Phytochemistry, Vol. 28, No. 9, pp. 2281–2284, 1989.
Printed in Great Britain.

0031-9422/89 $3.00+0.00
Maxwell Pergamon Macmillan plc.

PURIFICATION AND PARTIAL CHARACTERIZATION OF UDP-GLUCOSE: PHENOL-β-D-GLUCOSYLTRANSFERASE FROM PAPAYA FRUIT

URSULA KEIL and PETER SCHREIER

Lehrstuhl für Lebensmittelchemie, Universität Würzburg, Am Hubland, D-8700 Würzburg, F.R.G.

(*Received in revised form* 21 *February* 1989)

Key Word Index—*Carica papaya*; Caricaceae; papaya fruit; UDP-glucose; phenol-β-D-glucosyltransferase.

Abstract—UDP-Glucose: phenol-β-D-glucosyltransferase was isolated from *Carica papaya* fruit pulp and purified *ca* 660-fold to electrophoretic homogeneity. The procedure used protamine sulphate and ammonium sulphate fractionation followed by chromatography on Phenyl-Sepharose CL-4B, QAE-Sephadex A-25 and Sephacryl S-200 to separate glycosidases and UDP-galactose: phenol-β-D-galactosyltransferase activity. The transferases had pI of 4.6 and 5.3. M_rs of 56 000 and 61 000 were determined by gel filtration. The M_r of phenol-β-D-glucosyltransferase estimated by SDS-PAGE was 28 000 suggesting that it consisted of two subunits; the optimum pH and optimum temperature were 7.5 and 50°, respectively, and the enzyme followed typical Michaelis kinetics with K_m and V_{max} of 0.17 mM and 0.04 nkat for UDP-glucose, and 0.07 mM and 0.10 nkat for *p*-nitrophenol, respectively (40°). The energies of activation and inactivation were 38 and 175 kJ/mol, respectively. The activity was stimulated by the divalent cations Ca^{2+}, Mg^{2+}, and Mn^{2+}; $HgCl_2$, $Hg(CN)_2$, AgCl, $CuSO_4$ and UDP showed strong inhibitory effect. For UDP, competitive inhibition with K_i 0.2 mM was found.

INTRODUCTION

Several enzymes have been purified and partially characterized from *Carica papaya*, such as thioglucosidase [1], β-galactosidase and α-mannosidase [2] from seeds as well as β-fructofuranosidase [3], catalase [4], polygalacturonase [5], acid phosphatase [6], pectinesterase [7] and β-glucosidase [8, 9] from fruit pulp. Information about UDP-sugar transferases [10] from papaya is still lacking. This paper concerns the purification and partial characterization of UDP-glucose: phenol-β-D-glucosyl transferase from papaya fruit pulp.

RESULTS AND DISCUSSION

Among the phenol-specific UDP-sugar transferases present in *C. papaya* UDP-glucose: phenol-β-D-glucosyltransferase (**1**) showed the highest activity in crude extracts of the fruit pulp. UDP-galactose: phenol-β-D-galactosyl transferase (**2**) exhibited *ca* 40% of the activity determined for (**1**). Phenol-specific UDP-glucuronosyltransferase was not detectable.

As the enzyme assay selected was influenced by glycosidase activities (cf. Experimental), their inhibition in papaya fruit pulp [8, 9] had to be established. This could be achieved using Tris–maleate buffer (pH 7.5) [9, 11] for enzyme extraction and purification. Due to loss of transferase activity using ammonium sulphate fractionation, pretreatment of the crude extract with protamine sulphate was carried out. After addition of neutralized protamine sulphate solution to a final concentration of 0.2% to the crude extract (A) and centrifugation, an extract (B) was obtained exhibiting higher activity of transferases than the untreated extract A (Table 1). Similar effects after pretreatment of crude extracts have been observed for a number of UDP-sugar transferases from plants [12–18]. Using protamine sulphate pretreatment, 90% of the activity of **1** was recovered in the subsequent ammonium sulphate cut (Table 1).

When the redissolved (750 mM ammonium sulphate in Tris–HCl, pH 7) preparation was applied to Phenyl-Sepharose CL-4B column (Fig. 1), both the transferase activities (**1** and **2**) were eluted with 10 mM Tris–HCl (pH 8.0) buffer. The pooled fractions, enriched 51-fold in activity (**1**) (Table 1), were concentrated by ammonium sulphate precipitation. After centrifugation, redissolving and buffering to 10 mM Tris–HCl (pH 7.5) using Bio Gel P-10, the transferase active protein was applied to a QAE-Sephadex A-25 column. The elution profiles of the transferases **1** and **2** are shown in Fig. 2. While **2** was completely washed out with the unbound protein, **1** was eluted using a sodium chloride gradient. Subsequent gel filtration of the ultrafiltered transferase (**1**) fractions obtained by ion exchange chromatography led to 660-fold enrichment with a recovery of 31% (Table 1). Electrophoretic homogeneity of the purified enzyme (**1**) was demonstrated by ultrathin-layer isoelectric focusing (UIEF); pI 4.6 was determined. For transferase **2**, UIEF of Phenyl-Sepharose CL-4B fraction showed pI 5.3.

M_rs of 56 000 and 61 000 were recorded after gel filtration on Sephacryl S-200 for the transferases **1** and **2**, respectively. Similar M_rs have been established previously for various UDP-glucosyltransferases from different plant origin [19–22]. The M_r of **1** estimated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was 28 000 suggesting that **1** consisted of two subunits.

The purified transferase **1** showed half maximal activity at pH 5.3 and 10.4 and its pH optimum (7.5) was similar to that of some other plant UDP-glucosyltransferases [19–22]. The observed increased stability of purified **1**

Table 1. Purification of UDP-glucose:phenol-β-D-glucosyltransferase from papaya fruit

Purification step	Vol. (ml)	Total act. (nkat)	Total protein (mg)	Spec. act. (nkat/mg protein)	Purification factor*	Recovery* (%)
Crude extract A	780	16	3140	0.005	1	100
Crude extract B	850	34	3020	0.011	2.2	212
$(NH_4)_2SO_4$ prec.	22	30	335	0.090	18.0	188
Phenyl-Sepharose CL-4B	120	25	98	0.255	51.0	156
$(NH_4)_2SO_4$ prec.	3	22	88	0.250	50.0	138
Bio Gel P 10	30	21	82	0.256	51.2	131
QAE-Sephadex A 25	93	9	6	1.50	300	56
Ultrafiltration	2	6	5.5	1.09	218	38
Sephacryl S-200	27	5	1.5	3.33	666	31

*Calculated with respect to the crude extract A.

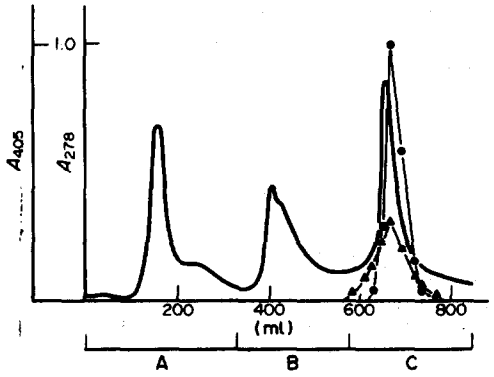

Fig. 1. Hydrophobic chromatography of phenol-specific UDP-sugar transferases from papaya fruit pulp on Phenyl-Sepharose CL-4B. A, 750 mM ammonium sulphate in 200 mM Tris–HCl (pH 7); B, 200 mM ammonium sulphate in 100 mM Tris–HCl (pH 7.5); C, 10 mM Tris–HCl (pH 8.0). (—) $A_{278\,nm}$; (●) UDP-glucose:phenol-β-D-glucosyltransferase; (▲) UDP-galactose:phenol-β-D-galactosyltransferase.

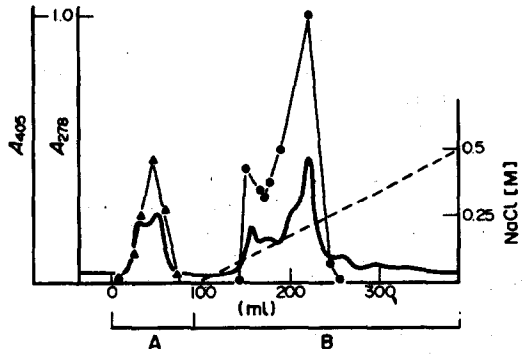

Fig. 2. Chromatographic separation of UDP-glucose:phenol-β-D-glucosyltransferase (●) and UDP-galactose:phenol-β-D-galactosyltransferase (▲) from papaya fruit pulp on QAE-Sephadex A-25. A, 10 mM Tris–HCl (pH 7.5); B, linear gradient from 0 to 0.5 M NaCl in equilibration buffer. (—) $A_{278\,nm}$.

under basic conditions (half-life 5 days at pH 8) also corresponded to previously published data.

For **1** a temperature optimum of 50° was determined; with a Z value of 10.6°, high temperature stability was found. Graphical evaluation of the Arrhenius plot led to 38 and 175 kJ/mol for energies of activation and inactivation of **1**, respectively.

The values of apparent K_m and V_{max} for UDP-glucose and *p*-nitrophenol were 0.17 mM and 0.04 nkat, and 0.07 mM and 0.10 nkat, respectively (40°). Similar data have been found for various plant UDP-glucosyltransferases [12, 15, 19, 21, 23, 24]. In addition to *p*-nitrophenol, using purified **1**, other substrates were checked qualitatively. HRGC-MS analysis of per-*O*-methylated derivatives revealed the formation of β-D-glucosides of phenol, benzyl alcohol, 2-phenylethanol and 4-hydroxyphenylethanol, whereas *o*- and *m*-nitrophenol, 2,4-dinitrophenol as well as monoterpene alcohols, such as linalool, nerol and geraniol, were not accepted as substrates. Using UDP-galactose and UDP-glucuronic acid instead of UDP-glucose, no reaction with *p*-nitrophenol was observed. This high specificity of **1** towards the glycosyl donor corresponded to previously published observations [20, 25–27].

The influence of a variety of effectors was studied under standard enzyme assay conditions. Using Mn^{2+}, Mg^{2+} and Ca^{2+} in concentrations from 10^{-3} to 10^{-2} M, activation of **1** was observed. Activating effects caused, in particular, by Ca^{2+} and Mg^{2+} have been described previously for UDP-glucosyltransferases from plant origin [14, 23, 26, 28–30]. Inhibition of **1** was observed by addition of $HgCl_2$, $Hg(CN)_2$, AgCl, $CuSO_4$ and UDP (cf. [14, 16, 20, 23, 30]). For UDP, competitive inhibition with K_i 0.2 mM was found.

Due to analytical difficulties (i.e. limited availability of formed β-D-glucosides; lack of appropriate enzymic test) kinetic data for the above mentioned, additionally checked substrates could not be determined. Thus, all physical constants obtained in this work are limited to the artificial phenolic substrate. Furthermore, the question whether in 4-hydroxyphenylethanol the glucosyl residue was transferred to the phenolic or the aliphatic hydroxyls or both cannot be answered.

EXPERIMENTAL

Fruits. Fresh papaya fruits (Solo variety) were obtained by air-freight from Brazil. Ripe fruits (green-yellow colour; pink, soft pulp) were used.

Enzyme assays. The activity of transferases (**1**, **2**) was determined by measuring the colourless *p*-nitrophenol glycoside (PNP-Gly) formed at pH 7.5 from yellow *p*-nitrophenol (PNP) and UDP-activated sugar (UDP-Glc; UDP-Gal). Assays containing 10–100 μl enzyme preparation and 0.1 ml 0.4 mM PNP and 1 mM UDP-activated sugar in 50 mM Tris–HCL buffer (pH 7.5) were incubated at 40°. After an appropriate time (5–20 min) the reaction was stopped by the addition of 1 ml 200 mM borate buffer (pH 9.8). Blank tests, in which the addition of enzyme was carried out after making alkaline, were performed accordingly. The reactions were linear for at least 20 min and were directly proportional to the amount of enzyme present. The A_{405} was measured and the amount of PNP-Gly formed was determined indirectly by difference measurements, considering the amount of PNP in blank tests and enzyme assays (ε = 18.5 M/mN/cm).

Enzyme extraction and purification. All steps were carried out at 0–5°. Papaya fruits were peeled and cut into two pieces; the seeds were carefully separated. The initial extraction was carried out by placing 500 g fruits and 500 ml 50 mM Tris–maleate buffer (pH 7.5) in a blender and homogenizing for 30 sec. The extract was centrifuged at 18000 *g* for 30 min and the supernatant decanted (crude extract A). After addition of neutralized protamine sulphate soln to a final concn of 0.2% and centrifugation (18000 *g*, 30 min), crude extract B was obtained. Solid $(NH_4)_2SO_4$ was added to 2.8 M and the material precipitated was collected by centrifugation (18000 *g*, 90 min). The $(NH_4)_2SO_4$ pellet was suspended in 200 mM Tris–HCl buffer (pH 7) containing 750 mM $(NH_4)_2SO_4$. Insoluble material was removed by centrifugation and discarded.

The supernatant was loaded onto a Phenyl-Sepharose CL-4B column, 2.5 × 20 cm, equilibrated with 200 mM Tris–HCl buffer (pH 7) containing 750 mM $(NH_4)_2SO_4$. The gel was treated with equilibration buffer until unbound protein was completely washed out. Subsequent washing was carried out using five colum vol. 200 mM $(NH_4)_2SO_4$ in 100 mM Tris–HCl buffer (pH 7.5). Elution of transferases **1** and **2** was performed using five column vol. 10 mM Tris–HCl buffer (pH 8).

The transferase (**1**, **2**) active fractions from the Phenyl-Sepharose CL-4B column were pooled, and solid $(NH_4)_2SO_4$ added. The material pptd at 2.8 M was collected by centrifugation (18000 *g*, 30 min). The $(NH_4)_2SO_4$ pellet was suspended in 50 mM Tris–HCl buffer (pH 7). After buffering the enzyme soln at 10 mM Tris–HCl (pH 7.5) using gel filtration on Bio Gel P-10 (2.5 × 20 cm), the eluate was applied to a QAE-Sephadex A-25 column equilibrated with 10 mM Tris–HCl (pH 7.5). Using equilibration buffer, unbound protein containing the complete activity of **2** was washed out. Elution of **1** was achieved employing a linear gradient of six vol. of 0–0.5 M NaCl in the equilibration buffer.

The transferase **1** active fractions of ion exchange chromatography were pooled and ultrafiltered to 2 ml using an Amicon cell (Kalle TU-AN-4045.380 membrane). The ultrafiltrate was applied to a Sephacryl S-200 column, 2.5 × 60 cm, equilibrated with 50 mM Tris–HCl buffer (pH 7.5). Gel filtration was performed using the same buffer.

M_r *determinations.* The M_r of **1** and **2** were determined separately by gel filtration [31] on Sephacryl S-200 as described above. The column was calibrated and a standard curve V/V_0 vs log M_r for the calibration proteins was used to determine the M_rs of **1** and **2**. SDS-PAGE of purified **1** was carried out according to ref. [32].

Ultrathin-layer isoelectric focusing (UIEF). UIEF in 50 μm PAGE gels was carried out as described in ref. [33]. For the visualization of enzymes after UIEF the substrates for ultrathin-layer agarose gels on polyester films [34] were prepared as follows. Agarose soln (2%) in 50 mM Tris–HCl buffer (pH 7.5) was prepared by heating. After cooling to *ca* 40°, to each of 50 ml agarose solns, 1 ml substrate soln (2.5 mM PNP and 10 mM activated sugar in 50 mM Tris–HCl buffer, pH 7.5) was added. After UIEF, the PAGE gel was immersed in satd $(NH_4)_2SO_4$ soln (5 min), washed with H_2O, covered with the substrate containing agarose gel and incubated at 40° for 10–30 min. Light bands exhibiting enzymic activity (PNP-Gly) were detected after NH_3 treatment.

Substrate specificity. The formation of aryl β-D-glucosides was studied after per-*O*-methylation by HRGC and HRGC-MS as recently described [35].

Influence of effectors. The influence of a variety of effectors (glucose, ascorbic acid, dithiothreitol, $MgCl_2$, $MnCl_2$, $CaCl_2$, $HgCl_2$, $Hg(CN)_2$, AgCl, $CuSO_4$, NaN_3, $ZnCl_2$, SDS, EDTA, UDP, iodoacetamide, *p*-chloromercuribenzoate) was studied using standard enzyme assay and concn of effectors from 10^{-8} to 10^{-1} M. If necessary, the pH was corrected to pH 7.5.

K_i *determination.* Enzyme activity was determined under standard conditions using different PNP (0.02, 0.04, 0.08, 0.16 mM) and UDP concns (0.01, 0.05 and 0.1 mM). The incubation time was 20 min. The determination was performed graphically using Lineweaver–Burk plot. The resulting K_i values were used for a secondary Lineweaver–Burk plot leading to graphical evaluation of K_i.

Protein measurements. Protein content was determined according to the method of ref. [36] with bovine serum albumin as standard.

Acknowledgements—Dr Jenny Hartmann-Schreier's assistance is gratefully acknowledged. We thank Prof. Dr Rüdiger's group for performing the SDS-PAGE determinations.

REFERENCES

1. Tang, C. S. (1973) *Phytochemistry* **12**, 769.
2. Ohtani, K. and Misaki, A. (1983) *Agric. Biol. Chem.* **47**, 2441.
3. Chan Jr, H. T. and Kwok, S. C. M. (1976) *J. Food Sci.* **41**, 320.
4. Chan Jr, H. T., Tam S. Y. T. and Koide, R. T. (1978) *J. Food Sci.* **43**, 989.
5. Chan Jr, H. T. and Tam, S. Y. T. (1982) *J. Food Sci.* **47**, 1478.
6. Carreno, R. and Chan Jr., H. T. (1982) *J. Food Sci.* **47**, 149.
7. Lourenco, E. J. and Catutani, A. T. (1984) *J. Sci. Food Agric.* **35**, 1129.
8. Hartmann-Schreier, J. and Schreier, P. (1986) *Phytochemistry* **25**, 2271.
9. Hartmann-Schreier, J. and Schreier, P. (1987) *Food Chem.* **26**, 201.
10. Watkins, W. M. (1986) *Carbohydr. Res.* **149**, 1.
11. Dahlqvist, A. (1961) *Biochem. J.* **80**, 547.
12. Saleh, N. A. M., Poulton, J. E. and Grisebach, H. (1976) *Phytochemistry* **15**, 1865.
13. Tabata, M., Ikeda, F., Hiraoka, N. and Konoshima, M. (1976) *Phytochemistry* **15**, 1225.
14. Fleuriet, A., Macheix, J. J., Suen, R. and Ibrahim, R. K. (1980) *Z. Naturforsch.* **35c**, 967.
15. Saleh, N. A. M., Fritsch, H., Witkop, P. and Grisebach, H. (1976) *Planta* **133**, 41.
16. Ibrahim, R. K. (1977) *Z. Pflanzenphysiol.* **85**, 253.
17. Müller, W. U. and Leistner, E. (1978) *Phytochemistry* **17**, 1739.

18. Ibrahim, R. K. and Boulay, B. (1980) *Plant Sci. Letters* **18**, 177.
19. Mizukami, H., Terao, T. and Ohashi, H. (1985) *Planta Med.* **51**, 104.
20. Matsuo, M. and Underhill, E. W. (1971) *Phytochemistry* **10**, 2279.
21. Barz, W. and Köster, J. (1981) *Arch. Biochem. Biophys.* **212**, 98.
22. Bajai, K. L., De Luca, V., Khouri, H. and Ibrahim, R. K. (1983) *Plant Physiol.* **72**, 891.
23. Van Brederode, J. and Van Wigterecht, G. (1973) *Mol. Gen. Genet.* **122**, 215.
24. Poulton, J. E. and Kauer, M. (1977) *Planta* **136**, 53.
25. Jourdan, P. S. and Mansell, R. L. (1982) *Arch. Biochem. Biophys.* **213**, 434.
26. Teusch, M., Forkmann, G. and Seyffert, W. (1986) *Z. Naturforsch.* **41c**, 699.
27. Kerscher, F. and Franz, G. (1987) *Z. Naturforsch.* **42c**, 519.
28. Ingold, E. and Seitz, H. K. (1986) *Z. Naturforsch.* **41c**, 409.
29. Steyns, J. M. and Van Brederode, J. (1986) *Biochem. Genet.* **24**, 349.
30. Kalinowska, M. and Wojciechowski, Z. A. (1986) *Phytochemistry* **25**, 45.
31. Whitaker, J. R. (1963) *Analyt. Chem.* **35**, 1950.
32. Laemmli, U. (1970) *Nature* **227**, 680.
33. Radola, B. J. (1975) *Biochim. Biophys. Acta* **386**, 181.
34. Höfelmann, M., Kittsteiner-Eberle, R. and Schreier, P. (1983) *Anal. Biochem.* **128**, 217.
35. Schwab, W. and Schreier, P. (1988) *Phytochemistry* **27**, 1813.
36. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.