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PURIFICATION AND PROPERTIES OF AN ENZYME FROM CELL SUSPENSION CULTURES OF PARSLEY CATALYZING THE SYNTHESIS OF UDP-APIOSE AND UDP-D-XYLOSE FROM UDP-D-GLUCURONIC ACID

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SUMMARY

1. An enzyme from cell cultures of parsley catalyzing the synthesis of UDP-apiose and UDP-D-xylose from UDP-D-glucuronic acid has been purified at least 24-fold by $(NH_4)_2SO_4$ fractionation and Sephadex and DEAE-cellulose column chromatography.

2. The ratio apiose/xylose formed did not change significantly with increasing purification of the enzyme. It was also not possible to separate the enzymatic activities for apiose and xylose synthesis by isoelectric focusing or analytical disc electrophoresis.

3. The enzyme has a requirement for NAD^+ and is inhibited by NADH. NH_4^+ inhibits apiose synthesis and stimulates xylose synthesis in the pH range 8.2–7.5 and stimulates both activities at pH 7.0. The effect of other ions, nucleotides, and SH reagents is about the same on both enzymatic activities.

4. The molecular weight of the enzyme was estimated by thin-layer chromatography on Sephadex G-200 to be about 115 000. The apparent K_m value for UDP-D-glucuronic acid is $2 \cdot 10^{-6}$ M for apiose and xylose synthesis.

5. The enzyme from parsley is very similar in its properties to the corresponding enzyme from *Lemna minor*.

INTRODUCTION

In earlier papers[1,2] we have briefly reported on an enzyme preparation from cell suspension cultures of parsley (*Petroselinum hortense*) catalyzing the synthesis of UDP-apiose and UDP-D-xylose from UDP-D-glucuronic acid. A similar enzyme from *Lemna minor*[3] has also been partially purified.

We now wish to report some of the properties of the enzyme from parsley and compare it with the enzyme from *Lemna minor*.

MATERIALS AND METHODS

Materials

UDP-D-[$^{14}C_6$]glucuronic acid (302 mC/mmole) was obtained from Radiochemical Centre, Amersham. Reference proteins for the molecular weight determination were purchased from Boehringer, Mannheim.

Cell cultures

Cell suspension cultures of *Petroselinum hortense* were grown as described previously[1] and illuminated for 25 h with white light before enzyme extraction[1,2].

Enzyme assay

This was carried out as described previously[2] with the following changes: 0.12 nmole UDP-D-[$^{14}C_6$]glucuronate and 0.1 μmole dithioerythritol were used.*

Enzyme purification

The enzyme purification was carried out as described previously[2] with the following modification: The column chromatography on Sephadex G-200 and DEAE-cellulose was carried out in reverse order.

Protein was determined by the biuret method[8] or by the method of WARBURG AND CHRISTIAN[9].

Molecular weight determination

The molecular weight was determined with the apparatus of Boehringer Mannheim[6,7] according to their instructions with $5 \cdot 10^{-2}$ M Tris–HCl buffer (pH 7.5) containing $3 \cdot 10^{-3}$ M dithioerythritol. Approximately 2 μg protein from the Sephadex G-200 column were applied to a length of 4 cm on the plate (20 cm $\times$ 20 cm). Chromatography was run for 10 h at 4°. During that time the cytochrome migrated a distance of 4–4.5 cm.

RESULTS

Partial purification of the enzyme(s)

The purification procedure for the enzyme is summarized in Table I. In the crude extract the enzymatic reaction is partially inhibited. After treatment of the crude extract with Dowex the apiose/xylose ratio does not change significantly upon further purification of the enzyme. Reaction with the enzyme from the Sephadex G-200 column was linear with protein concentration up to 0.1 mg/ml and with time (Fig. 1).

Attempts to separate the enzymatic activities for apiose and xylose formation

It was not possible to separate the enzymatic activities for apiose and xylose formation on either DEAE-cellulose or Sephadex G-200. The elution patterns from these columns have already been published[2].

The enzyme from the Sephadex G-200 column was subjected to isoelectric

* See NOTE ADDED IN PROOF, p. 318.

TABLE I

PURIFICATION OF UDP-APIOSE/UDP-XYLOSE SYNTHETASE FROM CELL CULTURES OF PARSLEY

One enzyme unit is defined as the enzyme quantity which catalyzes the conversion of 1 μmole UDP-glucuronic acid per min at 30° in the enzyme assay. For financial reasons the substrate concentration used (10^{-6} M) was much lower than the optimal concentration (approx. $4 \cdot 10^{-5}$M).

Purification step	*Protein (mg)*	*Specific activity (units × 10^{-6}/mg protein)*		*Apiose/xylose*	*Enzyme purification*	
		Xylose	*Apiose*		*Xylose*	*Apiose*
Crude extract	1000	—	—	—	—	—
Treatment with Dowex 1-X2	760	7.0	10.3	1.47	1	1
$(NH_4)SO_4$ fractionation (0.4–0.5)*	135	22.2	35.0	1.58	3.2	3.4
DEAE-cellulose column	63	91.0	145.0	1.60	13	14
Sephadex G-200 column	40**	167	247.0	1.48	24	24

* After removal of salts with Sephadex G-25.

** This protein value determined by the method of WARBURG AND CHRISTIAN[9] is too high.

focusing on Sephades G-75 thin-layer plates[4] at a pH range of 4–7. The result is shown in Fig. 2. The enzymatic activity was located in a 1.0-cm broad zone. A separation of apiose and xylose activity was not observed. The isoelectric point for both activities is about 5.

It was also not possible to separate the two enzymatic activities by analytical disc electrophoresis at pH 9.

Properties of the enzyme

All experiments were carried out with the enzyme from the Sephadex G-200 column.

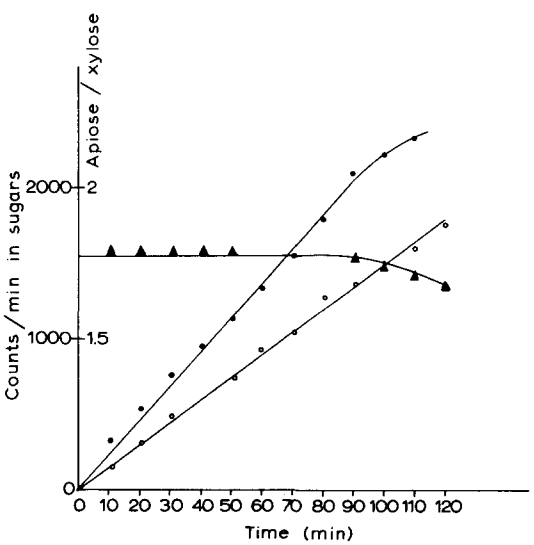


Fig. 1. Dependence of reaction rate and of the apiose/xylose ratio (▲—▲) on time. ●—●, apiose synthesis; ○—○, xylose synthesis.

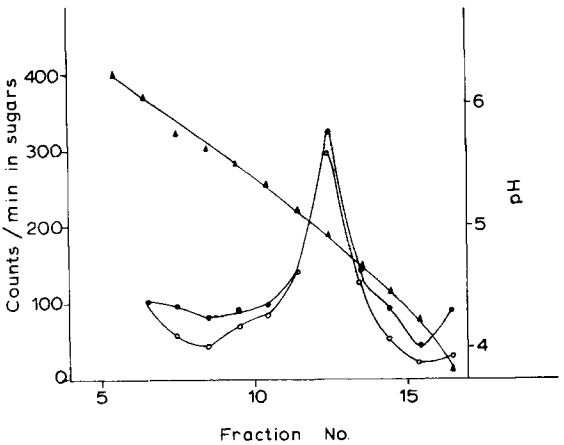
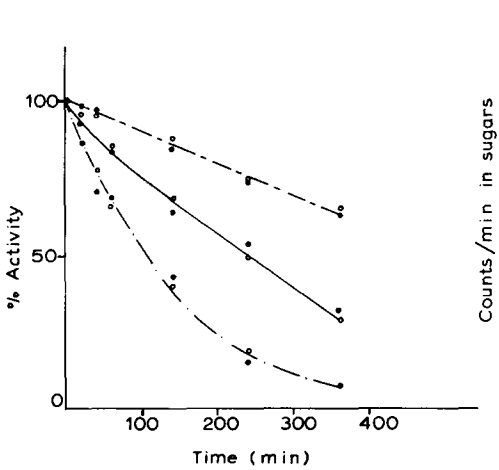


Fig. 2. Isoelectric focusing of enzyme from Sephadex G-200 column on Sephadex G-75 thin-layer plate[3]. ●—●, apiose synthesis; ○—○, xylose synthesis; ▲—▲, pH gradient.

Stability. The influence of SH compounds on the stability of the enzyme at 30° is shown in Fig. 3. Dithioerythritol has a stabilizing effect, whereas mercaptoethanol inhibits the reaction. Both enzymatic activities are affected in the same manner. In the presence of 1 mM dithioerythritol the enzyme is stable for about a month at −35°, and at 2° it loses about 50% of its activity after 96 h.

pH optima. The pH optima for apiose and xylose synthesis are shown in Fig. 4. Both enzymatic activities have a pH optimum of about 8.2. The ratio of apiose to xylose formed depends on the buffer used and on the pH. At the pH optimum this ratio is about 1.5 whereas with potassium phosphate buffer at lower pH this ratio can increase to about 1.8.



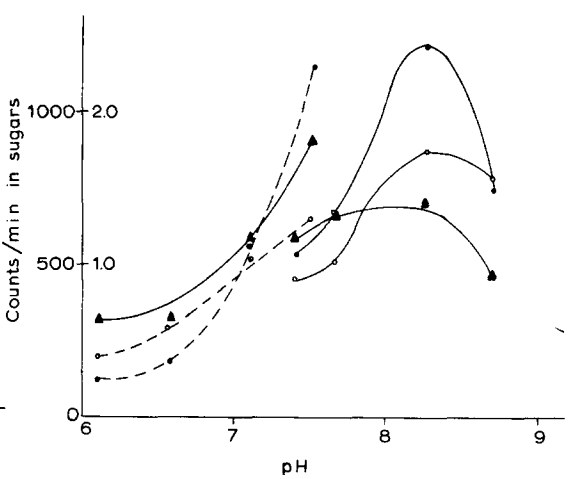


Fig. 3. Influence of SH compounds on enzyme stability at 30°. ●, apiose synthesis; ○, xylose synthesis. ———, without addition of SH compound, – – –, at 1 mM dithioerythritol; — · —, at 3 mM 2-mercaptoethanol.

Fig. 4. Dependence of activity on pH. ●, apiose synthesis; ○, xylose synthesis. — — —, 0.2 M potassium phosphate buffer; ———, 0.2 M Tris–HCl buffer; ▲—▲, apiose/xylose ratio.

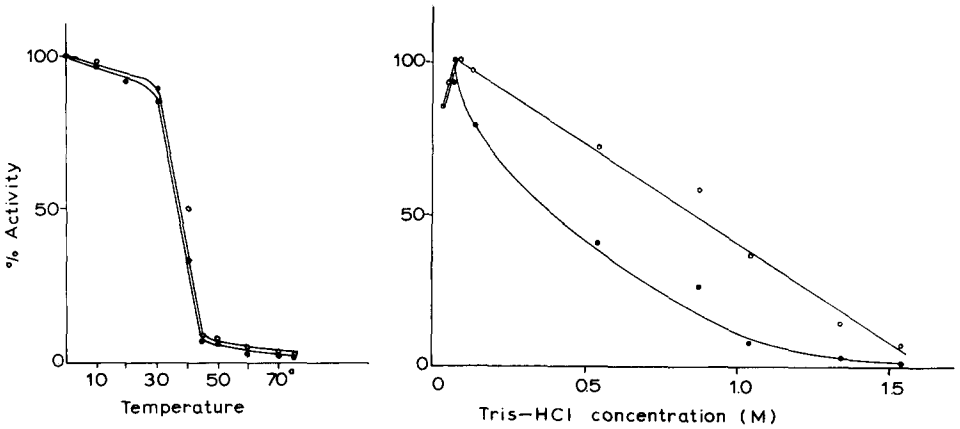


Fig. 5. Heat stability of the enzyme. The enzyme was kept for 10 min at the indicated temperature. ●—●, apiose synthesis; ○—○, xylose synthesis.

Fig. 6. Effect of Tris–HCl buffer (pH 8.0) on enzyme activity. The activity at the optimal concentration of 0.1 M Tris–HCl is taken as 100%. ●—●, apiose synthesis; ○—○, xylose synthesis.

Heat stability. The heat stability of the enzyme was tested between 0 and 75°. The result is shown in Fig. 5. Above 30° a sharp drop in activity occurs, which is exactly the same for apiose and xylose synthesis.

Effect of Tris–HCl buffer. The effect of increasing concentrations of Tris–HCl buffer (pH 8.0) on enzyme activity is shown in Fig. 6. Optimal activity for both apiose and xylose synthesis is observed at 0.1 M; at higher concentrations of buffer both enzymatic activities are inhibited, but to a different degree.

Effect of inorganic ions. The effect of some ions on apiose and xylose synthesis is seen in Table II. The two enzymatic activities are influenced in about the same manner by these ions. As in the case of the enzyme from Lemna[3] a pronounced difference is found with NH_4^+. This effect was studied more carefully at different pH values and at different concentrations of NH_4^+. The results are shown in Fig. 7. Whereas between pH 8.2 and 7.5 the inhibitory effect of NH_4^+ on apiose synthesis and the stimulating effect on xylose synthesis differs only slightly and the sum of apiose *plus* xylose is constant, at pH 7.0 NH_4^+ does not inhibit but stimulates apiose formation.

TABLE II

EFFECT OF INORGANIC SALTS ON APIOSE AND XYLOSE SYNTHESIS

Additions were made to the enzyme assay.

Addition	*Concentration (M)*	*Apiose (%)*	*Xylose (%)*
Water	—	100	100
KCl	$1 \cdot 10^{-2}$	84	95
	$1 \cdot 10^{-1}$	69	87
KCN	$1 \cdot 10^{-2}$	95	125
NaCl	$1 \cdot 10^{-2}$	76	82
$MgCl_2$	$1 \cdot 10^{-2}$	61	87

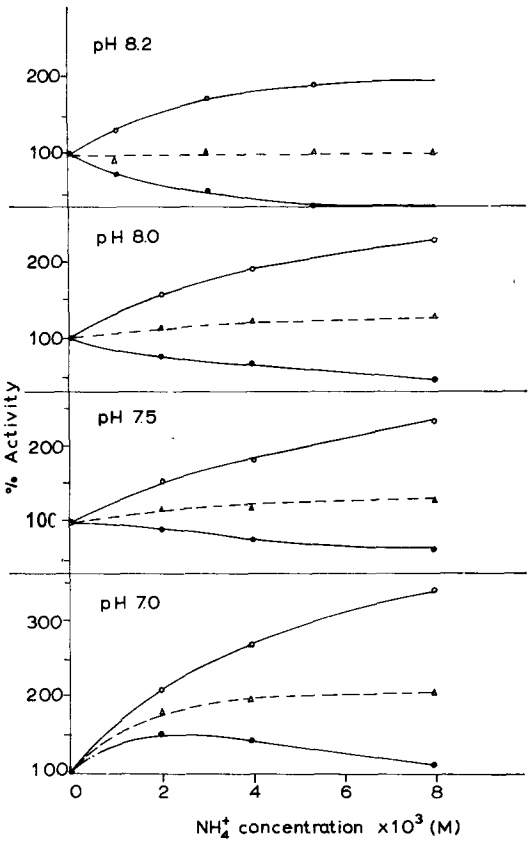
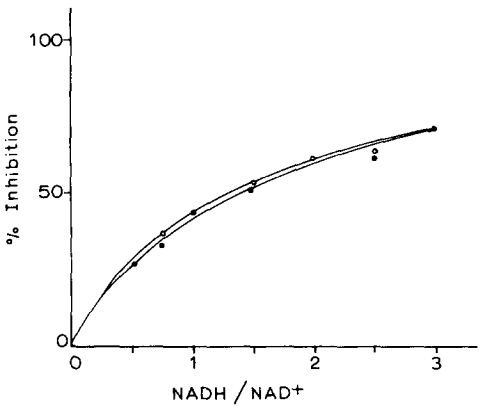


Fig. 7. Effect of NH_4^+ on apiose and xylose formation. The values are related to the incubation without NH_4^+ as 100%. ●—●, apiose synthesis; ○—○, xylose synthesis; △---△, sum of apiose *plus* xylose.

TABLE III

INFLUENCE OF NUCLEOTIDES AND NUCLEOTIDE SUGARS ON ENZYME ACTIVITY

Addition	*Concentration (M)*	*Apiose (%)*	*Xylose (%)*
Water	—	100	100
UMP	$1 \cdot 10^{-3}$	74	107
UDP	$1 \cdot 10^{-5}$	34	54
	$1 \cdot 10^{-4}$	21	44
	$1 \cdot 10^{-3}$	14	28
UTP	$1 \cdot 10^{-3}$	17	45
CDP	$1 \cdot 10^{-3}$	92	107
UDP-D-glucose	$1 \cdot 10^{-5}$	86	88
	$1 \cdot 10^{-4}$	62	71
	$1 \cdot 10^{-3}$	26	35
UDP-D-xylose	$1 \cdot 10^{-5}$	58	72
	$1 \cdot 10^{-4}$	22	28
	$1 \cdot 10^{-3}$	9	14



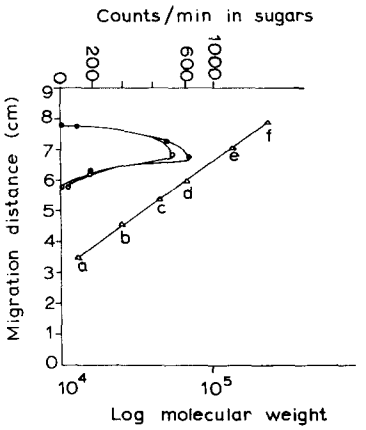


Fig. 8. Influence of the NADH/NAD+ ratio on enzyme activity. NAD+ was kept at a concentration of 3 mM whereas the concentration of NADH was increased from 1 to 3 mM. ●—●, apiose synthesis; ○—○, xylose synthesis.

Fig. 9. Molecular weight determination by thin-layer chromatography on Sephadex G-200 superfine. (a) Cytochrome (13 000), (b) chymotrypsinogen (25 000), (c) albumin (egg) (45 000), (d) albumin (bovine) (67 000), (e) aldolase (147 000), (f) catalase (230 000). ●—●, apiose; ○—○, xylose.

Influence of SH reagents and EDTA. 10^{-4} M *p*-chloromercuribenzoate and 10^{-3} M iodoacetamide inhibit apiose and xylose formation to about 70%. EDTA at a concentration of 1 mM activates both enzyme activities slightly (approx. 20%).

Influence of nucleotides and nucleotide sugars. The influence of nucleotides and nucleotide sugars on apiose and xylose synthesis is shown in Table III. Both apiose and xylose formation are inhibited in the same manner by UDP, UTP, UDP-xylose, and UDP-glucose. The strongest inhibitors are UDP and UDP-D-xylose. UDP-apiose was not available in large enough quantities to test its effect on enzymatic activity.

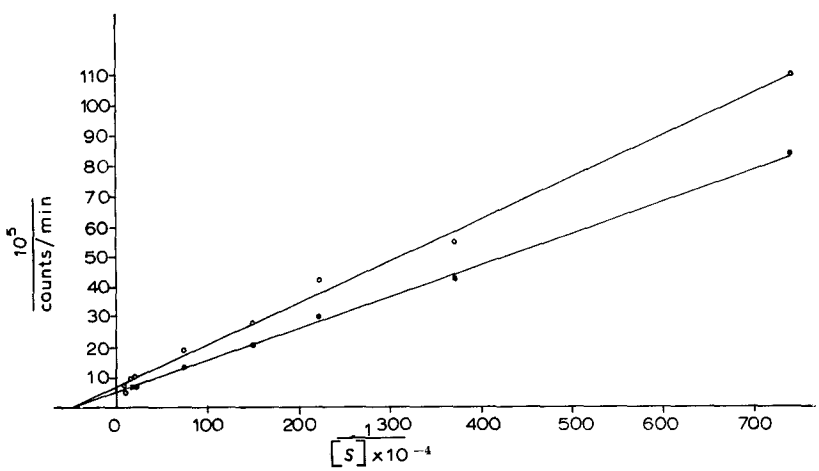


Fig. 10. Effect of UDP-D-glucuronic acid concentration on reaction rate for apiose and xylose synthesis. ●—●, apiose synthesis; ○—○, xylose synthesis.

NAD^+ requirement. Like the Lemna enzyme[5], the parsley enzyme has a requirement for NAD^+. The optimal NAD^+ concentration is about $3 \cdot 10^{-3}$ M. In the absence of NAD^+ a residual activity of about 10% of the activity in the presence of NAD^+ is observed. Whether this remaining activity is due to bound or to absorbed NAD^+ cannot be determined at the moment.

Influence of the $NADH/NAD^+$ ratio on enzyme activity. NADH inhibits the Lemna enzyme[5]. The dependence of enzyme activity on the ratio $NADH/NAD^+$ is shown in Fig. 8. Both enzyme activities are inhibited in the same manner by an increasing $NADH/NAD^+$ ratio.

Molecular weight. An estimation of the molecular weight of the enzyme was obtained by thin-layer chromatography on Sephadex G-200 (ref. 6). The peak of the enzymatic activity corresponds to a molecular weight of about 115 000 (Fig. 9).

Effect of UDP-D-glucuronic acid on the reaction rate. From the effect of UDP-D-glucuronic acid on the reaction rate of apiose and xylose synthesis, the apparent K_m value at 30° was determined according to Lineweaver and Burk to be about $2 \cdot 10^{-6}$ M at pH 8.2 (Fig. 10).

DISCUSSION

The enzyme from cell cultures of parsley has a remarkable similarity to the corresponding enzyme from *Lemna minor*[3,5]. Some of the properties of these two enzymes are summarized in Table IV. As in the case of the Lemna enzyme it was not possible to separate the enzymatic activities for apiose and xylose synthesis. The influence of SH compounds (Fig. 3), the heat stability (Fig. 5), and the influence of the $NADH/NAD^+$ ratio (Fig. 8) are the same for both enzymatic activities. The influence of nucleotides and nucleotide sugars (Table III) and of some inorganic salts (Table II) can be slightly different for apiose and xylose synthesis, but the direction of the effect is the same. A remarkable exception is the effect of NH_4^+, which stimulates xylose and inhibits apiose synthesis in the pH range from 8.2 to 7.5 and stimulates both activities at pH 7.0 (Fig. 7).

The finding that between pH 7.5 and 8.2 the sum of apiose and xylose formed remains constantly independent of NH_4^+ concentration is further support for the assumption[3] that we are dealing with a multifunctional enzyme. It can be assumed

TABLE IV

SOME PROPERTIES OF UDP-APIOSE/UDP-XYLOSE SYNTHETASE FROM *Lemna minor* AND CELL CULTURES OF PARSLEY

	pH optimum	*Isoelectric point*	*K_m (M)*	*NAD^+ requirement*	*NADH*	*NH_4^+*	*UDP*
Lemna enzyme	~8	~5	$\sim 5 \cdot 10^{-6}$	+	−	apiose − xylose +	−
Parsley enzyme	~8.2	~5	$\sim 2 \cdot 10^{-6}$	+	−	apiose − xylose +	−

+stimulation. − inhibition.

that NH_4^+ channels a common intermediate for apiose and xylose in the direction of xylose synthesis. At pH 8.0, 7.5 and 7 the ratio $[NH_4^+]/[NH_3]$ is 25, 90 and 250, respectively. If a nucleophilic reaction with an intermediate is assumed NH_3 rather than NH_4^+ should be the reaction species.

NOTE ADDED IN PROOF: (Received December 8th, 1971)

In contrast with the enzyme from Lemna, a small amount of arabinose ($R_{xylose} = 0.7$) may be present because of the incomplete separation from UDP-arabinose epimerase (EC 5.1.3.5).

ACKNOWLEDGEMENTS

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