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GLUCOSE TRANSFERASE IN ROOTS OF *GEUM URBANUM*

MIKULÁŠ PŠENÁK, PETER KOVÁCS and ANTONÍN JINDRA

Department of Biochemistry and Microbiology, Faculty of Pharmacy, Comenius University, Bratislava, Czechoslovakia

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Abstract—Acetone powders from roots *Geum urbanum* plants catalysed the intramolecular transfer of glucose; the phenolic glucosides salicin and arbutin were transformed into mono-, di- and probably tri- and tetra-glucosides of saligenin and oligoglucoside of quinol respectively. These derivatives were formed without any energy-rich donor of glucose being added to the system. Among newly formed products gentiobioside of saligenin and quinol were identified.

The mechanism of isosalicin and saligenin gentiobioside formation is discussed.

INTRODUCTION

THE FORMATION of isosalicin (*o*-hydroxybenzyl-β-D-glucoside) was first mentioned by Bourquelot[1]. Later, Rabaté[2] reported the transglucosylation of salicin to the isomeric isosalicin by an enzyme preparation from leaves of *Salix purpurea*. The β-glucosidases from *Prunus amygdalus* and *Vicia faba* also catalyse this reaction.[3,4] Isosalicin has been isolated from blossoms of *Filipendula ulmaria* (Maxim.)[5] and also reported to occur in the flower oil of a number of species of *Spireae* and in tissues of several willows (*Salix fragilis*, *S. babylonica* and *S. vitellina*[6]).

The mechanism of the intramolecular transfer of glucose from the phenolic hydroxyl group to alcoholic one in saligenin has now been studied in detail. We used for our studies enzyme preparations from roots of *Geum urbanum*.[7]

RESULTS

Glucose Transferase Activity in vivo

Saligenin and salicin were infiltrated into both seedlings of *Geum urbanum* through the root system, and isolated leaves of old plants. Methanolic extracts of the plant materials were then analysed by paper chromatography and phenol-positive spots detected. The newly formed product, isosalicin, was found in both saligenin and salicin-fed plants and isolated according to Porter.[8] It was separated from salicin on a polyamide column. Fractions containing isosalicin were combined, concentrated and hydrolysed with acid or almond

[1] E. BOURGUELOT and H. HERISSEY, *C. R. Acad. Sci.* (*Paris*) **156**, 1790 (1913).
[2] J. RABATÉ, *Bull. Soc. Chim. Biol.* **17**, 572 (1935).
[3] J. D. ANDERSON, L. HOUGH and J. B. PRIDHAM, *Biochem. J.* **77**, 564 (1960).
[4] J. B. PRIDHAM and M. J. SALTMARSH, *Biochem. J.* **87**, 218 (1963).
[5] H. THIEME, *Pharmazie* **21**, 123 (1966).
[6] J. B. PRIDHAM and M. YOUNG, *Phytochem.* **6**, 462 (1967).
[7] M. PŠENÁK, A. JINDRA, P. KOVÁCS and H. DULOVCOVÁ, *Phytochem.*, in press.
[8] W. L. PORTER, *Anal. Chem.* **23**, 412 (1951).

emulsin: glucose and saligenin were identified in both cases. The isosalicin had absorption maximum at 274 nm and in the presence of 0·001 N sodium hydroxide at 294 nm.

Glucose Transferase Activity in vitro

When salicin or arbutin was treated with enzyme preparations of seedlings or roots of *Geum urbanum*, various phenol-positive spots were detected by paper chromatography which were not formed in the untreated controls . The R_f values of newly formed products in these experiments are given in Table 1. Diazotized *p*-nitroaniline gave the same colour with all spots as that with saligenin or arbutin respectively.

TABLE 1. R_f VALUES OF PHENOL-POSITIVE COMPOUNDS FORMED FROM SALICIN AND ARBUTIN *in vitro*

Products from	R_f solvent	
salicin	A	B
Saligenin	0·84	0·81
Isosalicin	0·59	0·54
E	0·41	0·33
D	0·35	0·27
C	0·29	0·22
B	0·17	0·09
A	0·12	0·01
A_1	0·04	—
Products from arbutin		
Quinol	0·85	0·82
Arbutin	0·51	0·44
I	0·34	0·24
H	0·25	0·15
G	0·21	0·12
F	0·09	0·03

A: *n*-butanol–acetic acid–water (4:1:2).
B: ethyl acetate–acetic acid–water (9:2:2).

The newly formed products were purified on a column of active charcoal and the colourless eluate was applied on a column of Sephadex G-10 giving four main peaks. Fractions corresponding to individual peaks were analysed by u.v. spectra with addition of sodium hydroxide: all products from salicin were shown to be derivatives of saligenin. Paper chromatography showed that the first peak contained compound A_1, the second two compounds (A and B) and the third three compounds (C, D and E). The fourth peak contained isosalicin and unreacted salicin. In the same way, the products formed from arbutin as substrate also gave four peaks on Sephadex G-10. No phenol-positive compounds were detected by diazotized *p*-nitroaniline in fractions corresponding to the first peak. The second peak contained compounds F and the third three compounds (G, H, I). Unchanged arbutin was found in the fourth peak.

Compounds A, C and G were separated on paper[8] and eluates hydrolysed with acid or almond emulsin. Compound C gave saligenin and glucose. By partial acid hydrolysis, a

reducing disaccharide and isosalicin were obtained. The unknown reducing sugar was tentatively identified chromatographically as gentiobiose in three solvent systems (*n*-butanol–pyridine–water (6:4:3); ethyl acetate–acetic acid–water (9:2:2); *n*-butanol–pyridine–water–benzene (5:3:3:1)). Compound C is therefore *o*-hydroxybenzyl-β-gentiobioside. In an analogous way, compound G was identified as *p*-hydroxyphenol-β-gentiobioside.

Partially hydrolysed samples of compound A showed the presence of saligenin, isosalicin, saligenin gentiobioside, glucose, gentiobiose and an unknown reducing sugar. The unknown sugar was separated and partially hydrolysed: glucose and gentiobiose were identified. Compound A is therefore very probably *o*-hydroxybenzyl-β-gentiotrioside.

Compounds A_1 and F, present only in low concentration, were not fully identified. However, from their chromatographic, u.v.-spectrophotometric and elution (from Sephadex G-10) data, it appears they are higher oligosaccharide derivatives. Compounds B, D, E, H and I decomposed spontaneously to glucose and saligenin or quinol respectively under the experimental contitions used and were not examined further.

Mechanism of Glucose Transfer

Quantitative experiments. The mechanism of glucose transfer was studied using salicin as substrate. During incubation, samples were analysed at several intervals and the formation of phenol-positive spots noted. Quantitative determinations were made of glucose, saligenin and isosalicin during the reaction and are given in Fig. 1. It was found that saligenin gentiobioside was only formed after 30 min of incubation.

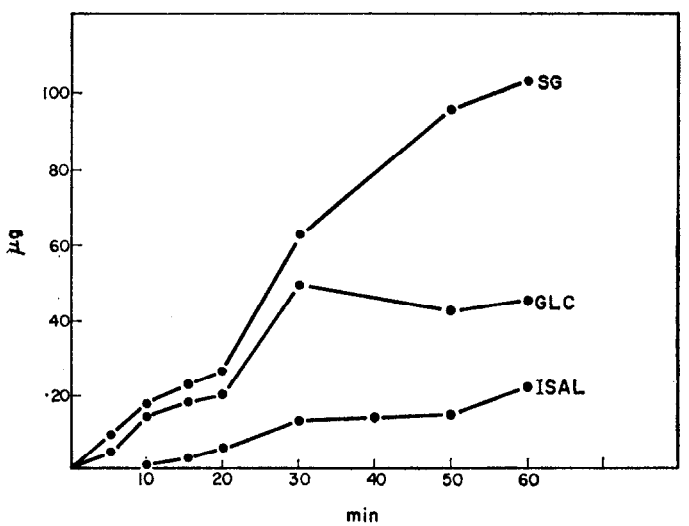

FIG. 1. ESTIMATION OF *glucose* (GLC), *saligenin* (SG) AND *isosalicin* (ISAL) FORMED FROM SALICIN *in vitro* (RESULTS ARE GIVEN IN µg/l mg ACETONE POWDER).

δ-Gluconolactone and $HgCl_2$ inhibited the production of saligenin (Table 2). If δ-gluconolactone was added to samples after 30 min incubation (at which time saligenin, glucose and isosalicin were present), it inhibited the formation of saligenin gentiobioside. When glucose-[U-^{14}C] was added to the medium, it was incorporated significantly only into saligenin gentiobioside and not into isosalicin (Table 3).

TABLE 2. EFFECTS OF INHIBITORS ON SALIGENIN FORMATION *in vitro*

Inhibitors	Concentration (mmolar)	Saligenin* (μg/l mg AP†)	Inhibition (%)
δ-Gluconolactone	25	11	66
δ-Gluconolactone	10	13	59
$HgCl_2$	25	4	87
$HgCl_2$	10	7	78
Control	—	32	—

* After 30 min incubation.
† AP: acetone powder.

TABLE 3. INCORPORATION OF [U-^{14}C]-GLUCOSE INTO ISOSALICIN AND SALIGENIN GENTIOBIOSIDE

Products	Concentration (μmoles)	Counts/min	Specific activity
Isosalicin	19·7	15	0·76
Saligenin gentiobioside	4·2	96	23·00

DISCUSSION

Infiltration of saligenin or salicin into *Geum* plants yielded a new phenol-positive compound identified as isosalicin. Enzyme preparations of the roots catalysed the formation of several phenol-positive compounds from salicin, all having λ_{max} at 274 nm (like saligenin) shifting to 294 nm with sodium hydroxide. The compounds were assumed to be derivatives of saligenin having a free phenolic group because they gave the same colour as saligenin with diazotized *p*-nitroaniline.

Elution from Sephadex G-10 gave six new substances (Table 1) and made it possible to get an idea of the molecular size of individual compounds. Compound A_1, which is eluted from the column before compound A (which is most probably the triglucoside of saligenin), might be saligenin tetraglucoside. Compound B is eluted together with A and might be also a saligenin triglucoside, but its sugar component remains unknown. Compounds C, D and E are molecules of the same size; since compound C was identified as saligenin gentiobioside, and by analogy with other work with β-glucosidase,[9] compounds D and E might contain β-1→3 and β-1→4 linked disaccharides.

Products formed from arbutin showed maximum absorption at 280 nm; they gave the same colour with diazotized *p*-nitroaniline as arbutin did. Elution of these products from the column of Sephadex G-10 revealed sizes of their molecules in the following decreasing sequence: F, G, H, I and arbutin. Compound G was identified as gentiobioside of quinol, compound F is assumed to be a triglucose derivative. By analogy with compound D and E it is possible that compounds H and I contain β-1→3 and β-1→4 linked disaccharides. The

[9] C. G. C. CHESTERS and A. T. BULL, *Nature* **202**, 454 (1964).

partially purified enzyme preparation from a young aspen[10] (*Populus grandidentata*) catalysed the formation of *p*-hydroxyphenyl-β-gentiobioside, if arbutin was used as substrate.[3]

The compounds were formed from salicin or arbutin using acetone powders without any energy-rich donor being added. It appears likely that isosalicin and saligenin gentiobioside are formed in a step wise manner (Table 3 and Fig. 1). Isosalicin must be produced by direct transfer of glucose from salicin to saligenin (Table 3). The formation of saligenin gentiobioside involves the addition of free glucose from the medium. On the other hand, in the formation of *p*-hydroxyphenyl-β-gentiobioside, Pridham[10] proposed a transfer of glucose from one molecule of arbutin to the second. It seems possible to us, however, that the gentiobioside of quinol might be formed in an analogous way, at least in the first phase of reaction, as found for saligenin-β-gentiobioside.

The work on purification of the enzyme system is in progress.

EXPERIMENTAL

Plant Material

Geum urbanum plants were grown in the Botanical Gardens of the Faculty of Pharmacy, Bratislava, under normal soil and climate conditions. Seedlings were grown in a greenhouse at 25° and 3-week-old plants were used for experiments. Acetone powders were prepared from seedlings and from the roots of 2-year-old plants in the flowering stage.

Paper Chromatography

The following solvent systems were used: *n*-BuOH saturated with H_2O,[11] *n*-BuOH–acetic acid–H_2O (4:1:5),[12] *n*-BuOH–pyridine–H_2O–benzene (5:3:3:1),[9] *n*-BuOH–acetic acid–H_2O (4:1:2),[13] ethyl acetate–acetic acid–H_2O (9:2:2)[4] and *n*-PrOH–ethyl acetate–H_2O (7:1:2)[14] (Whatman No. 1, descending technique). For the detection of sugars, diphenylamine[15] and silver nitrate[16] were used; for phenols, diazotized *p*-nitroaniline[17] in 20% Na_2CO_3.

In vivo *Experiments*

Salicin and saligenin (1% in H_2O) were infiltrated into both seedlings, through the roots, and isolated leaves of *Geum* plants (3 hr into seedlings, 6 hr into leaves).

After infiltration the plant material was homogenized in boiling methanol (25 ml/g) the supernatant centrifuged, evaporated to approx. 0·5 ml and analysed paper chromatographically.

The products formed were separated[8] (Whatman No. 3, with *n*-BuOH–H_2O) and the areas corresponding to individual compounds eluated with 50% EtOH.

Salicin and isosalicin were separated on a polyamide column with dil. MeOH. Fractions were analysed at 274 nm and 269 nm respectively.

Acidic hydrolysis was carried out at 100° with 1 N H_2SO_4 for 90 min; partial hydrolysis by heating for only 10 min. The hydrolysate was neutralized with $BaCO_3$, centrifuged and analysed chromatographically.

For enzymic hydrolysis, almond emulsin was used (in 0·05 M acetate buffer, pH 4·6) for 1 hr.

In vitro *Experiments*

The mixtures (total volume 5·0 ml) containing 100 mg of salicin or arbutin and 0·1 mg acetone powder in 0·05 M acetate buffer, pH 4·6, were incubated at 27° for 6 hr. Samples were inactivated by heat, centrifuged and applied to a column (20 × 1·5 cm) of active charcoal[18] and Celite 535 (1:1). The column was washed with H_2O (500 ml) and the phenolic glucosides eluted with 50% EtOH (600 ml); the EtOH eluate was concentrated to 5 ml and applied to a Sephadex G-10 column (60 × 2 cm) which was eluated by H_2O. The fractions

[10] J. B. PRIDHAM, *Biochem. J.* **76**, 13 (1960).
[11] M. A. JERMYN and F. A. ISHERWOOD, *Biochem. J.* **44**, 402 (1949).
[12] S. M. PARTRIDGE, *Biochem. J.* **42**, 238 (1948).
[13] S. HATTORI and M. SATÔ, *Phytochem.* **2**, 385 (1963).
[14] D. S. FEINGOLD, G. AVIGAD, and S. HESTRIN, *Biochem. J.* **64**, 351 (1956).
[15] J. L. BUCHAN and R. J. SAVAGE, *Analyst* **77**, 401 (1952).
[16] W. E. TREVELYAN, D. P. PROCTER and J. S. HARRISON, *Nature* **166**, 444 (1950).
[17] T. SWAIN, *Biochem. J.* **53**, 200 (1953).
[18] G. SCHRAMM and J. PRIMOSIGH, *Ber. Dtsch. Chem. Ges.* **76**, 379 (1943).

(4 ml) were estimated by u.v. at 274 nm (salicin) or 280 nm (arbutin). Fractions corresponding to individual peaks were bulked and analysed as before.[8]

Estimation of Free Glucose, Saligenin and Isosalicin

Samples (0·2 ml) were taken at intervals and 0·3 ml ethanol added. Saligenin and isosalicin were estimated as described by Pridham[20] and glucose determined by the anthrone method after separation on paper.[19]

Incorporation of [U-^{14}C]-*Glucose*

To the mixture (see *in vitro* experiments) [U-^{14}C]-glucose (0·001 μc) was added. Incubation, purification, separation and estimation of individual products was carried out as described. For determination of ^{14}C the samples were dried on planchets and assayed in a gas-flow counter (NHZ-619, Tesla).

Experiments with Inhibitors

The mixture (see *in vitro* experiments) contained δ-gluconolactone and $HgCl_2$ (final concentration $2{\cdot}5 \times 10^{-2}$ M and 1×10^{-2} M).

[19] M. PŠENÁK, D. WOITOWITZ, P. KOVÁCS and A. JINDRA, *Československ. Farm.* **14**, 397 (1965).
[20] J. B. PRIDHAM, *Anal. Chem.* **29**, 1167 (1957).