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# ISOLATION AND CHARACTERIZATION OF AMYLOPLAST ENVELOPE MEMBRANES FROM *SOLANUM TUBEROSUM*

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**Key Word Index**—*Solanum tuberosum*; Solanaceae; potato; tuber; amyloplast; membranes; lipids; enzymes.

**Abstract**—Amyloplasts were separated from other subcellular organelles by sedimentation through a discontinuous sucrose density gradient. Purified amyloplast envelope membranes were similar in most characteristics to those from other types of plastids. A substantial proportion of the carotenoid content of these membranes was present in the esterified form. In contrast to published work on chloroplasts of photosynthetic tissue, our results showed that the acylase (acyl-CoA:sn-glycerol 3-phosphate acyltransferase) is firmly bound to the envelope membranes. Evidence was obtained to indicate that digalactosyldiacylglycerol, phosphatidylglycerol and sulpholipid, but not monogalactosyldiacylglycerol, are exclusively found in the cell as amyloplast lipids.

## INTRODUCTION

Methods are readily available for the isolation and purification of intact chloroplasts and more recently for the characterization of purified chloroplast envelope membranes [1–3]. Amyloplasts represent a stage on the basic pathway of chloroplast development [4], as illustrated by the ability of potato amyloplasts to 'green' when exposed to light, and little information is available on the relationship of these membranes with those from other plastids. At least half of the amyloplasts present in mature potato tuber consist of a large starch grain (25–100 $\mu$m) tightly enclosed by the double membrane system of the envelope [5]. The total loss of envelope membrane that occurs when attempts are made to isolate amyloplasts by methods involving centrifugation is most likely due to the presence of this dense starch grain. In the present study, a method has been employed to separate amyloplasts from other subcellular organelles by utilizing the high density of the starch grain. The isolation of amyloplasts from dormant potato tuber should allow at a subcellular level investigations into the mechanism of low temperature and 'senescent' sweetening [5].

## RESULTS

### *Fractionation of amyloplast envelope membranes*

Initially, envelope material was prepared by subjecting the amyloplast preparation to a gentle osmotic shock which gave a quantitative yield of membranes in the supernatant after centrifugation of the starch residue. The same result was later achieved by gently swirling the amyloplasts for 30 sec at 1° in medium containing sucrose (15% w/w). The latter method was preferred as intact nuclei could then be pelleted with the starch grains. The envelope suspension was either used directly for experimental work or the membranes were collected as a yellow pellet following centrifugation at 100 000 g. Analysis of the chemical constituents of the membranes were generally carried out on the pellet.

### *Envelope purity*

The purity of fractions was checked by light and electron microscopy. Light microscopy indicated that the amyloplast preparation consisted mainly of starch grains or amyloplasts (30–70 $\mu$m) with occasional nuclei or tracheid elements. Electron micrographs of the envelope pellet showed a high degree of purity consisting of single unit membrane-bound vesicles and large folded sheets of membrane with the presence of only occasional mitochondria and nuclear fragments.

### *Carotenoid composition*

Envelope lipid contained 0.7–1.0% (w/w) pigment, the actual amount being dependent upon the previous storage history of the tuber source. Analysis of the pigment fraction gave the carotenoid composition shown in Table 1. However, 17–22% of these carotenoids were present in the esterified form as judged by TLC and phase behaviour before and after saponification. Atheroxanthin (58%), neoxanthin (29%), lutein and zeaxanthin (6%) and cryptoxanthin (7%) were identified as components of the esterified fraction. Analyses of whole tuber pigments (163 ± 8(5) $\mu$g/100 g fr. wt) gave a similar composition of carotenoids and the same proportion esterified as in envelope membranes.

### *Lipid analysis*

Analysis of pelleted envelope fractions for total protein and polar lipid gave a mean value for the lipid/protein ratio of 1.61 ± 0.15 (7). Associated with the polar lipids (Table 1) were smaller amounts of

Abbreviations: MGDG = monogalactosyldiacylglycerol, DGDG = digalactosyldiacylglycerol, TGDG = trigalactosyldiacylglycerol, SL = sulpholipid, PG = phosphatidylglycerol.

Table 1. Composition of pigments and polar lipid fractions of the amyloplast envelopes

| Lipid* | μg/mg Protein | % Lipid by weight |
|---|---|---|
| Polar lipid | 1610 | 100 |
| Monogalactosyldiglyceride | 221 | 13.7 |
| Digalactosyldiglyceride | 725 | 45.0 |
| Trigalactosyldiglyceride | 90 | 5.6 |
| Sulpholipid | 86 | 5.3 |
| Phosphatidylcholine | 264 | 16.4 |
| Phosphatidylethanolamine | 80 | 5.0 |
| Phosphatidylinositol | 86 | 5.3 |
| Phosphatidylglycerol | 38 | 2.4 |
| Sterol acylmonoglucoside | 20 | 1.2 |
| Pigment† | 14.5 | 100 |
| β-Carotene 5,6-monoepoxide | 1.0 | 7 |
| Cryptoxanthin | 1.1 | 8 |
| Lutein + zeaxanthin | 3.5 | 24 |
| Antheroxanthin | 1.6 | 11 |
| Violaxanthin | 4.3 | 30 |
| Neoxanthin | 2.9 | 20 |

*Contains 1,2-diacylglyceride (42 μg/mg), 1,3-diacylglyceride (11 μg/mg) and triacylglycerol (18 μg/mg).

†Ratio of epoxide/non-epoxide is 2.1.

acylglycerides: 1,2-diacylglycerol (2.6%, w/w), 1,3-diacylglycerol (0.7%, w/w) and triacylglycerol (1.1%, w/w). The fatty acid composition of individual lipids is shown in Table 2.

*Contribution of amyloplast lipids to tuber lipids*

Four different samples of whole tuber each equivalent to tissue used for amyloplast preparations were analysed for individual lipid content and for total pigment. The contribution of each amloplast structural lipid to that present in the tuber was calculated by simple proportion (Table 3), assuming that carotenoids are restricted to amyloplast membranes.

*Galactolipid synthesis*

Envelope fractions incubated with UDP-[U-$^{14}$C] galactose incorporated radioactivity into MGDG (66%), DGDG (26%), TGDG (9%) and tetragalactosyldiacylglycerol (<1%). Membranes pelleted at 100 000 g for 30 min and incubated with labelled substrate contained 48% of the label present in similar membrane fractions which were not centrifuged. Reconstitution of the 'whole extract' by addition of the supernatant back to the pellet had no effect upon the amount of label incorporated by the pellet. A more detailed comparison of the labelling pattern of individual lipids present in pelleted and non-pelleted fractions revealed that in the pellet MGDG retained 61% of its label whilst DGDG and TGDG retained 33 and 38%, respectively (Table 4).

*Phosphatidic acid and diacylglycerol synthesis*

The incorporation of *sn*-[U-$^{14}$C] glycerol 3-phosphate into membrane lipids was studied with envelope fractions obtained before and after high speed centrifugation. In both cases radioactivity was present in the following lipids: monoacylglycerol and diacylglycerol (23%), phosphatidic acid (56%) and lysophosphatidic acid (20%). Membranes pelleted at 100 000 g retained 84% of their ability to stimulate phosphatidic acid and diacylglycerol synthesis.

## DISCUSSION

The preparation of intact amyloplasts free from other subcellular organelles was achieved by eliminating all centrifugation steps from the extraction method. The fragility of the amyloplast to centrifugal forces has been confirmed by the ease with which a suspension of envelope membranes is obtained by gently swirling the amyloplast preparation in a suitably buffered sucrose solution. The method makes use of two techniques to protect the amyloplast from disruptive external forces. First, the tuber core is infiltrated with buffered sucrose medium in an attempt to equalize the pressure difference across the vacuolar membrane prior to cell disruption and, secondly, following cell breakage the dense amyloplast is separated from other organelles by a relatively gentle descent under gravity through the medium. Even so, the yield of intact amyloplasts present in preparations rarely exceeded 16% on a starch weight basis. The calculation of this yield is based upon the assumption that the surface area of the amyloplasts is equivalent to that of the starch grains, whose total surface area may be calculated from the weight of starch present in the preparation and a knowledge of the distribution of grain size. Using a value of 75 Å$^2$ for the area occupied by a mole of hydrated galactolipid [6], the maximum amount of membrane lipid expected from

Table 2. Fatty acid composition of amyloplast envelope lipids

| Lipid class | Fatty acid (% peak areas) | | | | | |
|---|---|---|---|---|---|---|
| | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | 18:3 |
| Monogalactosyldiglyceride | 1.6 | 0.4 | 0.7 | 0.6 | 73.1 | 23.5 |
| Digalactosyldiglyceride | 6.3 | 0.2 | 5.1 | 1.5 | 68.9 | 18.0 |
| Trigalactosyldiglyceride | 26.1 | 0.5 | 7.8 | 2.8 | 48.2 | 14.7 |
| Sulpholipid | 25.4 | 0.4 | 6.8 | 2.5 | 51.4 | 13.5 |
| Phosphatidylcholine | 21.2 | 0.3 | 3.6 | 1.4 | 58.0 | 15.5 |
| Phosphatidylethanolamine | 21.0 | 0.4 | 3.4 | 1.6 | 58.5 | 14.5 |
| Phosphatidylinsitol | 38.4 | 0.2 | 3.8 | 1.4 | 40.7 | 14.5 |
| Phosphatidylglycerol | 47.4 | 1.0 | 3.9 | 2.5 | 33.4 | 11.8 |
| Sterol acylmonoglucoside | 32.2 | 1.1 | 6.5 | 4.9 | 37.4 | 17.9 |
| 1,2-Diacylglycerol | 4.4 | 1.6 | 1.2 | 1.7 | 64.5 | 26.6 |

Table 3. Contribution of amyloplast envelope lipids to total lipids of tuber tissue

| Sample | Amyloplast lipid as % of tuber lipid* | | | |
|---|---|---|---|---|
| | MGDG | DGDG | SL | PG |
| A | 60 | 92 | 89 | 81 |
| B | 84 | 105 | 100 | 85 |
| C | 66 | 115 | 102 | 98 |
| D | 57 | 90 | 83 | 92 |
| Mean±S.D. | 67±12 | 100±12 | 94±9 | 89±8 |

*PC, PE, PI, ESG, TGDG values are all less than 30%. Amyloplast lipids comprised 30–35% of cellular polar lipids.

the preparation could be calculated and compared with that found by analysis. No attempt has been made to maximize amyloplast yield by varying the density of the sucrose solutions or the dimensions of the column, both of which were chosen initially to minimize the effects of turbulence. However, a significant saving in preparation time without loss of amyloplast yield has been achieved by the use of a coarse grater in place of the original method of cutting thin discs of potato by hand.

The high purity of the envelope fraction obtained from the amyloplast preparation is indicated by electron micrographs and supported by pigment composition, lipid compositions and protein/lipid ratio all of which are similar in content to that previously reported for chloroplast and etioplast envelope preparations [1, 7, 8]. However, electron micrographs revealed only the presence of single membranes instead of the expected double membranes, an observation which together with the loss of lipid synthesizing ability in the 100 000 g pellet suggest that some irreversible physical changes have taken place in the membranes during the final purification stage.

All the carotenoids identified in envelope preparations are present in the flesh of a number of tuber varieties [9] and the amount of total pigment and qualitative composition of the envelope pigment (Table 1) is similar to that found in spinach chloroplast envelopes [1, 10]. Minor variations from chloroplast membrane compositions are the replacement of $\beta$-carotene by the corresponding 5,6-epoxide derivative and the partial replacement of violaxanthin by the corresponding monoepoxide, antheraxanthin. As might be expected for a dark-grown tuber, the ratio of epoxide/non-epoxide (2.1) is similar to that found in dark-adapted chloroplast envelopes [10].

Table 4. Incorporation of UDP-[U-$^{14}$C] galactose into lipids by amyloplast envelope

| Envelope fraction* | UDP-[U-$^{14}$C] galactose incorporated into lipids (dpm)†$\times 10^3$ | | | |
|---|---|---|---|---|
| | MGDG | DGDG | TGDG | Total |
| 500 g supernatant | 49.4 | 35.7 | 9.6 | 95.5 |
| 100 000 g pellet | 30.3 | 11.8 | 3.6 | 46.1 |

*For details, see Experimental.

†TLC plate showed traces of radioactivity (<1%) corresponding to tetragalactosyldiglyceride.

The presence of a substantial amount of carotenoid in the ester form has not been previously reported either in potato tuber flesh or isolated chloroplast envelope preparations. The reason for this oversight may lie in the fact that most workers routinely saponify the crude pigment which removes fatty material and facilitates the later separation and identification of individual carotenoids. We have not attempted to identify the fatty acid component of the ester due to the small amounts involved and the presence of other lipids as fatty acid-containing impurities, but have merely inferred the presence of esters from TLC and phase behaviour before and after saponification.

The polar lipids which constitute the major portion of the lipid phase of the membrane are qualitatively similar to those present in etioplast [8] and chloroplast envelopes [1, 2, 7] of other plant species. However, there are different distinct quantitative differences. Although the galactolipids, including SL, represent about 70% of the polar lipid as in other plastid envelopes, the DGDG/MGDG ratio of three is much greater than in chloroplasts (1–2). Also, the lipid/protein ratio of 1.6 is very high compared to the 1.0–1.3 of published values [1, 7], though the low protein value may only reflect the dormant state of the potato tuber.

The fatty acid compositions of the lipids (Table 2) are different from those found in chloroplast lipids [7, 8] but are not unusual in that the high content of linoleic acid present merely reflects the characteristic pattern found in whole tissue lipids [11] and in mitochondrial membrane lipid of potato tuber [12]. *Trans*-$\Delta_3$-hexadecenoic acid which is found as a major fatty acid in the PG from choroplast envelopes [8, 13] and as a minor component (4%) in etioplasts [8] was not present in the PG from amyloplast envelopes which suggests that this acid is confined to plastids of photosynthetic tissues. The fatty acids of 1,2-diacylglycerol are highly unsaturated and similar in composition to those of MGDG and DGDG which confirms the role of this lipid as a substrate for the synthesis of galactolipids by enzymes present in envelope membranes.

Plastids, such as the chloroplast and chromoplast present in cells of photosynthetic tissue, contain carotenoid pigments in the stroma and internal membrane structures as well as in envelope membranes. In a non-photosynthetic tissue such as potato tuber, pigment is likely to be confined to the lipid portion of amyloplast envelopes and this afforded an excellent opportunity to use the carotenoids as an internal standard in determining the contribution to cell lipids of each lipid present in the envelope, provided that the composition of envelope lipid and tissue lipid is known. Such data were collected from four groups of Desirée potato tubers, all of which had different lipid compositions as a result of past storage history. The amyloplast envelope membranes' contribution to the total cellular membrane of potato tuber tissue was found to be 30–35%. Three tissue lipids, DGDG, SL and PG, appeared to be associated exclusively with the amyloplast whilst a substantial proportion of MGDG was located elsewhere (Table 3). A ratio of DGDG/MGDG of three in amyloplast lipid to two in

tissue lipid confirms this view. The exclusiveness of the above three lipids to plastids is not surprising as a substantial body of indirect evidence has favoured this viewpoint. There is good evidence to exclude the presence of MGDG in potato tuber mitochondria [12, 14] and, as electron micrographs show a scarcity of endoplasmic reticulum and golgi bodies in dormant potato cells, the most likely membranes to contain MGDG would appear to be the plasmalemma or tonoplast. There is evidence to suggest that MGDG is present in membrane-enriched fractions from potato tuber which contain a potassium-stimulated magnesium-dependent ATPase [15]. However, MGDG might have been present due to contamination of the plasmalemma with plastid envelope membrane which also contains a magnesium stimulated ATPase [13].

The plastid envelope is well established as the major and probably the sole site for the biosynthesis of galactolipids [16, 17], and more recently this membrane has been shown to be the site for phosphatidic acid and diacylglycerol synthesis in spinach chloroplasts [18]. We have confirmed that this is also the case for the amyloplast.

Joyard and Douce [18], during investigations into phosphatidic acid biosynthesis with *sn*-glycerol 3-phosphate, found a soluble acylase (acyl-CoA: *sn*-glycerol 3-phosphate acyltransferase) in spinach choroplast extract which was required by the envelope membrane in order to initiate enzyme synthesis. They presumed this enzyme to be loosely bound to the envelope membrane which had detached during the isolation procedure. Our studies show this enzyme to be firmly bound to the envelope membranes to the extent that 84% of the potential enzyme activity was retained in the isolated membrane pellet.

Studies on the synthesis of galactolipid from UDP-galactose and endogenous 1,2-diacylglycerol showed that the high speed centrifugation destroyed about 50% of the overall enzyme capability. A more detailed analysis (Table 4) revealed that retention of MGDG synthesis (61%) was about twice that of DGDG and TGDG synthesis (33, 38% respectively) suggesting that the former enzyme system was more stable to mechanical damage and confirming that two distinct galactosylation sites in the membrane are involved in the synthesis of galactolipids [18, 19].

## EXPERIMENTAL

*Materials.* Potato tubers (*Solanum tuberosum* var. Desirée) were grown at the Institute and used either after harvesting or following a storage period at 5°. UDP-D-[U-$^{14}$C] galactose and L-[U-$^{14}$C] glycerol-3-phosphate were purchased as their ammonium salts from the Radiochemical Centre (Amersham).

*Preparation of amyloplast membrance fraction.* Four potato cores (12 × 3.5 cm dia) were washed thoroughly in iced water, transferred to a medium of 0.3 M $K_2HPO_4$ containing 10 mM $MgCl_2$, 10 mM EDTA, 0.2% (w/v) BSA (essentially fatty acid free) and 12% (w/w) sucrose at 1° and vacuum infiltrated with medium for 30 min. The apparatus used for the isolation of amyloplasts consisted of a glass column (37 × 2.5 cm) topped with a glass funnel (9 cm dia, B24 joint) within which was a nylon cloth (300 μm mesh) overlaid with a wire gauge (1000 μm mesh). A glass ampoule (30 ml) was butt-jointed below the column's tap. The column and ampoule were completely filled (300 ml) with a medium of 0.3 M $K_2HPO_4$ containing 10 mM $MgCl_2$, 10 mM EDTA and 15% (w/w) sucrose at 1°, upon which was layered an equivalent vol. of infiltration medium. Thin discs of potato (0.5 mm) or fragments prepared with a coarse grater were quickly dipped into the upper layer, drained and discarded immediately. Amyloplasts were allowed to settle down the column for 30–45 min and collected in the ampoule which was then detached from the column. Envelope membranes were detached from amyloplasts by replacing the medium with 20 ml 0.3 M $K_2HPO_4$, 10 mM $MgCl_2$ adjusted to pH 7.2 with HCl with or without 15% w/w sucrose, followed by a gentle swirl (30 sec) in the former case or incubation for 10 min at 1° in the latter case. Starch was removed by centrifuging twice (500 g for 10 min) and the membranes obtained as a pellet by centrifugation (100 000 g for 30 min) of the supernatant.

*Microscopy.* Envelope membranes obtained by gently swirling amyloplasts in 15% sucrose medium were fixed for 2 hr at 20° following the addition of sufficient glutaraldehyde to give a 2% soln. Following centrifugation (30 000 g), the pellet was embedded in Agar and post-fixed with 1% osmium textroxide in Palades buffer (pH 7.4) for 90 min at 4°. After dehydration in a series of graded alcohols, specimens were embedded in Epon and sections cut and viewed on an AEI 801 electron microscope.

*Protein estimation.* Protein content of the membrane pellet was determined by the method of Lowry [20] with crystallized bovine serum albumin as the standard.

*Carotenoid analysis.* Diced potato tuber tissue was boiled for 10 min with *iso*-PrOH–M $K_2HPO_4$ (9:1), homogenized with an ultraturrox probe and the pigments extracted from the residue with MeOH (×1) and $Et_2O$ (×2). The extracts were combined, phased with $H_2O$ and the crude pigment fraction recovered from the $Et_2O$ phase. Pigments were extracted together with polar lipids from membrane material by a modified Bligh and Dyer method [21]. Carotenoids were separated into three groups: hydrocarbon and ester fraction, monohydroxy derivatives and polyhydroxy derivatives by partition between hexane–90% MeOH and hexane–95% MeOH [22]. Carotenoid esters were separated from carotenes by phase separation following saponification of the mixture with 6% (w/v) KOH in EtOH. Individual carotenoids were separated by quantitative TLC on Si gel with solvent systems MeOH–toluene (3:17) and $C_6H_6$–hexane (1:1). Identification relied upon data from visible absorption spectra and degree in shift of absorption maxima of epoxide derivatives in the presence of acid, phase separations and, where possible, TLC comparison with authentic carotenoids. Total carotenoid values were measured directly on a recording spectrophotometer using an absorbance value of $A^{1\%}_{1\,cm,445}$ 2200.

*Lipid analysis.* Lipids were extracted from potato tuber and amyloplast membrane preparations by a modified Bligh and Dyer method and analysed as previously described [23]. Fatty acid composition of individual lipids was obtained by direct transmethylation in the presence of Si gel of lipid separated by TLC, followed by GLC of the recovered methyl ester derivatives.

*Enzyme assays.* Galactolipid synthesis was measured at 25° in a 4.2 ml incubation mixture containing $K_2HPO_4$ buffer, 0.3 M, pH 7.4; $MgCl_2$, 10 mM; and known amounts of amyloplast membranes which contained adequate amounts of 1,2-diglyceride substrate. Reactions were initiated upon addition of UDP-[U-$^{14}$C] galactose (1.48 nmol, $1.11 \times 10^6$ dpm)

and the mixture incubated for 1 hr. The reaction mixture for phosphatidic acid and diacylglycerol synthesis contained: $K_2HPO_4$ buffer, 0.3 M, pH 7.4; $MgCl_2$, 10 mM; ATP, 4 mM; CoA, 0.2 mM and known amounts of amyloplast membranes in a final vol. of 4.2 ml. Reactions were initiated upon addition of *sn*-[U-$^{14}$C] glycerol 3-phosphate, 1 mM (sp. act $1.74 \times 10^9$ dpm/mmol) and incubated for 1 hr. The reactions were terminated by the addition of $CHCl_3$–MeOH (1:2) and the lipids extracted by a modified Bligh and Dyer method [23].

Lipids were separated by quantitative TLC on Si gel with the solvent system $CHCl_3$–MeOH–HOAc–$H_2O$ (85:15:10:2), the plate monitored for ratio activity by a TLC-scanner and radioactive spots scooped into vials to be counted by liquid scintillation.

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