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STERYL GLUCOSIDE AND ACYL GLUCOSIDE BIOSYNTHESIS IN MATURING PEA SEEDS

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Abstract—Sterol glucosyltransferase activity was found in a particulate fraction of pea seeds. The activity was stimulated by Ca^{2+} and Mg^{2+} and inhibited by Zn^{2+}, Cu^{2+}, Hg^{2+}, EDTA and EGTA. Iodoacetamide was without effect but *p*-chloromercuribenzoate completely inhibited the enzyme. *N*-Ethylmaleimide gave 60–70% inhibition over a wide range of concentrations. The activity was stimulated by ATP in the presence of Mg^{2+}. Under such conditions, steryl acyl glucoside was formed. The acyl derivative was barely detectable in the presence of Ca^{2+} either with or without ATP. Both oleyl CoA and palmityl CoA stimulated acyl glucoside synthesis. Of the four nucleoside triphosphates, ATP, GTP, UTP and CTP both ATP and CTP stimulated acylation in the presence of Mg^{2+}. The observations suggest that acyl donors other than digalactosyl diglyceride and phospholipids may function in steryl acyl glucoside synthesis in plants.

INTRODUCTION

There have been several reports of steryl glucoside and steryl acyl glucoside synthesis in plants [1–10]. The sequence of reactions occur according to the sequence: sterol → steryl glucoside → steryl acyl glucoside. A number of acyl donors have been found to be effective including phosphatidyl ethanolamine for an acetone powder preparation of wheat seedlings [9], phosphatidyl inositol for a partly solubilized enzyme preparation from *Calendula officinalis* [6] and digalactosyl diglyceride for a soluble enzyme from carrot root [8]. More recently phosphatidyl choline has been shown to acylate steryl glucoside in a solubilized membrane preparation from cotton fibres [11, 12].

We previously observed stimulation of the sterol glucosyl transferase of etiolated pea seedling axis tissue by ATP [5]. In another report ATP was shown to stimulate steryl glycoside synthesis in tobacco seedlings [1]. This communication reports the dependence of glucosylation on the presence of free sulphydryl and the participation of ATP in the acylation of the glucoside.

RESULTS AND DISCUSSION

The enzyme activity was isolated in a fraction sedimenting between 1000 and 18000*g*, designated the 18000*g* pellet, from an homogenate of pea seeds prepared in 50 mM Tris–HCl (pH 8) which was 0.25 M in sucrose. The glucosyltransferase activity of this fraction was assayed by measuring the amount of ^{14}C appearing in a $CHCl_3$–MeOH extract of the reaction mixture after its incubation with UDPglucose-[U-^{14}C]). TLC examination of the extract in three different solvent systems followed by ^{14}C scanning of the chromatograms revealed radioactive material chromatographically identical with steryl glucoside. It was the sole product of the reaction during the 15 min incubation. Acid hydrolysis of the product and isolation of the glycone and aglycone revealed glucose and sterol to be the products.

Like the glucosyltransferase of pea seedling axis tissue [5] the enzyme was stimulated by Ca^{2+} and Mg^{2+} and inhibited by Zn^{2+}, EDTA and EGTA (Table 1). The requirement for a free sulphydryl group for enzyme activity is suggested by the complete inhibition of the activity on exposure to Cu^{2+} and Hg^{2+}.

This requirement for free sulphydryl was examined further by use of other sulphydryl inhibitors. In the presence of 4 mM iodoacetamide, *N*-ethylmaleimide (NEM) and *p*-chloromercuribenzoate (*p*CMB), the inhibition of glucosyltransferase activity was 4, 66 and 96% respectively. The effect of a range of concentrations of NEM on the activity showed that the inhibition was maximally 60–70% from 1–20 mM of the inhibitor. We have found the glucosyltransferase from a similar

Table 1. The influence of metal ions on sterol:UDPglucose glucosyltransferase

Addition (10 mM)	Specific activity (nmol/hr/mg)
None	20.4
Boiled enzyme	0.1
Ca^{2+}	51.3
Mg^{2+}	31.2
Zn^{2+}	0.9
Cu^{2+}	0.2
Hg^{2+}	0.0
EDTA	16.5
EGTA	6.3

Reaction mixtures contained 0.3 ml enzyme (0.85 mg protein), 7.5 μmol of the metal ion and 0.5 μmol UDPglucose-[U-^{14}C] (0.5 μCi) in 50 mM Tris–HCl buffer (pH 8) in a total vol. of 0.75 ml. The reaction was run at 28° for 15 min.

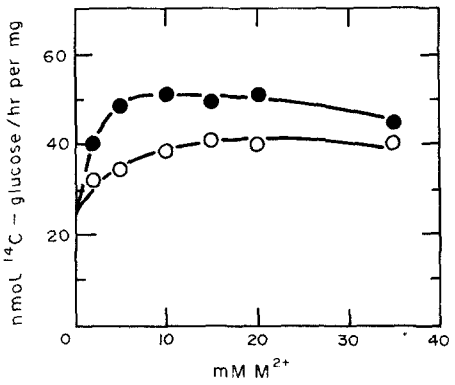

Fig. 1. Influence of a range of concentrations of Mg^{2+} (O—O) and Ca^{2+} (●—●) on glucosyltransferase. The reaction procedure for the assay is described in the Experimental.

fraction from pea seedling axis tissue to behave similarly. It is not clear what limits the inhibition by NEM unless there is more than one transferase only one of which is dependent on the presence of free sulphydryl. Alternatively, it is possibly a consequence of the distribution of the membrane-bound enzyme in a population of vesicles in which 60–70% have the enzyme located outside and accessible to the inhibitor. If this is the case then there must also be present in such a vesicle a system for transporting the substrate, UDPglucose, across the membrane as it is unlikely that such a molecule would enter the vesicle passively. If a transport system were involved it might partly explain the stimulation of the glucosylation reaction by ATP (see later). The different degrees of inhibition of the three inhibitors may be a result of their relative hydrophobicities. The glucosyltransferase is membrane-bound and must occupy a relatively hydrophobic environment. Inhibition by *p*CMB and not by iodoacetamide has been suggested as evidence for a hydrophobic pocket at the active site of the enzyme adenosine deaminase [13].

The transferase activity was stimulated over a broad range of concentrations of both Ca^{2+} and Mg^{2+} but was maximal for both metal ions in the 15–20 mM range (Fig. 1).

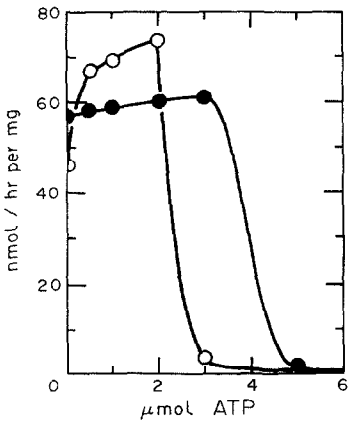

Fig. 2. Influence of ATP on glucosyltransferase activity. Reaction mixtures were as described in the Experimental but contained ATP and either Mg^{2+} (O—O) or Ca^{2+} (●—●) (15 μmol) The reactions were run at 28° for 15 min

We had previously observed that the glucosyltranferase from pea seedling tissue was slightly stimulated in the presence of ATP [5]. The same was true here (Fig. 2) but the effect was more pronounced in the presence of Mg^{2+} In each case, however, a precipitous loss of activity occurred at high concentrations of ATP. The influence of ATP on the product distribution in the $CHCl_3$–MeOH extracts obtained from incubations in the presence of either Ca^{2+} or Mg^{2+} was revealed in the time-course studies (Figs 3a and b). TLC of the extracts at each time interval allowed the separation of two radioactive products, the glucoside and a less polar compound. The latter compound was chromatographically identical with steryl acyl glucoside and, on base hydrolysis, produced the glucoside. It was clear that in the presence of ATP the production of acyl glucoside was only slightly increased with Ca^{2+} as the activator (Fig. 3a) but with Mg^{2+} acyl glucoside production was markedly increased (Fig. 3b).

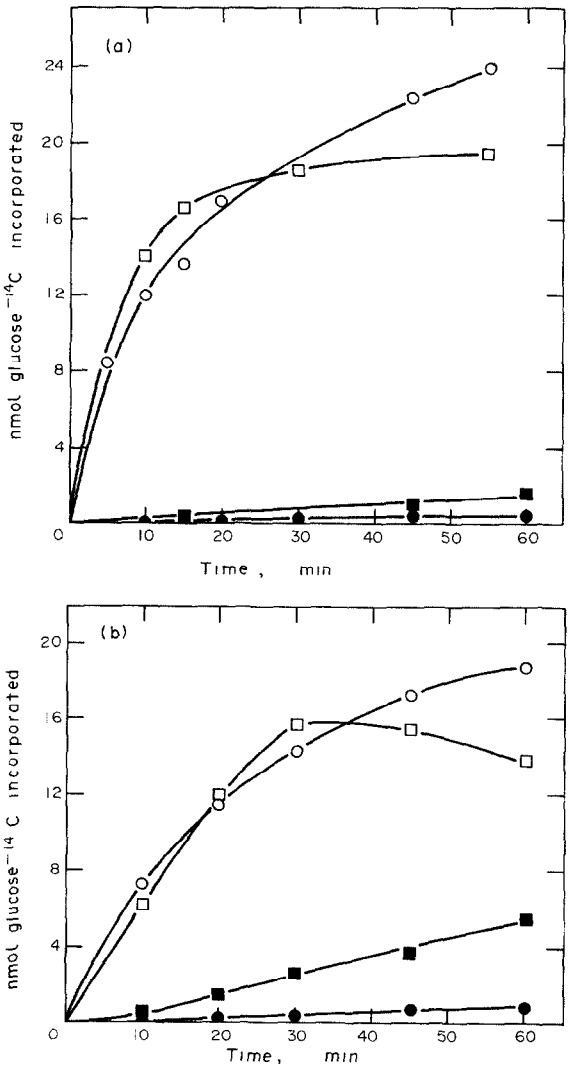

Fig. 3. Time-course of incorporation of ^{14}C rom UDPglucose-[U-^{14}C] into steryl glucoside and acyl glucoside in the presence of (a) Ca^{2+} and (b) Mg^{2+}. The reaction prodedure is described in the Experimental. Steryl glucoside is shown (O—O) in the absence, and (□—□) in the presence of ATP; acyl glucoside is shown (● —●) in the absence and (■—■) in the presence of ATP.

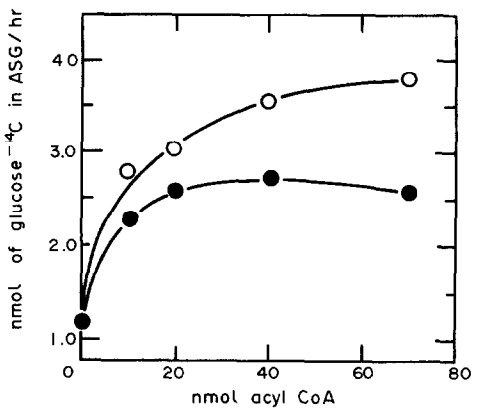

Fig. 4. Stimulation of acyl glucoside (ASG) synthesis by acyl CoA. Reaction mixtures contained enzyme (0.25 ml, 0.6 mg protein), UDPglucose-[U-^{14}C] (0.5 μmol, 0.05 μCi), Mg^{2+} (15 μmol), palmityl CoA (●—●) or oleyl CoA (○—○) and 2 mg of fatty acid-free BSA in a total vol. of 0.75 ml. Reactions were run at 28° for 1 hr.

The stimulation of acyl glucoside synthesis in the presence of Mg^{2+} and ATP suggested the participation of acyl CoA in the reaction. In the few cases that have been examined for steryl acyl glucoside synthesis in plants the acyl donor was either a phospholipid [6, 9, 11, 15] or digalactosyl diglyceride [8]. Elbein *et al.* [11] have also shown palmityl CoA can serve as an acyl donor, albeit a poor one, using a solubilized acyltransferase from cotton fibre membrane preparation. In Fig. 4 the stimulation of acyl glucoside synthesis in the presence of oleyl CoA and of palmityl CoA is shown. Attempts to substitute the acyl CoAs with ATP, CoA and either palmitic acid or oleic acid to generate the acyl coAs *in situ* were unsuccessful. The glucosylation reaction suffered 50–60% inhibition in the presence of such a mixture even though the assays were conducted in the presence of fatty acid-free BSA to minimize the detergent effects of such a mixture.

Although acylation may occur through the intermediacy of acyl CoAs, a contributing factor in the stimulation of the acylation reaction by ATP may be a consequence of the chelating properties of the triphosphate moiety in binding a metal ion inhibitor of the acylation reaction. To examine this possibility the enzyme reaction was conducted in the presence of Mg^{2+}, the labelled substrate, and each of the four nucleoside triphosphates, ATP, UTP, GTP and CTP. The results are shown in Table 2. It is clear that acylation was enhanced not only by ATP but also by CTP. The effect of GTP and UTP were minimal, UTP was slightly inhibitory to the incorporation of ^{14}C into glucoside and acyl glucoside combined. It is known that UDP is an inhibitor of the glucosyltransferase [14] and presumably UTP acts similarly or does so indirectly by undergoing hydrolysis to UDP. The stimulation of acylation by CTP suggests the participation of yet another acyl donor in the acylation reaction, one which is linked to CDP, e.g. CDP-glyceride.

Table 2. The influence of nucleoside triphosphates on the formation of acylated steryl glucoside

Nucleoside triphosphate	Steryl glucoside (nmol)	Acylated steryl glucoside (nmol)
None	18.2	0.8
ATP	15.6	3.8
UTP	16.0	1.0
GTP	19.1	1.4
CTP	15.5	3.3

The reaction mixtures contained 0.25 ml enzyme (0.70 mg protein), 15 μmol of Mg^{2+}, 1.5 μmol of each nucleoside triphosphate and 0.5 μmol of UDPglucose-[U^{14}C] in 50 mM Tris–HCl buffer in a total vol. of 0.75 ml. The reactions were run for 1 hr at 28°. The $CHCl_3$–MeOH extracts were analyzed by TLC.

EXPERIMENTAL

Materials. Pea seeds, *Pisum sativum* L. cv Alaska, were from W. Atlee Burpee Co. Riverside, California. UDPglucose-[U-^{14}C] (155 μCi/μmol) was obtained from International Chemical and Nuclear, Irvine, California. Unlabelled UDP-glucose was from Calbiochem, La Jolla, California. Nucleoside di- and tri-phosphates and the acyl CoAs were from Sigma Chemical Co., St. Louis, Missouri and from Calbiochem. They were freshly prepared in 50 mM Tris–HCl buffer (pH 8) immediately before use. Aquasol counting fluor (New England Nuclear Corp.) was used for radio assay.

Methods. TLC solvent systems and detection of spots were as previously described [5]. Separation of acyl glucoside from glucoside was accomplished by TLC on Si gel sheets activated at 105° for 15 min then run in an unsaturated tank containing $CHCl_3$–EtOH–H_2O (90:9:0.5). The glucoside had R_f 0.25 and the acyl glucoside 0.57. TLC of the hexane extract from acid hydrolysis of the steryl glucoside was in EtOAc–hexane (22:3) [15]. Paper chromatography of the aq. phase from acid hydrolysis of the steryl glucoside was in *n*-BuOH–HOAc–H_2O (12:3:5) [16]. Spots were detected with a *p*-anisidine spray followed by brief heating. Glucose had R_f 0.17.

Radioactivity measurements. $CHCl_3$–MeOH extracts were counted by evapn of the sample in a vial followed by addition of 4 ml scintillation fluor. ^{14}C counting of the glucoside and acyl glucoside, separated by TLC, employed a chromatogram scanner to locate precisely the zones corresponding to the two compounds. These areas were scraped into counting vials and mixed with Aquasol fluor for counting. Efficiency of counting was determined by spiking the samples with toluene-[^{14}C] for the $CHCl_3$–MeOH extracts. Overall efficiency of quantitating the radioactive materials after TLC was obtained by running known amounts of previously isolated radioactive steryl glucoside and acylated glucoside through the entire procedure of TLC, and removal and counting of radioactive zones.

Protein measurement. This was determined by modification of the Lowry procedure [17] using BSA as standard.

Maturing seed tissue. Pea plants were grown in a green-house on a cycle of 16 hr light/8 hr dark under Grolux lights. Night and day temp. were 10 and 24° respectively. The seeds were harvested at a late stage of development longer than 24 days after anthesis.

Preparation of the sterol UDPglucose glucosyltransferase. Seeds were frozen in liquid N_2 then were ground in a pestle and mortar similarly cooled. To the fine powder was added 50 mM Tris–HCl buffer (pH 8) which was 0.25 M in sucrose. Buffer to tissue ratio was 1.5 ml/g The well-mixed slurry was squeezed through Miracloth and the filtrate centrifuged at 1000 *g* for 5 min and the supernatant from this at 18000 *g* for 20 min. The 18000 *g* pellet was resuspended in 50 mM Tris–HCl (pH 8). The final vol. of enzyme was typically 25 ml from 20 g seed tissue and contained about 2.5 mg protein/ml. The samples were frozen in liquid N_2 and stored at −20°. Such preps were used to examine the metal ion and sulphydryl inhibitor effects on the transferase. Later preps employed a buffer with 10 mM EDTA and 10 mM mercaptoethanol for tissue maceration and the 18000 *g* pellet was resuspended in 50 mM Tris–HCl (pH 8) containing 10 mM mercaptoethanol. Such preps were usually about 10% more active than those prepared without the EDTA and mercaptoethanol.

Sterol·UPDglucose glucosyltransferase assay. Reaction mixtures contained 0.3 ml enzyme (*ca* 0.75 mg protein), 15 μmol of either Ca^{2+} or Mg^{2+}, 0.5 μmol UDPglucose-[U-^{14}C] (0.05 μCi) and 50 mM Tris–HCl (pH 8) to give a final vol. of 0.75 ml. The reaction was run at 28° and stopped after 15 min with $CHCl_3$–MeOH (2:1). The radioactive products were isolated as previously described [5]. To examine the influence of metal ions and chelating agents the reaction was conducted using 7.5 μmol of the appropriate addition. For the influence of ATP, a range of concentrations was added to the reaction mixture with appropriate decrease in the addition of buffer to maintain the final vol. at 0.75 ml.

Acid hydrolysis of steryl glucoside. Samples isolated by elution with $CHCl_3$–MeOH from TLC scrapings were evapd under N_2 at room temp. The combined samples were heated under reflux in 0.5% H_2SO_4 in EtOH–H_2O (19:1) for 20 hr. After neutralization with NaOH, sterol was extracted with hexane.

Reaction with sulphydryl inhibitors. 0.5 ml enzyme was incubated at 0° for 5 min with 2 μmoles each of either NEM, *p*CMB, iodoacetamide or the corresponding vol. of Tris–HCl buffer. The mixtures were centrifuged at 105000 *g* for 10 min at 4° and the pellets resuspended in buffer to give the original vol. for measurement of the glucosyltransferase. The transferase was assayed in the usual way in the presence of 20 mM Ca^{2+}. An identical procedure was adopted using a range of concns of NEM from 1 to 20 mM in the pre-incubation vol.

Time-course of glucosylation and acylation of glucoside. 4 separate tubes each containing 7× the vol. used for the glucosyltransferase assay were prepared. One pair of tubes contained 105 μmol of Ca^{2+}, the other 105 μmol of Mg^{2+}. One tube of each pair contained 10.5 μmol. of ATP. The mixtures were incubated at 28° for 1 hr. At several intervals during the incubation period 0.75 ml samples of the mixture were removed and added to 2 ml of $CHCl_3$–MeOH (2:1). The reaction products were isolated, separated by TLC and the ^{14}C in glucoside and acylglucoside quantitated.

Base hydrolysis of acyl glucoside. Samples isolated by elution with $CHCl_3$–MeOH from TLC scrapings were evapd under N_2 at room temp. and heated under reflux with 10% KOH in EtOH–H_2O (1:1) for 16 hr. The cooled soln was extracted with $CHCl_3$–MeOH and the concd extract subjected to TLC.

Reaction with acyl CoAs. To a series of tubes containing the reaction mixture for the glucosyltransferase using 15 μmol Mg^{2+} and 2 mg fatty acid-free BSA was added either palmityl CoA or oleyl CoA from 0–75 nmol. The reactions were run at 28° and stopped after 1 hr. The reaction products were isolated and the acyl glucoside separated and quantitated as above.

Influence of nucleoside triphosphates. The glucosyltransferase assay was run in 5 separate tubes in the presence of 15 μmol Mg^{2+} and either 1.5 μmol each of ATP, UTP, GTP, CTP or an equivalent vol. of buffer. The radioactive products were isolated, separated and quantitated as described.

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REFERENCES

1. Bush, P. B. and Grunwald, C. (1974) *Plant Physiol.* **53**, 131.
2. Kauss, H. (1968) *Z. Naturforsch.* **23b**, 1522.
3. Laine, R. H. and Elbein, A. D. (1971) *Biochemistry* **10**, 2547.
4. Ongun, A. and Mudd, J. B. (1970) *Plant Physiol.* **45**, 255.
5. Fang, T.-Y. and Baisted, D. J. (1976) *Phytochemistry* **15**, 273.
6. Wojciechowski, Z. A. and Zinowski, J. (1975) *Biochim. Biophys. Acta* **398**, 111.
7. Forsee, W. T., Laine, R. A. and Elbein, A. D. (1974) *Arch. Biochem. Biophys.* **161**, 248.
8. Eichenberger, W. and Grob, E. C. (1970) *Chimia* **24**, 394.
9. Péaud-Lenoël, C. and Axelos, M. (1972) *Carbohyd. Res.* **24** 247.
10. Eichenberger, W. and Siegrist, H. P. (1975) *FEBS Letters* **52**, 153.
11. Elbein, A. D., Forsee, W. T., Schultz, J. C. and Laine, R. A. (1975) *Lipids* **10**, 427.
12. Elbein, A. D. and Forsee, W. T. (1976) *Arch. Biochem. Biophys.* **172**, 410.
13. Ronca, G., Bauer, C. and Rossi, C. A. (1967) *European J. Biochem.* **1**, 434.
14. Hou, C. T., Umemura, U., Nakamura, M. and Funahashi, S. (1968) *J. Biochem.* **53**, 351.
15. Baisted, D. J. (1971) *Biochem. J.* **124**, 375.
16. Storm, D. L., Burri, R. C. and Hassid, W. Z. (1973) *Biochem. Biophys. Res. Commun.* **50**, 147.
17. Potty, V. H. (1969) *Anal. Biochem.* **29**, 535.