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Studies on UDPglucose-Fructose Glucosyltransferase in Potato Tubers *in vitro* and *in vivo*

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UDPglucose: D-fructose 2-glucosyltransferase ** from potato tuber tissue has been isolated using a modified method compared to the one described in Refs. 4 and 5. The enzyme activity was found to be 6 times higher than in the earlier investigations. The sucrose synthesis in the potato tuber tissue *in vivo* has been demonstrated.

Since the isolation of UDPglucose in 1949, the number of known analogous compounds has increased steadily and the different sugar nucleotides have been found to play an important role in cell metabolism.[1–3]

In 1960 Schwimmer and Rorem [4] reported the potato tuber to be a rich source of UDPG-transferase (EC.2.4.1.13). This enzyme catalyses the reaction: UDPG + fructose ⇌ UDP + sucrose.

In 1966 one of the present authors found an increased activity of this enzyme in γ-irradiated potato tubers.[5] The present paper is a report on further investigations of the enzyme.

In the preceding paper,[5] only the amount of sucrose formed in the reaction was determined; the UDP amount liberated was not measured. In the investigation reported here, the aim was to follow not only the sucrose synthesis but also the UDP formation by coupling the UDP to the pyruvate kinase-lactate dehydrogenase reaction and by measuring the decrease in fluorescence due to oxidation of NADH.[6,7] In addition a search was to be made for specific locations of the enzyme a) in the tuber, b) in the different parts of the cell. Our purpose was furthermore to investigate the sucrose synthesis *in vivo* by using C-14 labelled UDPG. Finally, we intended to reinvestigate the general kinetics of the enzyme and to examine different isolation procedures.

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** Abbreviated: UDPG-transferase.

MATERIALS AND METHODS

Potato tubers of the Bintje variety, stored after harvest for 2–8 months at +4 to +5°C were used throughout this investigation. The tubers were harvested in two successive years, 1967 and 1968.

The enzymes and the most important chemicals used. UDPG = uridine diphosphoglucose, sodium salt; UDPG carbon-14, ammonium salt, specific activity: 194 mC/mmole (Amersham); UDP = uridine-5-diphosphate, sodium salt; UTP = uridine-5-triphosphate, sodium salt; UMP = uridine-5-monophosphate, sodium salt; Uridine, crystalline; BSA = bovine serum albumin, Fraction V Powder; PEP = phospho(enol)pyruvic acid, trisodium salt; LDH = lactic dehydrogenase No. 340-UV; PK = pyruvate-kinase Type II; β-NADH = dihydronicotinamide adenine dinucleotide disodium salt (reduced); NAD^+ = nicotinamide adenine dinucleotide disodium salt (oxidized) Grade III; F-1-P = fructose-1-monophosphate, F-6-P = fructose-6-monophosphate, F-1,6-P = fructose-1,6-diphosphate, G-1-P = glucose-1-monophosphate, G-6-P = glucose-6-monophosphate (Boehringer).

Except where otherwise stated, the enzymes and chemicals listed above are Sigma-products.

Preparation of the enzyme. 200 g peeled potato tubers were rapidly cut in pieces and disintegrated in a Waring blendor for 30 sec at low speed in a medium containing 0.5 M mannitol, 5×10^{-3}M EDTA, 4×10^{-3}M cysteine, and 0.1% BSA. During the first seconds of homogenizing the pH was adjusted to pH 7.6–7.8. The homogenate was squeezed through double gauze and the turbid filtrate was centrifuged for 20 min at 800 *g* to remove starch and cell debris. The supernatant was made 70 % saturated with respect to ammonium sulphate. The precipitate, which was collected by centrifugation at 10 000 *g* and 0°C was dialysed against distilled water at 2–4°C for 20 h. The dialysed preparation was centrifuged at 10 000 *g* and the precipitate obtained was suspended in water. The protein content for each enzyme preparation was determined according to micro Kjeldahl (in duplicate).

The synthesis of sucrose was performed with different concentrations of UDPG, fructose, and enzyme. Tris buffer and $MgCl_2$ were included in the reaction mixture (final concentrations 0.33 M and 0.5 mM, respectively); the final volume was 1.015 ml.

The amount of sucrose synthesized after 1, 2, and 3 h incubation times at 25°C and 37°C and at various pH values was determined in three different ways: 1) spectrophotometrically according to Rorem and coworkers,[8] 2) paper chromatographically according to a quantitative method described earlier,[9] 3) using UDPG labelled with carbon-14 in the glucose moiety and measuring — after paper chromatographical separation of the sucrose — the incorporation of ^{14}C by liquid scintillation.

Qualitative determination of sugar phosphates, nucleosides, and nucleotides by paper chromatography. The solvent NH_3,propanol,H_2O (6:3:1) used by Hanes and Isherwood [10] was chosen as the best agent for the separation and the spots were developed according to the same authors.[10] The location of nucleotides and nucleosides was also visualized in UV as dark spots.

Determination of phosphatase activity in the enzyme suspension. Alkaline phosphatase using sodium glycerophosphate as substrate,[11,12] acid phosphatase according to Fishman and Davidson.[13]

Preparation of mitochondria and microsomes. The preparation of mitochondria was performed according to Jaarma.[14] The microsome fraction was obtained by centrifuging the supernatant from the isolation of mitochondria for 3 h at 140 000 *g*. The pellets of mitochondria and microsomes were washed with 0.5 M mannitol containing 0.1 % BSA and finally with pure 0.5 M mannitol.

In vivo incorporation of C-14 labelled UDPG. A cubical piece ($5 \times 5 \times 5$ mm) was cut out of the potato tissue. UDPG-^{14}C together with carrier UDPG was injected into the cube with a small capillary needle. The piece was carefully rinsed with Tris buffer pH 8.7 and put in a test tube containing a small amount of the same buffer — just enough to cover the piece. The incubation time was 1–3 h. After the incubation the piece was squeezed in a special small cylindrical press. About 80–90 % of the cell-sap could be extracted in this way. The cell-sap containing the reaction products was deproteinised and centrifuged. The sucrose was separated by paper chromatography and the amount synthesized determined by measuring the ^{14}C-labelling in the sucrose spot by liquid

scintillation. In some parallel experiments the cell-sap was used without previous deproteinisation and centrifugation, *i.e.* with the starch fraction present.

Determination of UDP. a) paper chromatographically as described above; b) by coupling the UDP to the pyruvate kinase lactic dehydrogenase reaction:

$$\text{1. UDP+PEP} \xrightarrow{\text{EC.2.7.1.40}} \text{UTP+pyruvate.}$$

$$\text{2. Pyruvate+NADH+H}^+ \xrightarrow{\text{EC.1.1.1.27}} \text{lactate+NAD}^+$$

and by measuring the decrease in fluorescence due to oxidation of NADH at 340 nm.[6,7]

pH-Measurement. The control of pH in the reaction mixture before and after the synthesis of sucrose was performed with microelectrodes, which made it possible to measure pH in a volume of 0.2 ml.

RESULTS AND DISCUSSION

Inasmuch as the authors were fully aware that the enzyme suspension used was not a pure UDPG-transferase, the investigation was started with measurements of other enzymes possibly present, which could perhaps interact with the compounds present in the substrate (reaction mixture). First of all the activities of alkaline and acid phosphatases were tested. The activity of alkaline phosphatase was negligible, whereas the activity of acid phosphatase was high. With disodium phenylphosphate as substrate, 0.03 μmoles per min were cleaved at pH 5.0. If, however, the pH was kept at 7.0, the influence on UDPG was insignificant. Even the activity of β-glucuronidase was determined and found to be nil at pH 6.0 (*cf.* Jaarma [15]).

The next step in the investigation was a search for a special location of the glucosyltransferase in the tubers; the enzyme was found to be equally distributed in the different parts — with the exception of the tissue including the vascular ring, where the concentration was somewhat lower. It must, however, be stated that it was rather difficult to isolate only vascular tissue. Differential centrifugation was performed to determine whether the enzyme is present also in the mitochondrial and microsomal fraction. The main activity was found in the soluble fraction.

In isolating the enzyme, Schwimmer and Rorem [4] used only phosphate buffer pH 7.0 as disintegrating and homogenizing medium. In an investigation reported earlier,[5] an isolation procedure was used according to the authors named above. By changing the medium to the one described under Materials and Methods, the enzyme activity was increased three times. The increase of activity can, of course, be only apparent and in fact be due to a higher amount of enzyme extracted by using the altered isolation procedure.

By reinvestigating the pH dependence of the glucosyltransferase, the optimum pH was found to be 8.7 instead of the earlier reported 8.1 (see Fig. 1). The amount of sucrose synthesized was not influenced by changing the reaction temperature from 37°C to 25°C.

The enzyme activity at different concentrations of fructose and UDPG was determined. The value for K_m with respect to fructose as substrate was 6.6 mM as shown in Fig. 2 and regarding UDPG as substrate K_m was 0.6 mM (Fig. 3). In spite of the unpurified state of the enzyme the K_m values found are in good agreement with those reported by Avigad [16] for glucosyltransferase

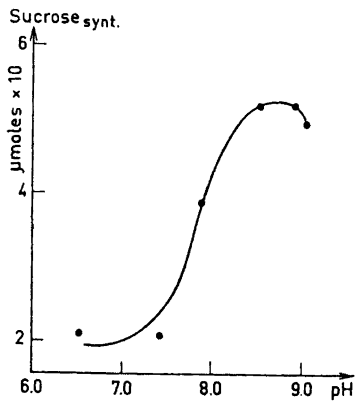

Fig. 1. Effect of pH on UDPG-transferase activity. Reaction mixture: 6.12 mM UDPG, 0.33 M Tris, 10.2 mM fructose, 0.5 mM $MgCl_2$, enzyme conc. 2.0 mg protein N/ml, 25°C, reaction time 1 h.

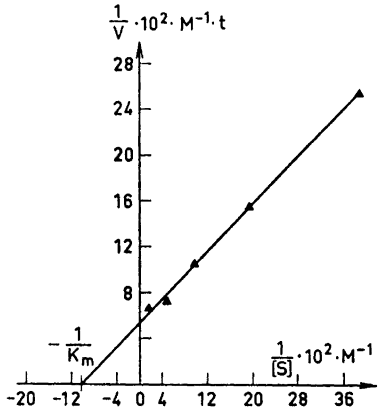

Fig. 2. Effect of fructose concentration on UDPG-transferase activity. Reaction mixture: 6.12 mM UDPG, 0.33 M Tris pH 8.7, 0.5 mM $MgCl_2$, enzyme conc. 2.1 mg protein N/ml, 25°C, time 2 h. Effect of substrate conc. plotted according to Lineweaver and Burk. $K_m = 6.6 \times 10^{-3}$M.

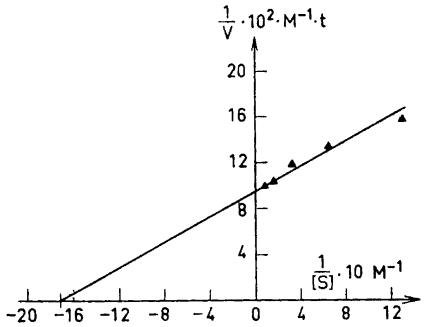

Fig. 3. Effect of UDPG concentration on UDPG-transferase activity. Reaction conditions as for Fig. 2 with 10.2 mM fructose. $Km = 6 \times 10^{-4}$M. (Lineweaver and Burk).

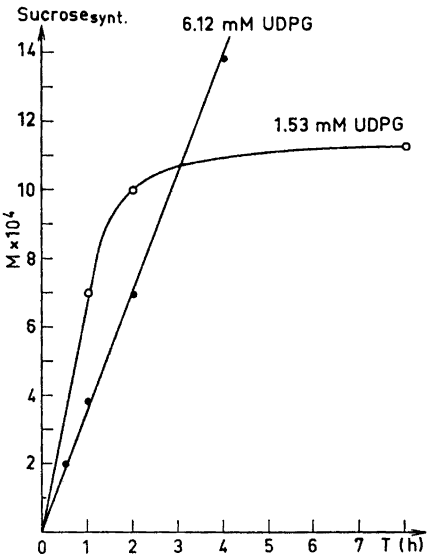

Fig. 4. Time dependence of UDPG-transferase at different concentrations of UDPG. ● 6.12 mM UDPG; ○ 1.53 mM UDPG. T (h) = time in hours. Reaction conditions as for Fig. 3.

from artichoke and by Cardini *et al.*[17] for the corresponding enzyme from wheat germs. The time dependence of glucosyltransferase at two different starting concentrations of UDPG is given in Fig. 4.

It is obvious that the sucrose synthesizing enzymes in different plants are very similar in character. The limiting factor for the sucrose synthesis seems to be the UDPG-concentration. By working at optimal conditions the activity of the potato enzyme was found to be about 6 times higher than in the earlier investigations performed according to Schwimmer and Rorem.[4] A maximum yield of about 90 % sucrose (calc. on UDPglucose) was obtained.

Fig. 5 illustrates the separation of reaction products by paper chromatography. Sucrose is synthesized only if UDPG and fructose are present. Uridine

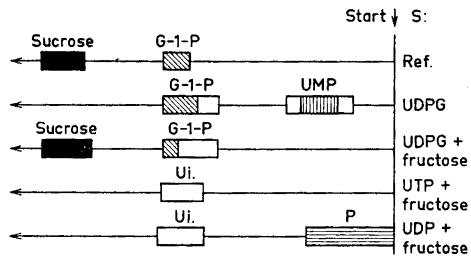

Fig. 5. Separation of reaction products by paper chromatography. Combined development: a) for reducing sugars, b) for sucrose, c) for phosphorus containing compounds, d) UV. Ui=unidentified, P=inorganic phosphate.

is always formed. In the running time necessary for separation of the other reaction products the uridine runs off the paper, which is the reason why the uridine spots are not given in the figure. When UTP or UDP were substituted for UDPG in the reaction mixture, the paper chromatographical separation and development revealed a reducing sugar phosphate which had the same R_F value as fructose-6-phosphate. Recent reports on an enzyme system, which catalyzes the transformation of UDP-D-glucose into UDP-L-rhamnose,[18] and the simultaneously presented evidence concerning the existence of an UDPfructose in germinating pea seeds,[19] support the interpretation that the reducing sugar phosphate observed in the present investigation in reality *is* F-6-P.

From the paper chromatographical examination it also appeared that UDP was missing. Furthermore, if UDP was added to the reaction mixture together with UDPG even this added amount of UDP disappeared.

In order to ascertain that this observation was correct and not due to some inadequate separation by the paper chromatography, the UDP concentration was determined fluorometrically according to Kornberg *et al.*[6] as described under Materials and Methods. The presence of UDP could not be demonstrated in this way either. Fully in accordance with the paper chromatographical results, an added amount of UDP disappeared also here, as shown in Fig. 6 a. When the reaction mixture was deproteinised before the pyruvate kinase-lactic-dehydrogenase test, the same result was obtained. No UDP could be observed. In this case, however, 10 μmole added UDP gave a decrease of the fluorescence, which means that the testing system functions *if* there is UDP present and our UDPG-transferase preparation is absent; see Fig. 6 b.

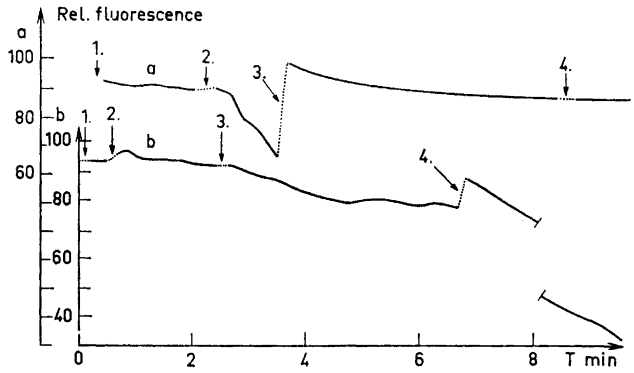

Fig. 6. Result of enzymatic determination of UDP. a. Additions: 1. 33 μM NADH, 20 μM UDP, PEP, Tris, Mg^{2+}, K^+; 2. LDH, PK; 3. 1 ml reaction mixture including enzyme; 4. UDP in excess. b. Additions: 1. 8.3 μM NADH, 1 ml deproteinised reaction mixture, Tris, Mg^{2+}, K^+; 2. PEP; 3. LDH; PK; 4. 10 μM UDP.

This must denote that there is another enzyme present in the transferase preparation which breaks down or converts the UDP.

The question was raised, however, of how to investigate the sucrose synthesis *in vivo*. As there is about one per cent sucrose present in the cell-sap of the tuber, a small increase in this amount is not so easy to observe. An

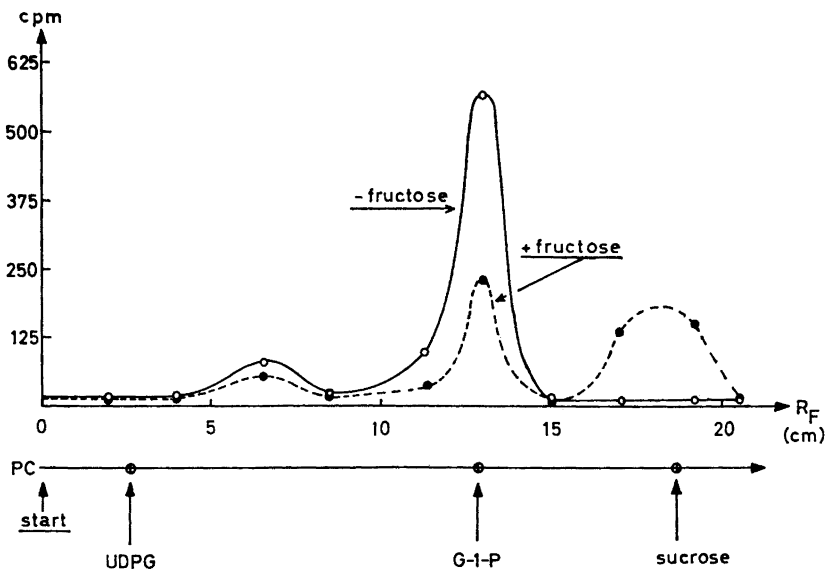

Fig. 7. Incorporation of ^{14}C from UDPG at the sucrose-synthesis *in vitro*. PC=Paper chromatography.

isotope technique using UDPG labelled with ^{14}C in the glucose moiety was chosen for this purpose. The experimental details are given under Materials and Methods. The complete reaction mixture with labelled UDPG was also used for *in vitro* experiments. The main activity was found in the sucrose fraction as shown in Fig. 7. When fructose was omitted, no sucrose was synthesized. Another labelled compound was, however, observed. It had the same R_F value as glucose-1-phosphate. In addition, activity could be measured also in another phosphate-containing compound, which is still unidentified. Without fructose in the reaction medium, the main activity was found in these two phosphorus containing compounds.

A complication in the *in vivo* experiments seems to be the absorption of activity on the starch present. Fig. 8 illustrates the incorporation *in vivo* of

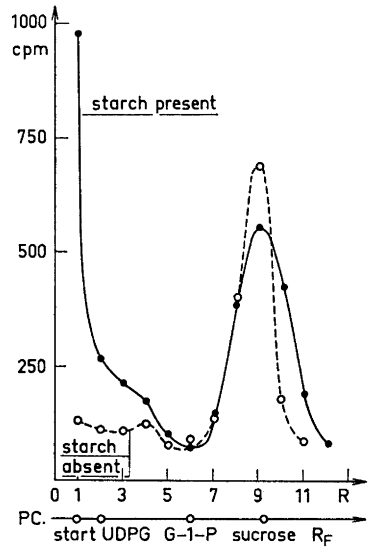

Fig. 8. Incorporation of ^{14}C from UDPG at the sucrose-synthesis *in vivo*. PC = Paper chromatography. R = relative distance.

^{14}C from UDPG labelled in the glucose moiety, in sucrose. As can be seen from the figure the highest ^{14}C-activity was found in the starch fraction, if it was present. By removing the starch before analyzing the reaction mixture a higher amount of labelled sucrose was obtained. When, however, starch-free sell-sap was used as substrate for the sucrose synthesis *in vitro*, (which is not illustrated in the figure) the labelling of the sucrose was about 4 times higher than in the corresponding *in vivo* experiments, where starch was present. Apparently, either UDPG-^{14}C or sucrose-^{14}C is absorbed on the starch. Another possible explanation for this phenomenon is an enzymatical incorporation of the labelled compound in the starch fraction. It must, however, be stated that the absorption of the labelled UDPG on pure starch was significant.

In summarizing the results of the present investigation it can be reported that: an increased activity of UDPG-transferase from potato tubers — as

compared with earlier reports [4,5] — was obtained by changing the disintegrating and homogenizing medium and by allowing the enzyme to react at pH 8.7 instead of 8.1 When optimal substrate concentration were used in addition to the changed pH, the yield of sucrose synthesized could be raised from 15 to 90 %. The sucrose synthesis *in vivo* from UDPG was demonstrated. It is difficult to say whether the injected UDPG has penetrated the cell-membrane or the synthesis has taken place in the cell-sap leaking through punctures in the cell-wall. The native sucrose in the potato tissue and the synthesized product were absolutely identical. R_F-Values on chromatograms, spectrophotometrical assay, X-ray films, and liquid scintillation measurements all gave perfect agreement.

The observed increase in enzyme activity or higher amount of enzyme obtained by using the described method for isolation might partly be caused by the presence of cysteine although no evidence is available that UDPG-transferase is an SH-enzyme.

The data obtained, however, indicate that further purification of the enzyme is needed. Work is in progress in this respect.

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