BBA 66302

PURIFICATION AND PROPERTIES OF AN ENZYME PREPARATION FROM *LEMNA MINOR* L. CATALYZING THE SYNTHESIS OF UDP-APIOSE AND UDP-D-XYLOSE FROM UDP-D-GLUCURONIC ACID

ECKARD WELLMANN AND HANS GRISEBACH

Lehrstuhl für Biochemie der Pflanzen am Biologischen Institut II der Universität, Freiburg i. Br., Schaenzlestr. 9/11 (Germany)

(Received December 4th, 1970)

SUMMARY

The enzyme(s) from *Lemna minor* L. catalyzing the synthesis of UDP-apiose and UDP-D-xylose from UDP-D-glucuronic acid has been partially purified. The ratio of apiose to xylose formed did not change with increasing purification of the enzyme. It was also not possible to separate the enzymatic activities for apiose and xylose formation by analytical disc electrophoresis at different pH-values or by isoelectric focusing on Sephadex G-75 thin-layer plates.

NH_4^+ inhibits apiose synthesis and stimulates xylose synthesis. The effect of other ions, nucleotides, and SH reagents is the same on both enzymatic activities. UDP and SH reagents like iodoacetamide strongly inhibit both reactions.

INTRODUCTION

In earlier papers[1,2] of this series we reported on an enzyme preparation from *Lemna minor* L. which catalyzes the synthesis of UDP-apiose and UDP-D-xylose from UDP-D-glucuronic acid. This reaction was shown to be NAD^+-dependent. GUSTINE AND KINDEL[3] also described the enzymatic formation of UDP-xylose and of an unidentified apiosyl derivative from UDP-D-glucuronic acid with an enzyme preparation from the same plant.

We have now further purified this enzyme(s) and wish to report some of its properties.

MATERIALS AND METHODS

Organism

Lemna minor L., Strain M 11, was the same as in the previous investigations[1].

Material

UDP-D-[$^{14}C_6$]glucuronic acid (273 μC/μmole) was synthesized as described previously[1] or bought from the Radiochemical Centre, Amersham.

UDP-xylose was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Purification of enzyme(s)

All steps were carried out at 4°. About 250 g (wet wt.) of plants were suspended in 500 ml of 0.1 M Tris–HCl buffer (pH 7.5) containing $1 \cdot 10^{-2}$ M 2-mercaptoethanol and $1 \cdot 10^{-3}$ M KCN (Buffer A) and homogenized with 250 g quartz sand in a precooled mortar. The resulting slurry was filtered through four layers of cheesecloth and the residue again extracted with 250 ml of the same buffer. The filtrate was centrifuged at 35 000 × *g* for 20 min. The supernatant was subjected to $(NH_4)_2SO_4$ fractionation at pH 7.5 by addition of the solid salt.

Protein precipitated between 35 and 70% saturation was collected by centrifugation at 35 000 × *g* for 10 min and dissolved in 5 ml of Buffer A.

The solution was chromatographed with $1 \cdot 10^{-2}$ M Tris–HCl buffer (pH 7.5) containing $1 \cdot 10^{-3}$ M dithioerythritol and $1 \cdot 10^{-3}$ M KCN on a column (20 cm × 3 cm) of Sephadex G-25, equilibrated before use with the same buffer.

5 ml of the fraction with the highest protein content was then chromatographed with $1 \cdot 10^{-2}$ M Tris–HCl buffer (pH 7.5) containing $1 \cdot 10^{-3}$ M dithioerythritol (Buffer B) on a column (50 cm × 2.5 cm) of Sephadex G-200 at a flow rate of 25 ml/h. The fractions with enzymatic activity (approx. 15 ml) were absorbed on a DEAE-cellulose column (2 g) equilibrated before use with Buffer B. The column was then washed with 50 ml 0.09 M KCl in the same buffer and the protein eluted with 3 ml 0.15 M KCl in the same buffer. The fractions with enzymatic activity were then desalted by chromatography on a column of Sephadex G-25 equilibrated before use with $5 \cdot 10^{-3}$ M Buffer B. This solution was used for disc electrophoresis, isoelectric focusing, and the other investigations. The solution was stored at −20°.

Analytical disc electrophoresis

Disc electrophoresis was carried out in a polyacrylamide gel of middle pore size (7.5%) at pH 7.5; 8.9 and 10.3 (spacer gel 0.5 cm, running gel 3.5 cm)[4]. The electrode solution contained $1 \cdot 10^{-3}$ M thioglycolic acid. After 2.5 h electrophoresis (2 mA/tube) the gel was cut into 1-mm thick discs which were each crushed in 100 µl 0.2 M Tris–HCl buffer (pH 8) containing $1 \cdot 10^{-3}$ M dithioerythritol and the enzymatic activity determined in the standard incubation mixture. The incubations were run for 60 min.

Isoelectric focusing

The electrofocusing was carried out on Sephadex G-75 thin layer plates (10 cm × 20 cm) in the pH-range 5–7 (ref. 7). The plates were prepared from about 30 ml Sephadex G-75 soaked in water containing 1 ml of the protein solution, 2% ampholine pH 5–7, and $1 \cdot 10^{-3}$ M dithioerythritol. Electrode solutions were 0.2 M H_2SO_4 and 0.4 M ethylenediamine, respectively. The electrofocusing was carried out at 15 V/cm for 15 h in a cooled (4°) apparatus for thin layer electrophoresis (Desaga, Heidelberg). For determination of enzymatic activity and pH, 0.5-cm broad zones of the Sephadex layer were each eluted with 1 ml of water.

Enzyme assay

The incubation mixture consisted of 0.13 nmole (0.025 µC) UDP-D-[$^{14}C_6$]glu-

curonate, 0.1 μmole NAD^+, 0.05 μmole dithioerythritol, and 5 μmoles Tris–HCl buffer (pH 7.8) in a total volume of 50 μl. The reaction was started by addition of protein. The protein concentration chosen was such that not more than 5% of the substrate reacted in 10 min. The mixture was incubated at 30° for 10 min. At the end of this period 20 μl of acetic acid was added. The solution was heated for 15 min at 100°. The reaction mixture was then applied to one sheet of Schleicher Schuell 2043 b and chromatographed with reference sugars for approx. 12 h with the solvent system pyridine–ethyl acetate–water (2:8:1, by vol.). The reference sugars were detected with aniline phtalate. The apiose and xylose zones were cut into strips 2 cm wide and counted in toluene–2,5-diphenyloxazole (5 g/l). The background was determined by counting a zone between the zones of apiose and xylose, which was equally as wide as the pentose zone.

Protein assay

Protein was determined in the crude extract by the biuret method[5] and otherwise according to the method of LOWRY *et al.*[6] using bovine albumin as standard.

RESULTS

Partial purification of the enzyme(s)

The purification procedure for the enzyme is summarized in Table I. It was not possible to determine exactly the enzymatic activity in the crude extract since even with high substrate concentrations only a weak reaction takes place, which is not linear with protein concentration. The enzymatic activity can also not be determined in the presence of $(NH_4)_2SO_4$, since apiose formation is inhibited and xylose formation stimulated by NH_4^+ (see below). After chromatography on Sephadex G-25 the reaction is still inhibited at high protein concentrations. Not until the protein concentration is reduced to less than 1 mg/ml in the standard incubation (see MATERIALS AND METHODS) and $1 \cdot 10^{-3}$ M KCN is added does the reaction become

TABLE I

PURIFICATION OF UDP-APIOSE/UDP-XYLOSE SYNTHETASE FROM *L. minor*

Incubations were carried out as described for enzyme assay. 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. The specific activity appears to be so low because for financial reasons the substrate concentration used ($2.6 \cdot 10^{-6}$ 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)*		*Enzyme purification*	
		Xylose	*Apiose*	*Xylose*	*Apiose*
Crude extract	1300	—	—	—	—
$(NH_4)_2SO_4$ fractionation (0.35–0.7)	530	—	—	—	—
Sephadex G-25 column	450	0.52	0.79	1	1
Sephadex G-200 column	39	4.5	6.5	8.6	8.3
DEAE-cellulose column*	7.8	14.8	20.6	29	26

* After removal of salts with Sephadex G-25.

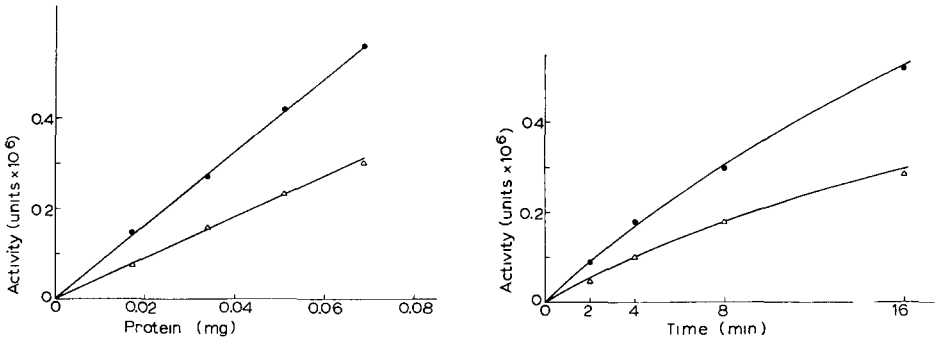

Fig. 1. Dependence of reaction rate on enzyme concentration. Experimental conditions are those described in the text for standard incubation. ●—●, apiose synthesis; △—△, xylose synthesis.

Fig. 2. Dependence of reaction rate on time. Experimental conditions are those described in the text for standard incubation. ●—●, apiose synthesis; △—△, xylose synthesis.

linear with protein concentration. After chromatography on Sephadex G-200 no inhibition was observed at high protein concentrations.

After the enzyme fraction from the DEAE-cellulose column was desalted on a Sephadex G-25 column the reaction was linear with protein concentration and time for apiose and xylose formation (Figs. 1 and 2).

Attempts to separate the enzymatic activities for apiose and xylose formation

The elution patterns on Sephadex G-200 and DEAE-cellulose columns for the enzymatic activities for apiose and xylose formation are shown in Figs. 3 and 4. On both columns both activities run exactly together.

The enzyme from the DEAE-cellulose column was desalted on a Sephadex G-25 column and was separated at pH 8.9 by analytical disc electrophoresis. About 6 protein bands were detected by staining the column with amido black. The gel was then cut into 1-mm-thick discs and the enzymatic activity for apiose and xylose formation determined in each disc. The result is shown in Fig. 5. The main enzymatic activity was located in a 1-mm-broad zone but no change in the ratio of apiose to

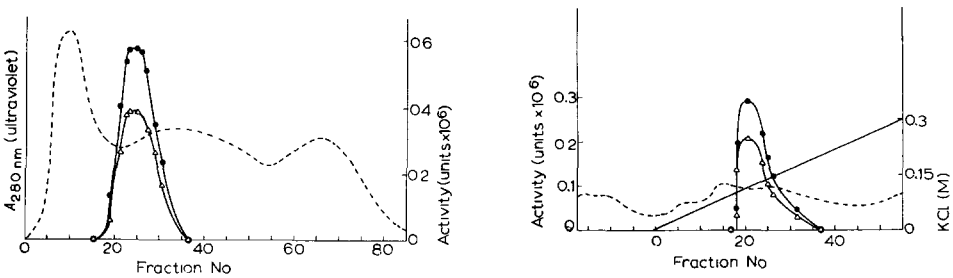

Fig. 3. Chromatography of Sephadex G-25 eluate on Sephadex G-200. -----, protein; ●—●, activity of apiose synthesis △—△, activity of xylose synthesis.

Fig. 4. Chromatography of enzymatically active fraction from Sephadex G-200 column on DEAE-cellulose column. -------, protein (absorbance at 280 nm); ●—●, activity of apiose synthesis, △—△, activity of xylose synthesis.

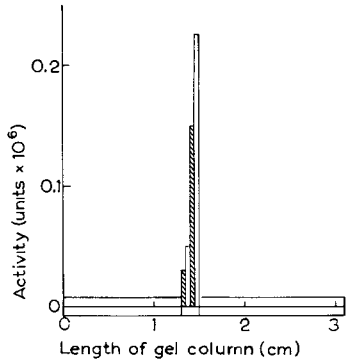

Fig. 5. Analytical disc electrophoresis of desalted enzyme from DEAE-cellulose column. Blank bars, activity of apiose synthesis; striped bars; activity of xylose synthesis.

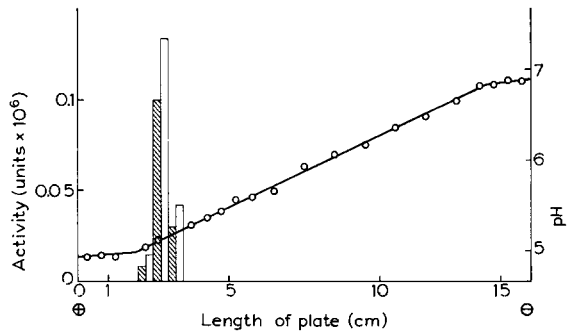

Fig. 6. Isoelectric focusing of desalted enzyme from DEAE-cellulose column. Blank bars, activity of apiose synthesis; striped bars, activity of xylose synthesis.

xylose formed compared to the ratio found with the enzyme put on the gel was observed. The same result was obtained when the disc electrophoresis was carried out at pH 7.5 or pH 10.3.

The desalted enzyme from the DEAE-cellulose column was also subjected to isoelectric focusing on Sephadex G-75 thin layer plates[7] at a pH-range of 5–7. The result is shown in Fig. 6. The bulk of the enzymatic activity was located in a 0.5-cm-broad zone at a pH of 5.1. The apiose to xylose ratio formed with the protein from this zone was the same as that formed with the enzyme before the focusing.

Stability of the enzyme

At −20° and in the presence of $5 \cdot 10^{-4}$ M dithioerythritol the enzymatic activity of the purified fractions is stable for about a month. Repeated thawing and freezing leads to strong losses of activity.

The presence of SH-compounds is essential for the stability of the enzyme. From the compounds tested (bisulfite, mercaptoethanol, cysteine, dithioerythritol) dithioerythritol shows the best effect. Addition of NAD^+ (up to $1 \cdot 10^{-2}$ M), glycerol (up to 50%), and saccharose (up to 50%) had no effect.

TABLE II

TEMPERATURE STABILITY OF THE ENZYMATIC ACTIVITY FOR APIOSE AND XYLOSE FORMATION

The desalted enzyme from the DEAE-cellulose column was incubated for 15 min at the indicated temperature, and the enzyme assay was then carried out.

Temperature	*Apiose (%)*	*Xylose (%)*
Control in ice water	100	100
15°	94	95
25°	89	88
35°	80	82
45°	48	44
55°	7	4

TABLE III

pH STABILITY OF THE ENZYMATIC ACTIVITY FOR APIOSE AND XYLOSE FORMATION

The enzyme from the Sephadex G-25 column was kept for 5 min at the pH indicated (by addition of acetic acid) and after neutralization with sodium bicarbonate the enzyme assay was carried out.

pH	*Apiose (%)*	*Xylose (%)*
8	100	100
7	100	103
6	98	95
5	89	84
4	8	0

The temperature and pH stability of the enzyme is shown in Tables II and III. Both apiose and xylose formation are influenced in the same manner.

pH optima

The pH optima were determined using 0.2 M phosphate buffer between 5 and 7.5 and 0.2 M Tris–HCl buffer between 7 and 8.8. As can be seen from Fig. 7 apiose synthesis has a pH optimum between 7 and 8, and xylose synthesis shows a broad maximum around 8. At basic and at acidic pH values xylose formation is favoured over apiose formation.

Effect of ions

The effect of ions on apiose and xylose synthesis is seen from Table IV. Whereas KCl and $MgCl_2$ at 0.1 M concentration have an inhibitory effect on both apiose and xylose synthesis, NH_4^+ inhibits apiose synthesis and stimulates xylose formation.

Influence of nucleotides and sugars

The influence of nucleotides and sugars on apiose and xylose synthesis is shown in Table V. Both apiose and xylose formation are inhibited in the same manner by UDP, UTP, UDP-xylose and UDP-glucose. The strongest inhibitor is UDP. UDP-

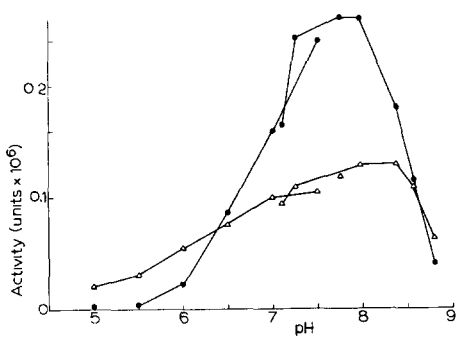

Fig. 7. Dependence of activity on pH. ●—●, apiose synthesis; △—△, xylose synthesis. The experimental conditions were the same as in the standard incubation except that the buffer and pH were varied as indicated in the text.

TABLE IV

EFFECT OF INORGANIC SALTS ON APIOSE AND XYLOSE SYNTHESIS

Experiments were carried out with desalted enzyme from DEAE-cellulose column. Additions were made to the enzyme assay.

Addition	*Concentration (M)*	*Apiose (%)*	*Xylose (%)*
Water	—	100	100
$(NH_4)_2SO_4$	$1 \cdot 10^{-2}$	24	210
	$1 \cdot 10^{-1}$	12	240
NH_4Cl	$1 \cdot 10^{-2}$	22	205
	$1 \cdot 10^{-1}$	15	265
KCl	$1 \cdot 10^{-2}$	95	98
	$1 \cdot 10^{-1}$	66	64
$MgCl_2$	$1 \cdot 10^{-2}$	62	88
	$1 \cdot 10^{-1}$	14	32

xylose does not inhibit the reaction at the concentration in which it is formed in the standard incubation.

Influence of SH reagents and other possible effectors

The influence of SH reagents and some other potential effectors on the formation of apiose and xylose is shown in Table VI. SH reagents inhibit apiose and xylose synthesis in the same manner.

Effect of UDP-D-glucuronic acid on reaction rate

From the effect of UDP-D-glucuronic acid on reaction rate for apiose and

TABLE V

INFLUENCE OF NUCLEOTIDES AND SUGARS ON APIOSE AND XYLOSE FORMATION

Incubations were carried out with the desalted enzyme from the DEAE-cellulose column in the enzyme assay.

Addition	*Concentration (M)*	*Apiose (%)*	*Xylose (%)*
Water	—	100	100
UMP	$1 \cdot 10^{-3}$	95	93
UDP	$1 \cdot 10^{-5}$	48	51
	$1 \cdot 10^{-4}$	16	14
	$1 \cdot 10^{-3}$	3	5
UTP	$1 \cdot 10^{-3}$	22	18
ADP	$1 \cdot 10^{-3}$	87	82
UDP-D-xylose	$1 \cdot 10^{-6}$	97	100
	$1 \cdot 10^{-4}$	45	59
	$1 \cdot 10^{-3}$	15	22
UDP-D-glucose	$1 \cdot 10^{-4}$	66	76
D-Xylose	$1 \cdot 10^{-3}$	99	102
D-Apiose	$1 \cdot 10^{-3}$	96	97
D-Glucuronic acid	$1 \cdot 10^{-3}$	105	98

TABLE VI

INFLUENCE OF SH REAGENTS AND OTHER POTENTIAL EFFECTORS ON APIOSE AND XYLOSE FORMATION

Incubations were carried out with the desalted enzyme from the DEAE-cellulose column in the enzyme assay.

Addition	*Concentration (M)*	*Apiose (%)*	*Xylose (%)*
Water	—	100	100
Dithioerythritol	$1 \cdot 10^{-2}$	128	112
KCN	$1 \cdot 10^{-3}$	100	105
EDTA	$1 \cdot 10^{-3}$	98	95
p-Chloromercuribenzoate	$1 \cdot 10^{-4}$	22	25
	$1 \cdot 10^{-3}$	0	4
Iodoacetamide	$1 \cdot 10^{-3}$	17	20
	$1 \cdot 10^{-2}$	5	8

xylose synthesis the apparent K_m value at 30° was determined according to Lineweaver and Burk to be about $5 \cdot 10^{-6}$ M at pH 7.8 (Fig. 8).

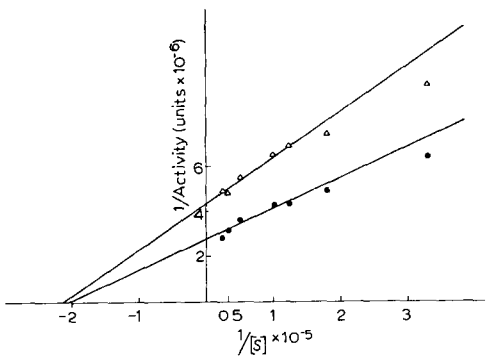

Fig. 8. Effect of UDP-D-glucuronic acid concentration on reaction rate for apiose and xylose synthesis. ●—●, apiose synthesis; △—△, xylose synthesis.

DISCUSSION

The reaction products formed from UDP-D-glucuronic acid with the enzyme from *L. minor* L. have been identified in our previous papers as UDP-D-xylose, UDP-apiose, and the cyclic apiose 1,2-phosphate (refs. 1, 8, 9; R. ORTMANN AND H. GRISEBACH, unpublished results). It was assumed that the latter compound is a nonenzymic breakdown product of UDP-apiose. This has now been verified by the finding that the formation of the cyclic apiose phosphate can be completely avoided if acidic solvents are used for purification of the UDP-pentose sugar fraction.

In the present investigation the UDP-pentoses were not isolated but were hydrolyzed at the end of the incubation and the enzymatic activity calculated from the radioactivity in the free sugars.

Since it was not possible to determine the specific activity in the crude extract, the exact degree of purification of the enzyme achieved is not known.

The most interesting result of our investigations is that we were not able to separate the enzymatic activities for apiose and xylose synthesis even by analytical disc electrophoresis or isoelectric focusing on thin layer plates (Figs. 5 and 6). Furthermore, the temperature and pH stability (Tables II and III) as well as the effect of KCl, $MgCl_2$, nucleotides, and SH reagents (Tables IV–VI) is the same on both enzymatic activities within the limits of experimental error. The only differences we have found between the enzymatic activities are the slightly different pH curves for apiose and xylose synthesis (Fig. 7) and the fact that apiose synthesis is remarkably inhibited by NH_4^+, whereas xylose synthesis is strongly stimulated by these ions (Table IV).

We can conclude from our results that either the syntheses of apiose and xylose are catalyzed by two different enzymes which have very similar properties or we are dealing with a multienzyme complex. The molecular weight of the corresponding enzyme from cell suspension cultures of parsley has been determined to be about 110 000–120 000 daltons (D. BARON AND H. GRISEBACH, unpublished results). This would indicate that the enzyme is a multichain protein.

It should also be mentioned that the corresponding apiose/xylose synthetase from parsley has the same properties as the enzyme from Lemna but that in parsley a second enzyme exists which catalyzes only xylose synthesis from UDP-D-glururonic acid (ref. 10; D. BARON AND H. GRISEBACH, unpublished results).

ACKNOWLEDGMENTS

The work was supported by Deutsche Forschungsgemeinschaft and by Fonds der Chemischen Industrie. The authors wish to thank Mrs. Elke Brutzer for her skilled technical assistance and Dr. K. Hahlbrock for his valuable advice.

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