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IMPROVED METHOD FOR PURIFICATION OF UDP-APIOSE/UDP-XYLOSE SYNTHASE FROM CELL CULTURES OF PARSLEY

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SUMMARY

1. An improved purification procedure is described for the UDPapiose/UDP-xylose synthase from cell suspension cultures of parsley.

2. The enzyme has been purified about 1000-fold by $(NH_4)_2SO_4$ fractionation and chromatography on DEAE-cellulose, hydroxylapatite and ω-aminoalkyl-Sepharose.

3. The ratio of apiose/xylose formed did not change significantly with increasing purification of the enzyme. The enzymatic activities for apiose and xylose synthesis were not separated by disc electrophoresis of the purified enzyme. Besides the enzyme peak, two other protein bands were visible on the disc column.

4. The molecular weight of the enzyme was determined by density gradient centrifugation, according to the method of Martin, R. G. and Ames, B. N. 1961), *J. Biol. Chem.* 236, 1372–1379) to be about 101 000. The enzymatic activities for apiose and xylose synthesis were also not separated in the density gradient centrifugation.

5. From these and previous results it can be concluded that the enzyme is a two-enzyme complex or a multifunctional protein probably with two catalytic sites.

INTRODUCTION

An enzyme catalyzing the synthesis of UDP-D-apiose and UDP-D-xylose from UDP-D-glucuronic acid was purified about 25-fold from *Lemna minor* L.[1] and from cell suspension cultures of parsley (*Petroselinum hortense* Hoffm.)[2]. The enzymatic activities for apiose and xylose synthesis were not separated by the purification procedure, isoelectric focusing or analytical disc electrophoresis[1,2]. The enzyme from parsley still contained some UDParabinose epimerase (EC 5.1.3.5.) (ref. 2). From the previous results it was concluded that the enzyme might be a two-enzyme complex

or a multifunctional protein. To clarify this question further purification of the enzyme was necessary.

In the present paper we report an improved purification procedure for the UDPapiose/UDPxylose synthase from cell suspension cultures of parsley leading to an approximately 1000-fold purification of the enzyme. The molecular weight of the enzyme was re-examined.

MATERIALS AND METHODS

Cell cultures

Cell suspension cultures of *P. hortense* were grown as described previously[3] and illuminated for 25 h with white light before harvesting. Cells were harvested by vacuum filtration through a porous glass filter and were then frozen with liquid N_2.

Enzyme assay

This was carried out as described previously[1].

Purification of the enzyme

Extraction of cells. All steps were carried out at 4 °C. 500 g of frozen cells were thawed in 1.2 l of 0.25 M Tris–HCl buffer (pH 8.0) containing $1 \cdot 10^{-2}$ M 2-mercaptoethanol (Buffer A). The cells were then homogenized in an ice bath for 2 min with an Ultra Turrax (Janke and Kunkel, Type T 45 N, 10 000 rev./min). Homogenization was then stopped for about 1 min in order to keep the temperature below 4 °C. The procedure was repeated for a total homogenization time of 10 min. The homogenate was centrifuged for 20 min at 10 000 × *g*.

Treatment with Dowex. The supernatant was stirred for 20 min with 50 g of Dowex 1X-2 (Cl^- form equilibrated with 0.25 M Tris–HCl, pH 8.0) and the Dowex filtered off through glass wool. The filtrate was centrifuged for 20 min at 10 000 × *g*.

$(NH_4)_2SO_4$-fractionation. The supernatant was subjected to $(NH_4)_2SO_4$ fractionation at pH 8.0 by addition of a saturated solution. Protein precipitated between 40 and 50% saturation was collected by centrifugation at 35 000 × *g* for 10 min and dissolved in Buffer A. The solution was then centrifuged at 10 000 × *g* for 15 min and the clear solution chromatographed on a column (10 ml gel per ml protein solution) of Sephadex G-25 with $2 \cdot 10^{-2}$ M Tris–HCl (pH 8.0) containing $1 \cdot 10^{-3}$ M dithioerythritol.

DEAE-cellulose chromatography. The protein fraction was applied to a DEAE-cellulose column (1.7 cm × 17 cm) pre-equilibrated with the Tris–HCl buffer. The column was first washed with 200 ml of $5 \cdot 10^{-2}$ M Tris–HCl (pH 7.3) containing $5 \cdot 10^{-2}$ M KCl and $1 \cdot 10^{-3}$ M dithioerythritol. Protein was then eluted with a linear KCl gradient in Tris–HCl, pH 7.3 ($5 \cdot 10^{-2}$ M KCl → 0.3 M KCl, 150 ml each) at a flow rate of 15–20 ml/h. The enzyme was eluted between $9 \cdot 10^{-2}$ and $18 \cdot 10^{-2}$ M KCl.

Chromatography on hydroxylapatite. Enzyme (180 ml) was then absorbed on a column (120 ml; 3.6 cm × 18 cm) of hydroxylapatite (Bio Gel HTP, Bio Rad Laboratories, Richmond, Calif.) equilibrated with $1 \cdot 10^{-3}$ M potassium phosphate buffer, pH 6.8. Protein was eluted in four fractions (90 ml each) with a $1 \cdot 10^{-2}$ M concentration of the same buffer at a flow rate of 20–25 ml/h. Enzyme was eluted in Frac-

tions 3 and 4. In contrast to the enzyme in Tris buffer, the enzyme in phosphate buffer cannot be frozen without inactivation.

Chromatography on ω-aminoalkyl-Sepharose. The enzyme was then absorbed on a column (3 ml gel per mg protein) of ω-aminoalkyl-Sepharose and protein eluted stepwise at a flow rate of 50 ml/h with the following solutions (twice the bed volume): (a) 10^{-1} M Tris–HCl, pH 8.2, containing $1 \cdot 10^{-3}$ M dithioerythritol and $1 \cdot 10^{-2}$ M EDTA; (b) $3 \cdot 10^{-2}$ M EDTA and (c) $5 \cdot 10^{-2}$ M EDTA in the Tris buffer with dithioerythritol. The enzyme was eluted in the last fraction.

Disc electrophoresis

The enzyme-containing protein fraction from the Sepharose column (40 ml) was concentrated to 1.3 ml by filtration through a "Diaflo" concentrator (Amicon, Model 50, ultrafiltration cell). The solution was then dialyzed for 16 h against $1 \cdot 10^{-2}$ M Tris–HCl, pH 7.6, containing $5 \cdot 10^{-3}$ M thioglycolic acid and then freeze-dried. The enzyme was dissolved in 300 μl of water.

Disc electrophoresis was carried out in a polyacrylamide gel of middle pore size (7%) at pH 8.8–9.0. The electrode solution contained $2 \cdot 10^{-3}$ M thioglycolic acid. 100 μl of solution containing about 10 μg of protein was applied to the column. After electrophoresis (2 mA per tube) the gel (7.5 cm × 0.4 cm) was cut into 2.5-mm thick discs and the discs were incubated in the enzyme assay with three times the normal substrate concentration for 31 h. Protein was determined in a parallel run with Coomassie brilliant Blue R-250 (ref. 5).

Sucrose density centrifugation[6]

A 5–20% sucrose gradient in 0.05 M Tris–HCl, pH 7.5, was used. 100 μl of enzyme solution with about 20–400 μg protein were layered on the gradient and the tubes were centrifuged in the Beckman SW 40 rotor for 15 or 5 h (with catalase as reference) at 40 000 rev./min. The tubes were then punctured and 32 fractions (five drops each) were collected. Reference proteins were purchased from Boehringer, Mannheim.

ω-Aminoakyl-Sepharose

The substituted Sepharose was prepared according to Cuatrecasas[4] from Sepharose 4 B (Pharmacia, Uppsala) and 3,3′-diaminodipropylamine (Th. Schuchardt, München).

5′-(4-Aminophenyl-pyrophosphoryl)-uridine

1 mmole of UMPmorpholidate[10] was treated with 3 mmoles of *p*-nitrophenylphosphate (Merck AG., Darmstadt) according to the procedure of Moffat and Khorana[11]. The resulting *p*-nitrophenyl ester of UDP was dissolved in dimethylformamide and reduced to the *p*-aminophenyl-derivative with H_2 and Pd on charcoal[12]. *p*-Aminophenyl-UDP was then coupled to succinoyl-ω-aminoalkyl-Sepharose with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Pierce Chemical Co., Rockford, Ill., USA)[4].

NAD–Sepharose

To 5 ml of the succinylated ω-aminoalkyl-Sepharose[4] in 4.5 ml of water, 0.5 mmole of NAD (370 mg) was added and the pH was adjusted to 4.7 with 0.1 M NaOH.

1.6 mmole (310 mg) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide·HCl dissolved in 1.2 ml water was then added and the pH kept for 2 h at 4.7 by addition of $2.5 \cdot 10^{-1}$ M HCl. The solution was then stirred at room temperature for another 20 h. The gel was washed with 2 l water for 3 h. About 8.5 μmoles NAD per ml gel were bound by this method. This was determined by decrease in the absorbance at 259 nm in the solution before and after coupling.

UDP-GlcUA–Sepharose

To 3 ml of the succinylated ω-aminoalkyl-Sepharose[4] in 2.4 ml water, 146 μmoles (90 mg) of UDP-D-glucuronic acid were added and the pH adjusted to 4.75 with 1 M HCl. 520 μmoles (100 mg) of the water soluble carbodiimide were then added and the pH kept at 4.75 for 3 h by addition of 0.1 M HCl. The slurry was stirred for another 24 h at room temperature. The gel was then washed with 800 ml water. About 11 μmoles UDPglucuronic acid per ml Sepharose were bound.

Protein assay

Protein was determined according to the method of Lowry *et al.*[13].

TABLE I

PURIFICATION OF UDP APIOSE/UDP XYLOSE SYNTHASE FROM CELL CULTURES OF PARSLEY

One enzyme unit is defined as the enzyme quantity which catalyzes the conversion of 1 μmole UDPglucuronic acid per min at 30 °C in the enzyme assay[1]

Purification step	*Protein (mg)*	*Spec. act. (units × 10^{-6} per mg protein)*		*Enzyme purification*		*Yield* (%)*	*Apiose/ Xylose*
		Apiose	*Xylose*	*Apiose*	*Xylose*		
Crude extract***	2520	0.27	0.41	—	—	—	—
Dowex 1-X2	2380	1.03	0.79	1	1	100	1.29
$(NH_4)_2SO_4$ fractionation (0.4–0.5)**	290	3.85	2.93	3.8	3.7	46	1.31
DEAE-cellulose	32	35.7	27.1	35	34	47	1.31
Hydroxylapatite	2.36	396	272	385	344	36	1.45
ω-Aminoalkyl-Sepharose	0.67	1107	780	1077	967	29	1.42

* Mean value from apiose and xylose.
** After removal of salts with Sephadex G-25.
*** Synthesis of UDPapiose is inhibited in the crude extract.

RESULTS

Purification of enzyme

With the improved purification procedure summarized in Table I, an approximately 1000-fold purification of the enzyme was achieved. No separation of the enzymatic activities for UDP-D-apiose and UDP-D-xylose synthesis was obtained during the purification and the changes in the ratio apiose/xylose observed were within the limits of experimental error. The purified enzyme was free of UDP-arabinose epimerase.

Compared with the previously published method[2] the following changes in the purification of the enzyme are important: (1) The parsley cells were first frozen with liquid N_2 and then thawed with the homogenization buffer. In this way a better yield of enzyme activity per g fresh wt of cells was obtained. The frozen cells could be stored at −20 °C for several months without significant loss of extractable enzyme activity. (2) Homogenization of cells was carried out with an Ultra Turrax below 4 °C with $2.5 \cdot 10^{-1}$ M Tris–HCl and a higher ratio of buffer to wet cells (2.5:1). (3) The ratio of Dowex to g of wet cells was increased to 1:10. (4) Elution of the DEAE column with $5 \cdot 10^{-2}$ M KCl removed a large amount of inactive protein and the linear KCl gradient applied with a lower flow rate gave a better resolution. (5) Gel filtration on Sephadex G-200 was replaced by chromatography on hydroxylapatite and ω-aminoalkyl-Sepharose. The substituted Sepharose, which has previously been used as an intermediate in the preparation of Sepharose derivatives for affinity chromatography[4], proved to be a very effective anion exchanger for enzyme purification with good enzyme recovery.

Analytical disc electrophoresis

The enzyme from the last purification step was subjected to disc electrophoresis at pH 8.8–9.0 on two columns. One column was used for detection of protein by staining with Coomassie brilliant Blue[5] and the other for enzyme detection (Fig. 1). No separation of activity for apiose and xylose synthesis occurred on the gel. One

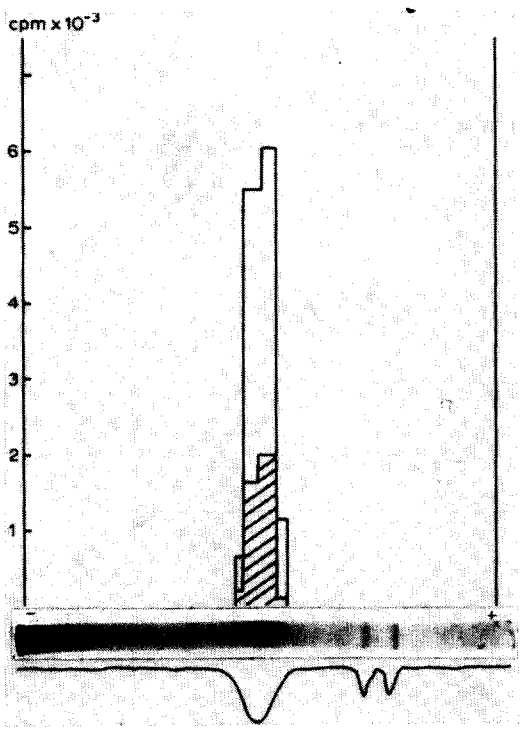

Fig. 1. Analytical disc electrophoresis of enzyme from ω-aminoalkyl-Sepharose column. Striped bar, apiose synthesis; open bar, xylose synthesis. Protein scan is shown in the lower part of the figure.

protein band coincided with enzymatic activity. Besides the enzyme, two further protein bands with higher mobility were visible. From the scan in the Joyce-Loebl Chromoscan it can be estimated that the enzyme is about 75% pure (Fig. 1).

In contrast to the normal behaviour (see Table I) the enzyme from the disc column showed a lower activity for apiose synthesis than for xylose synthesis. It had been found previously that the enzymatic activities for apiose and xylose synthesis can be influenced to a different degree by NH_4^+ (refs 1, 2). What causes the suppression of apiose synthesis or enhancement of xylose synthesis in the case of the enzyme from the gel column is not known.

Molecular weight

The molecular weight of the enzyme had been determined previously by thin-layer chromatography on Sephadex G-200 to be about 115 000 (ref. 2). The molecular weight of the enzyme after $(NH_4)_2SO_4$ fractionation and after DEAE-cellulose chromatography was now determined by sucrose density centrifugation according to the method of Martin and Ames[6] with haemoglobin, alcohol dehydrogenase, cytochrome *c* and catalase as reference proteins. An average molecular weight of 101 000 was obtained (Table II) which agrees reasonably well with the value obtained on Sephadex G-200. Again the enzymatic activities for apiose and xylose synthesis were not separated (Fig. 2).

TABLE II

MOLECULAR WEIGHT OF UDPAPIOSE/UDPXYLOSE SYNTHASE IN SUCROSE DENSITY CENTRIFUGATION

"Standard"	*Enzyme*
Haemoglobin	99 000*
Alcohol dehydrogenase	98 700*
Cytochrome *c*	104 000*
Catalase	103 800**
Average	101 000

* Enzyme after $(NH_4)_2SO_4$ fractionation.
** Enzyme from DEAE-cellulose column.

Affinity chromatography

An attempt was also made to purify the enzyme by affinity chromatography on substituted Sepharose[4]. Since UDP at 10^{-5} M inhibits the enzyme to about 50% (ref. 2) the 5'-(4-aminophenyl)-derivative of UDP was coupled to Sepharose *via* succinylated ω-aminoalkyl-Sepharose. However, no retardation of the enzyme on the UDP–Sepharose column occurred under the various conditions used. It was later found that UDP inhibits the enzyme strongly only above pH 7.5, whereas below pH 6.5 it even effects a slight stimulation of enzyme activity. Inhibition starts above pH 6.8, which is close to the pK_a value of UDP (pK_{a_1} 6.5; pK_{a_2} 9.4) (ref. 7).

For binding of UDP to the enzyme, the first dissociation of its terminal phosphate group seems necessary and this dissociation cannot occur in UDP-Sepharose. Further attempts to bind the enzyme selectively to Sepharose substituted with NAD or UDP-D-glucuronic acid also failed.

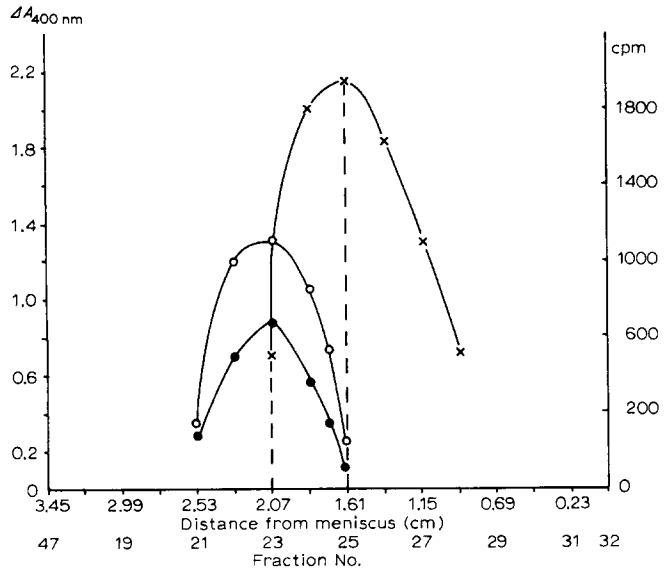

Fig. 2. Determination of molecular weight of enzyme by sucrose density centrifugation. ●—●, apiose synthesis; ○—○, xylose synthesis; ×—×, haemoglobin.

DISCUSSION

The separation of the enzymatic activities for formation of UDP-D-apiose and UDP-D-xylose from UDP-D-glucuronic acid has now been tried by the following methods: (a) ion-exchange chromatography on DEAE-cellulose, hydroxylapatite and ω-aminoalkyl-Sepharose; (b) gel filtration on Sephadex G-200 (ref. 2); (c) isoelectric focusing on Sephadex G-75 thin-layer plates[1,2]; (d) analytical disc electrophoresis at different pH values; (e) sucrose-density gradient centrifugation. In all cases the two enzymatic activities coincided exactly. It can therefore be assumed with confidence that the UDPapiose/UDPxylose synthase is either a two-enzyme complex which does not dissociate under the separation methods employed or a multifunctional protein[8,9].

With enzyme from all stages of purification the ratio apiose/xylose is constant within the limits of experimental error. However, this ratio can change under the influence of NH_4^+ (refs 1 and 2) and other conditions not yet defined. This suggests that the enzyme has two active sites which can be influenced differently.

Further investigations on the properties of this interesting enzyme are in progress.

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REFERENCES

1 Wellmann, E. and Grisebach, H. (1971) *Biochim. Biophys. Acta* 235, 389–397
2 Baron, D., Wellmann, E. and Grisebach, H. (1972) *Biochim. Biophys. Acta* 258, 310–318
3 Hahlbrock, K., Ebel, J., Ortmann, R., Sutter, A., Wellmann, E. and Grisebach, H. (1971) *Biochim. Biophys. Acta* 244, 7–15
4 Cuatrecasas, P. (1970) *J. Biol. Chem.*, 245, 3059–3065
5 Casjens, S., Hohn, Th. and Kaiser, A. D. (1970) *Virology* 42, 496–507
6 Martin, R. G. and Ames, B. N. (1961) *J. Biol. Chem.* 236, 1372–1379
7 Rauen, H. M. (1964) *Biochemisches Taschenbuch*, Part I, p. 601, Springer-Verlag, Berlin
8 Creighton, T. E. (1970) *Biochem. J.* 120, 699–707
9 Yourno, J., Kohno, T. and Roth, J. R. (1970) *Nature* 228, 820–824
10 Moffat, J. G. and Khorana, H. G. (1961) *J. Am. Chem. Soc.* 83, 649–658
11 Moffat, J. G. and Khorana, H. G. (1958) *J. Am. Chem. Soc.* 80 3756–3761
12 Wilchek, M. and Gorecki, M. (1969) *Eur. J. Biochem.* 11, 491–494
13 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193 265–275