Characterization of dolichol and dolichyl phosphate phosphatase from soya beans (*Glycine max*)

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A series of polyprenols, ranging in length from 15 to 22 isoprene units, has been isolated from soya beans (*Glycine max*) and purified by high-pressure liquid chromatography. N.m.r., i.r. and mass spectra of the compounds indicated that they are α -saturated polyprenols of the dolichol type. The amount present in dry seeds was about 9 mg/100 g, whereas dolichyl phosphate (Dol-P) was present only in trace amounts. Dol-P phosphatase activity was detected in the microsomal fraction of 5-day-old germinating soya-bean cotyledons. The Dol-P phosphatase activity was linear with respect to time and protein concentration and exhibited a broad pH optimum (pH 7–9). Triton X-100 was necessary for significant enzyme activity. Enzyme activity was slightly enhanced by EDTA, whereas dithiothreitol was without effect. An apparent K_m of 5 μ M was determined for Dol-P. Bivalent metal ions were not required for enzyme activity. A number of phosphorylated compounds tested as enzyme substrates (including a number of nucleoside phosphates, glucose 6-phosphate, sodium β -glycerophosphate and $\text{Na}_4\text{P}_2\text{O}_7$) did not compete with [1- ^3H]Dol-P as substrate. A number of phospholipids were also tested for their ability to act as Dol-P phosphatase substrates. At 1 mM concentration, phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid and lysophosphatidic acid each inhibited enzymic activity. However, at 0.1 mM concentration, phosphatidylcholine and phosphatidylethanolamine were slightly stimulatory, whereas phosphatidic acid and lysophosphatidic acid were still inhibitory. Phosphatidic acid showed competitive inhibition.

The presence of asparagine-linked oligosaccharides in plant glycoproteins has been established (Sharon & Lis, 1979). The participation of specific lipid sugar carriers in the biosynthesis of *N*-linked glycoproteins is also well known in animal, yeast, higher-plant and bacterial systems. In animals and in yeast, the lipid portion was characterized and found to be an α -saturated polyprenol containing 14–24 isoprene units, i.e. dolichol (Parodi & Leloir, 1979). The free lipid portion in bacteria was identified as bactoprenol, an unsaturated polyprenol containing 11 isoprenoid units (Hemming, 1974). There are several reports in the literature that suggest indirectly that the lipid carrier in plants is an α -saturated polyprenol (Pont Lezica *et al.*, 1975;

Lehle *et al.*, 1976; Brett & Leloir, 1977; Delmer *et al.*, 1978; Chadwick & Northcote, 1980; Staneloni *et al.*, 1980). In the present paper direct evidence is presented regarding the occurrence of dolichol and Dol-P in soya beans.

It has been suggested that availability of Dol-P may be a rate-limiting factor in the glycosylation process (Hemming, 1977; Carson *et al.*, 1981; Adrian & Keenan, 1981; Rip *et al.*, 1981). This means that the enzymes that influence Dol-P concentration (Dol-P phosphatase and dolichol kinase) could play an important role in the regulation of the biosynthesis of glycoproteins. A number of reports have appeared (Idoyaga-Vargas *et al.*, 1980; Wedgwood & Strominger, 1980; Adrian & Keenan, 1981; Burton *et al.*, 1981; Rip *et al.*, 1981; Belocopitow & Boscoboinik, 1982; Rupar *et al.*, 1982) regarding the occurrence and properties of Dol-P phosphatase. In the present study, we have observed for the first time an enzyme from a plant source that is active in the dephosphorylation of

Abbreviations used: Dol-P, dolichyl phosphate; DTT, dithiothreitol; PtdA, phosphatidic acid; lyso-PtdA, lyso-phosphatidic acid; PtdEtn, phosphatidylethanolamine, PtdCho, phosphatidylcholine; h.p.l.c., high-pressure liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Dol-*P*. Preliminary studies on the properties of this enzyme are presented.

Materials and methods

Chemicals

[1-³H]Dolichol (sp. radioactivity 12.5 Ci/mmol) was purchased from New England Nuclear, Boston, MA, U.S.A.; silica gel was obtained from E. Merck, Darmstadt, Germany; Tris base, bovine serum albumin and DTT were from Sigma, St. Louis, MO, U.S.A.; PtdA, PtdEtn, PtdCho and lyso-PtdA were from Sordary Research Laboratories, London, Ont., Canada; AMP, CMP, UMP, ADP, CDP, ATP, CTP and glucose 6-phosphate were from Boehringer, Dorval, PQ, Canada. Chromium(VI) oxide-pyridine complex, maleic acid and Triton X-100 were from Eastman Kodak Co., Rochester, NY, U.S.A.; dichloromethane was obtained from Aldrich Chemicals, Milwaukee, WI, U.S.A. All other chemicals used were from Fisher Scientific Co., Toronto, Ont., Canada.

Isolation of soya-bean polyprenol

Finely ground soya beans (200 g; First Line Seeds, Guelph, Ont., Canada) were digested by refluxing overnight in a mixture of 2 litres of 30% KOH, 400 ml of ethanol and 10 g of pyrogallol. The solution was then extracted three times with diethyl ether and the diethyl ether extracts were washed with water (Burgos *et al.*, 1963). Diethyl ether was removed by rotary evaporation and the residue was dissolved in a small amount of hexane. This solution was chromatographed on a column of Florisil, deactivated by 7% (w/v) water, as described by Carroll (1976). The polyprenol-rich fraction was eluted with 15% diethyl ether in hexane (Carroll *et al.*, 1973). The solvent was removed by evaporation and the residue was taken up into a small volume of hexane.

H.p.l.c. was performed on a Hewlett-Packard 1084A dual pump instrument equipped with a C₁₈ reverse-phase column (Brownlee Labs, Santa Clara, CA, U.S.A.). The column temperature was maintained at 45°C and the eluates were monitored at 210 nm with a Hewlett-Packard 1030B variable-wavelength detector. Sequential gradients of h.p.l.c.-grade methanol and propan-2-ol at a flow rate of 2 ml/min were used as mobile phase. The concentration of propan-2-ol was increased from 10 to 35% over the first 5 min and from 35 to 80% over the next 15 min. The partially purified polyprenol fraction from the Florisil column was injected repeatedly and the h.p.l.c. fractions corresponding to authentic human liver dolichol were collected and pooled. The 18-isoprene homologue corresponding to standard human dolichol was also isolated using h.p.l.c.

Analysis of soya-bean polyprenol

N.m.r. spectra of the fraction corresponding to standard dolichol were recorded in [²H]chloroform (as solvent) using a Varian T-60 spectrometer. Tetramethylsilane was used as internal reference standard. The compound was also oxidized to the aldehyde form, by the method of Ratcliffe & Rodehorst (1970) and purified using a Florisil column (Rupar & Carroll, 1978). I.r. spectra were recorded on a Beckman spectrophotometer. Mass spectrometry of the 18-isoprene homologue was carried out on a Varian MAT-311A instrument under conditions described previously (Carroll *et al.*, 1973).

Phosphorylation of [1-³H]dolichol and soya-bean polyprenol

[1-³H]Dolichol and the polyisoprenoid alcohol isolated from soya beans were phosphorylated by the method of Danilov & Chojnacki (1981). A Dol-*P* fraction was isolated from dry soya beans and 3-day-old cotyledons, by the method previously described for rat liver (Chaudhary *et al.*, 1982).

Preparation and analysis of microsomes from germinating soya beans

Soya-bean seedlings were grown in moist vermiculite in the dark at room temperature. On day 5 of germination, the cotyledons were removed and used for isolation of microsomes, essentially as described by Cherry (1974). Cotyledons were homogenized in 0.5 M-sucrose containing 10 mM-Hepes, pH 7.4. The homogenate was filtered through Miracloth (Calbiochem, La Jolla, CA, U.S.A.) and centrifuged at 40 000 *g* for 20 min. The resulting supernatant was again centrifuged (100 000 *g*) for 1 h and the pellet was washed and suspended in 0.5 M-sucrose/10 mM-Hepes (pH 7.4). The protein content of the microsomes was estimated on portions washed with cold 5% HClO₄, using the method of Lowry *et al.* (1951). Bovine serum albumin was used as standard.

The activity of Dol-*P* phosphatase was measured by monitoring the conversion of [1-³H]Dol-*P* into [1-³H]dolichol (Rip *et al.*, 1981). The standard 200 μl Dol-*P* phosphatase assay contained a final concentration of 100 mM-Tris/maleate, pH 7.4, 0.05% (w/v) Triton X-100, 1 μM-Dol-*P* (200 000 d.p.m.), 10 mM-EDTA and 50 μg of microsomal protein/ml. Mixtures were incubated in duplicate at 37°C for 30 min and the reaction was terminated by the addition of 4 ml of chloroform/methanol (2:1, v/v). Reaction mixtures were washed sequentially with 0.6 ml of water and 1.6 ml of chloroform/methanol/water (3:47:48, by vol.) and taken to dryness under N₂. The residue was dissolved in chloroform/methanol (2:1, v/v) and run on Silica gel H thin-layer-chromatographic plates in a solvent system of hexane/diethyl ether/acetic acid (65:35:1,

by vol.). The bands corresponding to the dolichol standard were located by exposing plates to I_2 vapour. Areas corresponding to standard dolichol

were scraped into vials and counted in 10ml of acidified Scintiverse. All experiments were repeated three times and the variation between replicate assays was less than 10%.

Results

Identification and quantification of soya-bean dolichol

The non-saponifiable soya-bean lipid fraction obtained after Florisil chromatography and h.p.l.c. gave a single spot with an R_F value similar to that of standard human liver dolichol on t.l.c. (Silica gel H) developed with hexane/diethyl ether/acetic acid (65:35:1, by vol.). Eight homologues (corresponding to 15 to 22 isoprene units) were observed on h.p.l.c. (Figs. 1a and 1b). The four major peaks were collected (Fig. 1c) and an n.m.r. spectrum was taken of the pooled material. It was very similar to the spectrum of human dolichol (Rupar, 1979). In order to confirm the α -saturation, a portion of the same compound was oxidized to the corresponding aldehyde and the i.r. spectrum was recorded. A C=O stretch bond was observed at $5.78\mu\text{m}$ (Fig. 2), indicating the absence of a double bond in the position $\alpha\beta$ to the aldehyde (Burgos *et al.*, 1963).

One of the soya-bean homologues (same retention time as human dolichol-18) was isolated by h.p.l.c. and was shown by mass spectrometry to contain 18 isoprene units (mass ion = 1244).

The concentration of dolichol in dry soya beans determined by h.p.l.c. was $8.6 \pm 0.5\text{mg}/100\text{g}$ ($n=6$). These values were corrected for losses incurred during isolation through the use of [$1\text{-}^3\text{H}$]dolichol as a tracer. Dol-P also appeared to be present in dry seeds and cotyledons but at very low concentrations. Similar types of h.p.l.c. profiles were observed with chemically phosphorylated soya-bean Dol-P and a fraction isolated from dry seeds and from cotyledons, using a recently described h.p.l.c. method for the quantification of Dol-P (Chaudhary *et al.*, 1982).

Fig. 1. H.p.l.c. profiles of dolichol and soya-bean poly-prenol

(a) shows results for human liver dolichol. The numbers above the major peaks represent, from left to right, the retention times for dolichols containing 18, 19, 20 and 21 isoprene units respectively. (b) shows the fraction from soya beans eluted from Florisil with 15% diethyl ether in hexane. (c) shows the four major peaks separated in (b) by h.p.l.c. with retention times similar to those of human dolichols-16-19.

Fig. 2. I.r. spectrum of aldehyde of the polyprenol identified by n.m.r.

Dol-P phosphatase

Kinetics. An enzyme that dephosphorylates Dol-P was detected in microsomes from soya-bean cotyledons. Activity was linear with respect to time (up to 60 min) and protein concentration (up to 100 $\mu\text{g}/\text{ml}$) (Figs. 3a and 3b). An apparent K_m of 5 μM was calculated for Dol-P, using a double-reciprocal plot (Fig. 3c). A broad pH optimum, ranging from pH 7 to 9, was observed (Fig. 4).

Fig. 3. Dol-P phosphatase kinetics

(a) shows the effect of incubation time on Dol-P phosphatase activity. The assay solution and experimental conditions were as described in the Materials and methods section except that the reaction was terminated at different time intervals. (b) shows the effect of microsomal protein on Dol-P phosphatase activity. Conditions are described in the Materials and methods section, except for variations in microsomal protein content. (c) shows a Lineweaver-Burk plot of Dol-P phosphatase activity. Assays were as described in the Materials and methods section, with Dol-P at concentrations of 1–10 μM .

Effect of detergent, bivalent metal ions, DTT and fluoride. In the absence of detergent, enzyme activity was very low (less than 10% of activity at optimal detergent concentration). The optimum detergent concentration was 0.05% when protein was used at 50 $\mu\text{g}/\text{ml}$ in the reaction mixture (Fig. 5). Bivalent metal ions do not appear to be required for enzyme activity, since at 10 mM concentration Ca^{2+} and Mn^{2+} were inhibitory (14 and 40% respectively) and Mg^{2+} had no effect. Activation by EDTA (128% of control at 10 mM) was observed, as has been reported in rat liver by Belocopitow and Boscoboinik (1982). DTT (10 mM) had no effect on enzyme activity, whereas 20 mM-fluoride was slightly inhibitory.

Substrate specificity. Water-soluble phosphates. A number of phosphate-containing compounds were tested as possible Dol-P phosphatase substrates. None of them competed with Dol-P as substrate (Table 1).

Phospholipids. Each of the phospholipids tested inhibited the enzyme activity at least 90% at 1 mM concentration. However, at 0.1 mM concentration

Fig. 4. Effect of pH on Dol-P phosphatase activity. The standard assay mixture was used except that the pH of the buffer was varied as indicated. Symbols: ●, sodium acetate/acetic acid; ▲, Tris/maleate; ○, Tris/HCl.

Fig. 5. Effect of different detergent concentrations on Dol-P phosphatase activity

Assays contained various detergent concentrations, at several protein concentrations, as indicated. Detergent concentration: ○, 0%; ●, 0.025%; ▲, 0.05%; □, 0.15%; △, 0.5%. All other experimental conditions were as described in the Materials and methods section.

Fig. 6. Competitive inhibition of Dol-P phosphatase activity by PtdA

Dol-P concentration was held constant while PtdA was used at several different concentrations: ○, no PtdA; △, 1.6 μ M-PtdA; □, 2.4 μ M-PtdA; ●, 3.2 μ M-PtdA.

Table 1. The effect of various phosphorus-containing compounds on soya-bean microsomal Dol-P phosphatase activity

Control assays were carried out as indicated in the Materials and methods section. Each value represents the average of three experiments, each using three assays. Water-soluble phosphates tested as substrate were present at 5 mM, whereas phospholipids were present at 0.1 mM.

Addition	Activity(%)
Water-soluble phosphates	
None (control)	100
Na ₄ P ₂ O ₇	98
Sodium β -glycerophosphate	104
Glucose 6-phosphate	98
AMP	121
CMP	111
UMP	88
CDP	90
ADP	90
ATP	104
CTP	84
Phospholipids	
None (control)	100
PtdA	2
PtdCho	152
PtdEtn	145
Lyso-PtdA	15

PtdEtn and PtdCho activated the enzyme, whereas PtdA and lyso-PtdA were still inhibitory (Table 1). With PtdA a competitive type of inhibition was observed (Fig. 6). Similar observations concerning the effect of phospholipids have been reported by Belocopitow & Boscoboinik (1982), using rat liver enzymes.

Soya-bean Dol-P. Since the addition of chemically phosphorylated dolichol to assays containing [1-³H]Dol-P as substrate resulted in a proportional decrease in [1-³H]Dol-P hydrolysis, it appears that the soya-bean material competes with pig liver Dol-P as substrate. This suggests that endogenous Dol-P in soya-bean microsomes is the 'in vivo' substrate for the enzyme.

Discussion

Hemming *et al.* (1963) first reported the presence of dolichol in the spadix of *Arum maculatum*, based on a comparison of i.r. spectra of standard dolichol and a polyisoprenoid alcohol obtained from this plant. We have now obtained sufficient amounts of highly purified polyprenol from soya beans to allow characterization by n.m.r., and i.r. spectroscopy and mass spectrometry.

The present results clearly establish the presence of dolichol in dry soya beans. It consists of a mixture of homologues (containing 15 to 22 isoprene units). Dolichols-17 and -18 together make up some 60% of the total material present. Previous reports (see the introduction) indicated that lipid isolated from plants could act as a glucose acceptor and that this glucosylated acceptor behaved very similarly to glucosylated Dol-P in a number of chromatographic properties. The plant lipid also resembled Dol-P-glucose in its resistance to phenolic treatment and its behaviour on hydrogenation. Therefore it was concluded by these authors that the lipid that participated in glycoprotein synthesis in plants is dolichol.

The major dolichyl phosphates present in seeds and in germinated cotyledons also appear to contain 17 and 18 isoprene units, as indicated by Brett &

Leloir (1977). In our experiments, the polyprenol phosphate isolated directly from soya beans behaved on h.p.l.c. like dolichol phosphorylated chemically.

Several authors (Hemming, 1977; Carson *et al.*, 1981; Rip *et al.*, 1981) have suggested that the amount of Dol-P available for glycoprotein formation may be influenced by the relative amounts of Dol-P phosphatase and dolichol kinase present. Some of the properties of a Dol-P phosphatase present in the microsomal fraction of soya-bean cotyledons were therefore investigated.

Dephosphorylation of [^3H]Dol-P was not due to the action of non-specific phosphatases, since a number of phosphate-containing compounds, including glucose 6-phosphate, $\text{Na}_4\text{P}_2\text{O}_7$, sodium β -glycerophosphate and several nucleoside phosphates, did not compete with Dol-P. Fluoride, which has been used as a general inhibitor of phosphatase activity, did not inhibit the Dol-P phosphatase activity significantly at 20 mM concentration. Endogenous Dol-P appears to be the natural substrate for the soya-bean Dol-P phosphatase, since chemically phosphorylated soya-bean dolichol competes with [^3H]Dol-P, and since the plant enzyme has a high affinity for Dol-P. The effect of phospholipids on Dol-P phosphatase is of considerable interest. The possibility that PtdA phosphatase acts primarily on Dol-P appears unlikely, since the reported PtdA phosphatase activities had pH optima of about 5 and were inactive at pH 7 (Mudd, 1980).

Concentrations of phospholipids (especially PtdA) are known to increase during germination (Harwood, 1980). Increased PtdA concentrations may inhibit the Dol-P phosphatase activity present, thereby maintaining the concentrations of Dol-P at a higher level. These data suggest that phospholipids may play an important, although indirect, role in the regulation of glycoprotein synthesis during germination.

A dolichol kinase activity has also been observed in soya-bean cotyledons, but it has not as yet been characterized. The measurement of both dolichol kinase and Dol-P phosphatase throughout the germination process will be helpful in understanding how plants regulate glycoprotein biosynthesis.

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References

- Adrian, G. S. & Keenan, R. W. (1981) *Biochem. J.* **197**, 233–238
- Belocopitow, E. & Boscoboinik, D. (1982) *Eur. J. Biochem.* **125**, 167–173
- Brett, C. T. & Leloir, L. F. (1977) *Biochem. J.* **161**, 93–101
- Burgos, J., Hemming, F. W., Pennock, J. F. & Morton, R. A. (1963) *Biochem. J.* **88**, 470–482
- Burton, W. A., Scher, M. G. & Waechter, C. J. (1981) *Arch. Biochem. Biophys.* **208**, 409–417
- Carroll, K. K. (1976) in *Lipid Chromatographic Analysis* (Marinetti, G. V., ed.), 2nd edn., vol. 1, pp. 173–214, Marcel Dekker Inc., New York and Basel
- Carroll, K. K., Vilim, A. & Woods, M. C. (1973) *Lipids* **8**, 246–248
- Carson, D. D., Earles, B. J. & Lennarz, W. J. (1981) *J. Biol. Chem.* **256**, 11552–11557
- Chadwick, C. M. & Northcote, D. H. (1980) *Biochem. J.* **186**, 411–421
- Chaudhary, N., Freeman, D. J., Rip, J. W. & Carroll, K. K. (1982) *Lipids* **17**, 558–560
- Cherry, J. H. (1974) *Methods Enzymol.* **31**, 583–589
- Danilov, L. L. & Chojnacki, T. (1981) *FEBS Lett.* **131**, 310–312
- Delmer, D. P., Kulow, C. & Ericson, M. C. (1978) *Plant Physiol.* **61**, 25–29
- Harwood, J. L. (1980) in *The Biochemistry of Plants* (Stumpf, P. K. & Conn, E. E., eds.), vol. 4, pp. 1–55, Academic Press, London and New York
- Hemming, F. W. (1974) in *Biochemistry of Lipids* (Goodwin, T. W., ed.), vol. 4, pp. 39–78, Butterworths, London
- Hemming, F. W. (1977) *Biochem. Soc. Trans.* **5**, 1223–1231
- Hemming, F. W., Morton, R. A. & Pennock, J. F. (1963) *Proc. R. Soc. London Ser. B* **158**, 291–310
- Idoyaga-Vargas, V., Belocopitow, E., Mentaberry, A. & Carminatti, H. (1980) *FEBS Lett.* **112**, 63–66
- Lehle, L., Fartaczek, F., Tanner, W. & Kausch, H. (1976) *Arch. Biochem. Biophys.* **175**, 419–426
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mudd, J. B. (1980) in *The Biochemistry of Plants* (Stumpf, P. K. & Conn, E. E., eds.), vol. 4, pp. 249–282, Academic Press, London and New York
- Parodi, A. J. & Leloir, L. F. (1979) *Biochim. Biophys. Acta* **559**, 1–37
- Pont Lezica, R., Brett, C. T., Romero Martinez, P. & Dankert, M. A. (1975) *Biochem. Biophys. Res. Commun.* **66**, 980–987
- Ratcliffe, R. & Rodehorst, R. (1970) *J. Org. Chem.* **35**, 4000–4002
- Rip, J. W., Rupar, C. A., Chaudhary, N. & Carroll, K. K. (1981) *J. Biol. Chem.* **256**, 1929–1934
- Rupar, C. A. (1979) Ph.D. Thesis, University of Western Ontario, Canada
- Rupar, C. A. & Carroll, K. K. (1978) *Lipids* **13**, 291–293
- Rupar, C. A., Rip, J. W., Chaudhary, N. & Carroll, K. K. (1982) *J. Biol. Chem.* **257**, 3090–3094
- Sharon, N. & Lis, H. (1979) *Biochem. Soc. Trans.* **7**, 783–799
- Staneloni, R. J., Tolmasky, M. E., Petriella, C., Ugalde, R. A. & Leloir, L. F. (1980) *Biochem. J.* **191**, 257–260
- Wedgwood, J. F. & Strominger, J. L. (1980) *J. Biol. Chem.* **255**, 1120–1123