′-epimerase from this source.

RESULTS AND DISCUSSION

Isolation and purification

It was found that the presence of 10% glycerol (v/v) in the extracting buffer resulted in maximum enzyme units in the crude extract. The inclusion of NaCl (0.1 M) in the extracting medium did not significantly increase the total enzyme units isolated; however, additional proteins were extracted giving a lower specific activity value. Thus, NaCl was excluded from the extraction procedure. The specific activity of the enzyme extract was nearly 20-fold higher than that of wheat germ extract [17]. As a first step of the purification procedure, nucleic acids were removed by precipitation with $MnSO_4$ (final concentration, 30 mM). Some proteins seemed to have coprecipitated at this step giving ~2-fold purification with 97% recovery of the enzyme. The clear supernatant was then subjected to $(NH_4)_2SO_4$ fractionation and the 30–55% fraction, after dialysis against the extraction buffer, showed an overall purification of 18-fold with a 70% recovery. This step was also useful in obtaining a concentrated enzyme preparation. The enzyme solution was then carried through acetone fractionation at −10° and the protein fraction obtained at 30–60% acetone (v/v) was found to possess 36% of the initial activity. The enzyme was enriched by about 66-fold. Temperature control and rapidity of both acetone mixing and centrifugation (3 min at 20 000 *g*) was crucial in obtaining a good recovery of the enzyme. It was also important to readily dissolve the acetone precipitated protein in water followed by dialysis against the enzyme extraction buffer.

The acetone fractionated enzyme when subjected to column chromatography using Sephacryl S-200 gave a major and two minor peaks of activity (Fig. 1A). The peaks were well within the resolving range of the column bed. The fractions containing only the major peak were pooled and concentrated by ultrafiltration. About 85% of the activity was recovered in this peak accompanied by *ca*

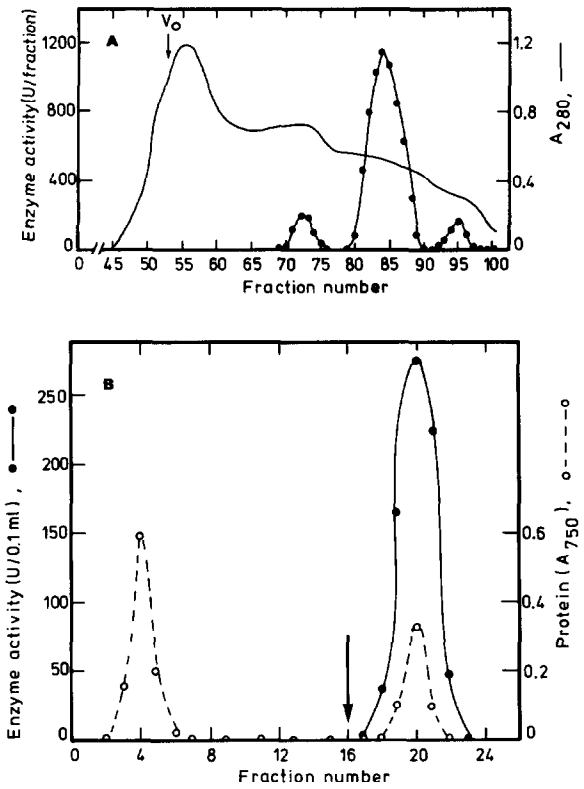

Fig. 1. (A) Gel-filtration of *V. faba* UDP-galactose 4'-epimerase preparation using Sephacryl S-200. The column (2.5 cm × 95 cm) was equilibrated with 0.05 M Tris–HCl buffer, pH 8 containing 10% glycerol (v/v) and 4 ml enzyme sample (step 4, Table 1) was applied, followed by elution with the same buffer; 3 ml fractions were collected. The void volume is expressed as V_0 and shown by the arrow. (B) Affinity chromatography of *V. faba* UDP-galactose 4'-epimerase. A column (1 × 5 cm) was prepared with NAD^+-agarose using 0.05 M Tris–HCl buffer, pH 8 containing 10% glycerol (v/v) and the enzyme (3 ml; step 5 of Table 1) was applied followed by elution with the same buffer. Fractions (2 ml) were collected until the washings were free of proteins. The enzyme was eluted (arrow) with 0.1 M NAD^+ solution made in the above buffer. A portion (0.5 ml) of each fraction was used for protein measurements [31] and expressed as absorbance of the developed colour at 750 nm.

10-fold enrichment. This is the first demonstration of the multiple forms of UDP-galactose 4'-epimerase (see Fig. 1A) from a plant source. Separation of two forms of the enzyme from goat-liver using a DEAE-cellulose column was earlier reported [8]. Attempts were made to further purify the *V. faba* enzyme by affinity chromatography using NAD^+-agarose; however, the enzyme was eluted unretarded. It was observed that the enzyme was able to catalyse epimerization without the need of exogenous NAD^+ indicating the presence of the cofactor in a bound form. The bound NAD^+ was therefore, removed by treatment with activated carbon as described by Fan and Feingold [17]. It was noted that the enzyme, after this treatment, was retarded by the affinity column. The active enzyme was recovered by eluting with buffered NAD^+ (Fig. 1B). The specific activity of the dialysed enzyme increased only slightly at this step. The protein removed from the enzyme preparation was nearly 50%; however, the accompanying loss (*ca* 32%) of enzyme activity was probably the cause of the observed small enhancement of specific activity.

A summary of the purification is presented in Table 1. The overall purification achieved was *ca* 800-fold with a 21% recovery. A purification of 1500-fold was reported for the wheat germ enzyme [17], but the final specific activity was one-tenth compared to *V. faba* enzyme. However, *V. faba* seeds are a better source of UDP-galactose 4'-epimerase and the specific activity of the crude extract is much higher (*ca* 20-fold) than the wheat germ extract.

Properties

The homogeneity of the enzyme from the final purification step was checked by disc gel electrophoresis; a main protein band was observed which corresponded with the enzyme activity. However, there were four slower migrating minor protein bands but no enzyme activity was associated with these. The enzyme was extracted (employing the buffer used for seed extraction) from the gel section corresponding to the main protein band. This preparation showed a specific activity of 660 units/mg protein. The low value compared to the enzyme applied (7900 units/mg protein) was presumably due to inactivation of the enzyme during the electrophoretic run.

The pH optimum of the enzyme was found to be 9.5; however, there was still nearly 25% of the optimal activity

Table 1. Purification of UDP-galactose 4'-epimerase from *Vicia faba* seeds*

Purification step	Volume (ml)	Protein (mg/ml)	Activity (units/ml)	Specific activity (units/mg protein) $\times 10^{-2}$	Recovery (%)	Purification factor (fold)
1. Crude extract	41	158	1650	0.10	—	—
2. $MnSO_4$ treatment	43	79	1520	0.19	97	1.8
3. $(NH_4)SO_4$ fractionation	16	16	2940	1.84	70	18.4
4. Acetone precipitation	12	3.1	2050	6.61	36	66.1
5. Sephacryl S-200	3	1.2	6970	73.33	31	733.3
6. NAD-Agarose	1	1.8	14218	79.00	21	790.0

*50 g of seed powder was taken for extraction; the recovery and the purification factor were calculated with respect to the values of the crude extract.

at pH 7.6. As the one-step enzyme assay involved measurement of UDP-glucose by using UDP-glucose dehydrogenase (pH optimum, 8.7), this continuous recording method could not be employed for the pH optimum experiments. The two-step method was, therefore, used and the pH of the assay mixture was adjusted to the optimal pH of the dehydrogenase in the second step. The pH optima of the wheat germ and the fenugreek enzymes were reported to be 9.0 and 8.5, respectively [17, 23].

The enzyme was found to lose activity on storage at 4° if glycerol was excluded from the buffer. At pH 7 or below, there were large losses of activity (50–75%) in 48 hr at 4° even in the presence of glycerol (10%, v/v). However, the enzyme could be stored at −20° for two months in a buffer of pH 8 containing glycerol. The requirement of NAD^+ for the activity of the enzyme is summarised in Table 2. The crude enzyme extract and the purified enzyme from the final purification step (see Table 1) were employed for the experiments. There was very little increase in eyzymic activity on addition of NAD^+ which shows that the cofactor is already present in the enzyme, probably in a bound form. However, charcoal-treatment of both enzyme preparations rendered them totally inactive towards action on UDP-galactose. The addition of exogenous NAD^+ at 10 μM to these inactive enzyme solutions caused regain of nearly 66–68% of the activity with full activity being restored at 100 μM or above this concentration. These experiments also explain the observed binding of the charcoal-treated (but not the untreated) enzyme to the NAD^+-agarose column. The affinity-purified enzyme showed nearly full activity without any addition of NAD^+ in the assay and this was presumably because the enzyme became saturated with the cofactor during its elution with NAD^+.

Table 2. The requirement of NAD^+ by *Vicia faba* UDP-galactose 4'-epimerase for its activity

Enzyme preparation	$(NAD^+) \times 10^4$ (M)	UDP-Glucose formed (n mol)
Crude extract	0	30.3
	0.1	35.6
	1.0	35.2
	2.5	35.1
Purified enzyme		
(step 6 Table 1)	0	38.4
	0.1	42.3
	1.0	45.8
	2.5	46.0
Charcoal-treated enzyme		
Crude extract	0	0
	0.1	19.3
	1.0	29.5
	2.5	29.1
Purified enzyme		
(step 6 Table 1)	0	0
	0.1	26.4
	1.0	38.2
	2.5	38.0

The two-step procedure (in the absence of NAD^+) was followed for each assay (see Experimental) using an appropriate amount of enzyme that fell in the linear time-course of the reaction. When NAD^+ was incorporated in the assay (concentrations shown), a 10 min preincubation was allowed before the addition of substrate. UDP-Glucose formed was estimated. The charcoal-treatment was carried out according to Fan and Feingold [17].

The equilibrium constant for the reaction, UDP-galactose ⇌ UDP-glucose, catalysed by the enzyme was determined under the conditions of the assay. Both UDP-[^{14}C]galactose and UDP-[^{14}C]glucose were employed in separate experiments for determining the constant from either direction. The enzyme from the final purification step (Table 1) was used and duplicate incubations for 4 hr and 8 hr were set-up using each substrate. The reactions were stopped by dipping test tubes in a boiling water-bath and the constituent sugars were analysed by TLC [27] after HCl-hydrolysis and neutralization. Following the separation of galactose and glucose, the respective zones were scraped out and radioactivity measured. The equilibrium constant was found to be 3.2 ± 0.15. The values reported for the enzymes from wheat germ and *E. coli* were 3.1 and 3.5, respectively [9, 17].

The effect of concentrations of UDP-galactose and UDP-glucose (in the range of 25–1000 μM) on the enzyme was examined under the assay conditions. Determination of the products by the two-step assay procedure and plotting the results according to Lineweaver and Burk [28] gave K_m values of 0.12 mM and 0.25 mM for UDP-galactose and UDP-glucose, respectively. The enzyme was found to be strongly inhibited by NADH with a competitive K_i of 5 μM. Under the normal enzyme assay conditions the amount of NADH generated will be well below the inhibiting concentration. In this regard the *in vivo* concentration ratio of NAD^+/NADH is likely to play a controlling role in the interconvertion of UDP-galactose and UDP-glucose.

The MW of the enzyme was found to be 78 000 as determined according to Andrews [29] by calibrating the gel filtration column using proteins of known MWs. The two minor peaks of the enzyme (Fig. 1A) showed M_r values of 159 000 and 39 800, respectively. These forms were rather unstable, thus their kinetic properties could not be studied. The MW of the wheat germ enzyme was reported to be nearly 100 000 [17]. The enzymes from *E. coli* and fenugreek seeds showed M_r values of 79 000 and 70 000, respectively [10, 23]. The former was a dimeric protein constituted of two identical sub-units.

EXPERIMENTAL

Mature and dry *Vicia faba* seeds (*var.* Bunyard Exhibition) were obtained locally. Sephadex column chromatography materials were from Pharmacia (Uppsala, Sweden) and the ion exchangers were from Whatman (U.K.). NAD^+-Agarose attached through ribose hydroxyls through a six carbon spacer was from Sigma (London); most other biochemicals and chemicals were also from Sigma. UDP-[^{14}C]galactose and UDP-[^{14}C]glucose (both 200 mCi/mmol) were from Radiochemical Centre (Amersham, U.K.). Galactose and glucose test-combinations were from Boehringer Corporation, London. UDP-Glucose dehydrogenase was prepared from calf-liver acetone-powder by following up-to purification step 4 of a method described earlier [30].

The extraction and purification of the enzyme were carried out at 4°. Testa-free seeds were finely powdered in a mechanical grinder and 50 g of the powder was stirred for 30 min in 0.05 M

Tris–HCl buffer, pH 8 containing 10% glycerol (v/v). The clear extract was obtained by centrifuging the slurry at 15000 *g* for 20 min. Two methods were employed for the assay of UDP-galactose 4′-epimerase: (a) 1 ml of the reaction mixture in a cuvette at 30° contained 640 μl H_2O, 100 μl glycine buffer (1 M, pH 9.5), 100 μl NAD^+ (0.01 M), 100 μl of the enzyme preparation and 50 μl of UDP-glucose dehydrogenase (400 units/ml). The *A* at 340 nm was monitored continually in a recording spectrophotometer until no change in *A* was visible (this took generally 5 min). The reaction was then started by adding 10 μl UDP-galactose (0.03 M) and the time course of *A* change was recorded; (b) in this two-step method the reaction mixture contained the buffer, H_2O, NAD^+, UDP-galactose and the 4′-epimerase extract. The reaction was terminated by dipping the test tube in boiling water-bath for 2 min. UDP-glucose formed was then assayed by adding an excess of UDP-glucose dehydrogenase and NAD^+. Alternatively, UDP-glucose and UDP-galactose in the terminated reaction mixture were estimated after hydrolysing with 0.1 M HCl at 100° for 15 min, neutralizing and determining glucose and galactose by using the respective test-combination kit. One unit of enzyme activity is expressed as the amount required to convert one nmol of the substrate per min at 30° under the given conditions. Sp. act. is defined as enzyme units per mg protein. Protein was determined by the method of ref. [31].

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