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ISOLATION OF AMYLOPLASTS FROM WHEAT ENDOSPERM

GAIL ENTWISTLE, R. HUW TYSON* and TOM AP REES

Botany School, University of Cambridge, Downing Street, Cambridge CB2, 3EA, U K

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Abstract—The aim of this work was to develop a method for the isolation of amyloplasts from the endosperm of developing grains of *Triticum aestivum*. Protoplasts were prepared by digesting endosperm with cellulase and pectinase. Procedures were developed for the lysis of the protoplasts by DEAE-dextran, and for fractionation of the lysate on a continuous gradient of Nycodenz. The latter gave amyloplast preparations that contained 20% of the plastid marker enzymes in the lysate, were not contaminated by microbodies or the endomembrane system, and showed less than 3% contamination by cytosol and 5% by mitochrondria When removed from the gradient 54% of the amyloplasts remained intact

INTRODUCTION

The pathway from translocated sucrose to storage starch in non-photosynthetic cells of plants is not known One of the most important unanswered questions is what compound(s) cross the amyloplast envelope to support starch synthesis therein Progress requires a procedure for the isolation of amyloplasts, and particular emphasis should be given to the development of methods for individual crops of economic importance, such as wheat Methods have been reported for the isolation of amyloplasts from soybean cells [1] and from maize endosperm [2]. Both these methods expose the amyloplasts to solutions of high osmolarity The soybean amyloplasts were recovered from 1.8 M sucrose, those from maize from a solution of Ficoll in 0 7 M sucrose, the latter has an osmolarity (1592 mosM) almost equal to that of 1 8 M sucrose. These treatments are likely to damage the transport mechanisms in the amyloplast envelope. Journet and Douce [3] avoided the use of high concentrations of sugars in a method that yielded starch-containing plastids from cauliflower florets. However, the yield was low and it is not clear that the method will be applicable to tissues where the starch content is very much higher and where the starch grains are much larger and almost fill the amyloplasts In this paper we report a method that does work for such a tissue, the endosperm of developing wheat grains, and avoids exposing the plastids directly to solutions of high osmolarity.

We used the following markers to monitor our fractions amyloplasts, alkaline pyrophosphatase (EC 3.6 1 1, PPase) [4], ADPglucose pyrophosphorylase (EC 2 7.7.27), starch synthase (EC 2 4.1 21) [1], cytosol, UDPglucose pyrophosphorylase (EC 2.7.7.9), alcohol dehydrogenase (EC 1.1 1 1) [1], pyrophosphate:fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.90, PFK(PP_i)) [5]; mitochondria, citrate synthase (EC 4.1 3.7) [6], cytochrome *c* oxidase (EC 1 9.3 1) [1], endomembrane systems, cytochrome *c* reductase (NADH dehydrogenase) (EC 1.6.99 3) [1].

RESULTS AND DISCUSSION

We tried to obtain amyloplasts by differential centrifugation of very gently prepared homogenates of the endosperm These homogenates were made by pressing the endosperm in a mortar with a pestle [7], and by squeezing endosperm through a 75 μm steel mesh. Centrifugation of such homogenates at forces ranging from 50 to 200 *g* showed no preferential sedimentation of amyloplast markers. Thus we turned to protoplasts as a source of amyloplasts.

Endosperm was digested overnight in the cold with cellulase and pectinase to release protoplasts. Attempts to purify protoplasts from the digest by the two-phase method of ref. [8], or by the use of sucrose gradients, up to 0.8 M, were unsuccessful because the media would not support the starch-laden protoplasts. We obtained our best results by allowing the digest to stand for 20 min and then centrifuging the sediment on a stepped gradient of Nycodenz, a non-ionic tri-iodinated derivative of benzoic acid with three aliphatic side chains that exerts a relatively low osmotic pressure [9] Examination of the purified protoplasts with a microscope showed that most of the cell debris had been removed The isolated protoplasts were spherical, excluded Evans Blue dye, showed cytoplasmic streaming under Nomarski optics, and accumulated neutral red dye into their vacuoles. When incubated in [U-^{14}C]glucose the purified protoplasts incorporated label into starch almost linearly for three hr at a rate of 3 2 nmol per mg starch/hr Triton, 0.1% (v/v) reduced this incorporation by 95% Endosperm halves incubated under comparable conditions incorporated ^{14}C at a rate of 18 nmol per mg starch/hr.

We had the utmost difficulty in devising a way of breaking the plasmalemma of the protoplasts without simultaneously rupturing the amyloplast envelope We assessed a range of methods by comparing latency and/or, protection of cytosolic and amyloplast markers,

* Present address Department of Horticulture and Landscape Architecture, Washington State University, Pullman, Washington 99164, U S A.

generally UDPglucose pyrophosphorylase and ADPglucose pyrophosphorylase, respectively Success was recognized if the latency or protection, in the protoplast lysate, of the cytosolic marker was low and that of the amyloplast marker was high We had no success with the following conventional methods osmotic shock, forcing the protoplasts through a fine needle [1], and passing the protoplasts through nylon meshes, ranging in gauge from 13 to 70 μm [10] Eventually we found that adding DEAE-dextran to protoplasts, followed three min later by dextran sulphate, and then incubating in ice for 90 min preferentially ruptured the plasmalemma This treatment broke the plasmalemma but did not disperse the contents The addition of the dextran sulphate led to the formation of a loose precipitate in which the ruptured protoplasts were entangled Consequently the whole preparation was next passed through a 70 μm mesh This led to dispersal of the contents of the protoplasts and gave a more homogeneous preparation, which we call the unfractionated protoplast lysate

The latency of ADPglucose pyrophosphorylase in the unfractionated protoplast lysate was $53 \pm 3\%$, that of UDPglucose pyrophosphorylase was $9 \pm 2\%$ values are means $\pm$ s.e. for 12 different lysates Thus the lysates contained an appreciable proportion of intact amyloplasts and relatively few unbroken protoplasts Most of the latter were broken in the subsequent fractionation of the lysate

Separation of the amyloplasts from the rest of the lysate was achieved by fractionation on a continuous density gradient of Nycodenz The gradient was not centrifuged but was merely stood in ice for four to six hr before being fractionated from the top An obvious starchy band of amyloplasts formed about 10–12 ml into the gradient The distribution of marker enzymes is exemplified by the gradients shown in Fig 1, and is summarized in Table 1 We paid particular attention to the markers for the cytosol their distribution reveals the extent to which the amyloplasts were contaminated by the cytosol and by unbroken protoplasts Of all the likely contaminants, the above would probably interfere most with studies of carbohydrate metabolism by the isolated amyloplasts. We emphasize that our procedure must be followed precisely to obtain the results shown. If older and larger grains are used in an attempt to obtain more protoplasts, some of the latter remain unbroken and copurify with the amyloplasts

Amyloplasts and protoplasts settle quickly and it is not easy to pipette replicate samples from a single suspension, nonetheless the agreement between replicates was satisfactory The wider variation in the absolute activities of the enzymes in the unfractionated lysate reflects variation in the precise yield of protoplasts in different experiments This variation did not affect the manner in which the enzymes were distributed throughout the gradient The sum of the enzyme activities in the different fractions of the gradient showed that little activity was lost during the fractionation It can be seen that about 20% of the amyloplast markers were recovered in the amyloplast fraction This is an appreciable yield that is not much less than that obtained when preparing chloroplasts [11]. Our amyloplast fractions were not seriously contaminated with cellular components likely to interfere with the study of starch metabolism *in vitro* There was good recovery of the cytosolic markers from the gradient and good agreement between the markers The contamination by cytosol and by unbroken protoplasts was less than 3% Contamination by mitochondria was only slightly greater and no contamination by the endomembrane system could be detected Contamination by microbodies is most unlikely as we could detect no activity of the appropriate markers, catalase and hydroxypyruvate reductase, in the unfractionated lysates The amyloplasts, though fragile, could be removed successfully from the gradient and such preparations were shown to exhibit 54% latency of the amyloplast marker, PPase (Table 1, Fig 1) This is double the value found previously [12] for soybean plastids taken from a sucrose gradient, and comparable to the 61% reported for the method for maize amyloplasts (average of values in Table 2, ref [2]) We suggest that our method results in the isolation of amyloplasts from starch-rich tissues without subjecting the amyloplasts to severe osmotic stress The preparations are sufficiently free from contamination to permit their use in studies of enzyme distribution and also transport of substrates

EXPERIMENTAL

Chemicals. DEAE-dextran (chloride form) and dextran sulphate were from Sigma Substrates enzymes, cofactors were from Sigma, or Boehringer Corp., Lewes, U K except that cellulase (Onozuka R_{10}) was from Yakult Pharmaceutical Co (Unwin Ltd, Welwyn U K), pectinase from Serva Feinbiochemica (Uniscience Ltd, London), and Nycodenz from Nycomed (UK) Ltd, Birmingham

Plants. Seeds of winter wheat, *Triticum aestivum* L. cv. Mardler, were sown in John Innes No 3 compost (25 to a 15-cm diam pot), kept for 1 week in a greenhouse (15–25), vernalized for 4–6 weeks at 4 in an 8-hr photoperiod of fluorescent light (PAR 100 $\mu E/m^2/s$), transplanted (2 to a 15 cm pot) and grown in the greenhouse (15–25) under a 16-hr photoperiod in daylight supplemented with sodium light (PAR 350-500 $\mu E \cdot m^2$ s) Plants were fed weekly with Phostrogen (Phostrogen Ltd, Corwen, Clwyd, U K) and watered as necessary Developing ears were harvested 8–12 days after anthesis (the first appearance of the anthers) The stems were cut off at ground level and kept in H_2O for no longer than 5 hr whilst 160 grains, of uniform size and fr wt 30–38 mg, were taken from the mid-ear region, cut in half through the crease and dissected to yield endosperm halves Eight samples, each of 40 endosperm halves, were prepared and kept on ice in 0 5 M sucrose 50 mM Hepes NaOH pH 7 5 (sucrose–Hepes) until used to make protoplasts

Protoplasts. The sucrose–Hepes was removed from the samples of endosperm and replaced with 3 0 ml 0 5 M sucrose, 10 mM KCl, 1 mM $CaCl_2$ 25 mM Mes–NaOH, pH 5 5 (sucrose–Mes) that contained 2% (w v) cellulase, 1% (w/v) pectinase and 0 1% (w v) bovine serum albumin The samples were the incubated at 10 for 14 16 hr This digestion does not release many protoplasts into the digestion medium Thus this medium may be removed with a Pasteur pipette and replaced with 0 5 ml ice-cold sucrose–Hepes Next the protoplasts were released by gently moving the tissue up and down a Pasteur pipette and the resulting suspension was filtered through nylon mesh (240 μm) Filtrates from pairs of samples were combined to give 4 samples from the original 8 samples of endosperm halves Each of the 4 samples was put in a 13-ml glass centrifuge tube and made up to 7 ml by gentle mixing with ice-cold sucrose–Hepes The tubes were stood in ice for 20 min and then the supernatant was removed with a pipette and discarded The sediment was very gently resuspended in 1 0 ml 60% (w v) Nycodenz and then covered with successive 2-ml layers of 40 20 and 10% (w v)

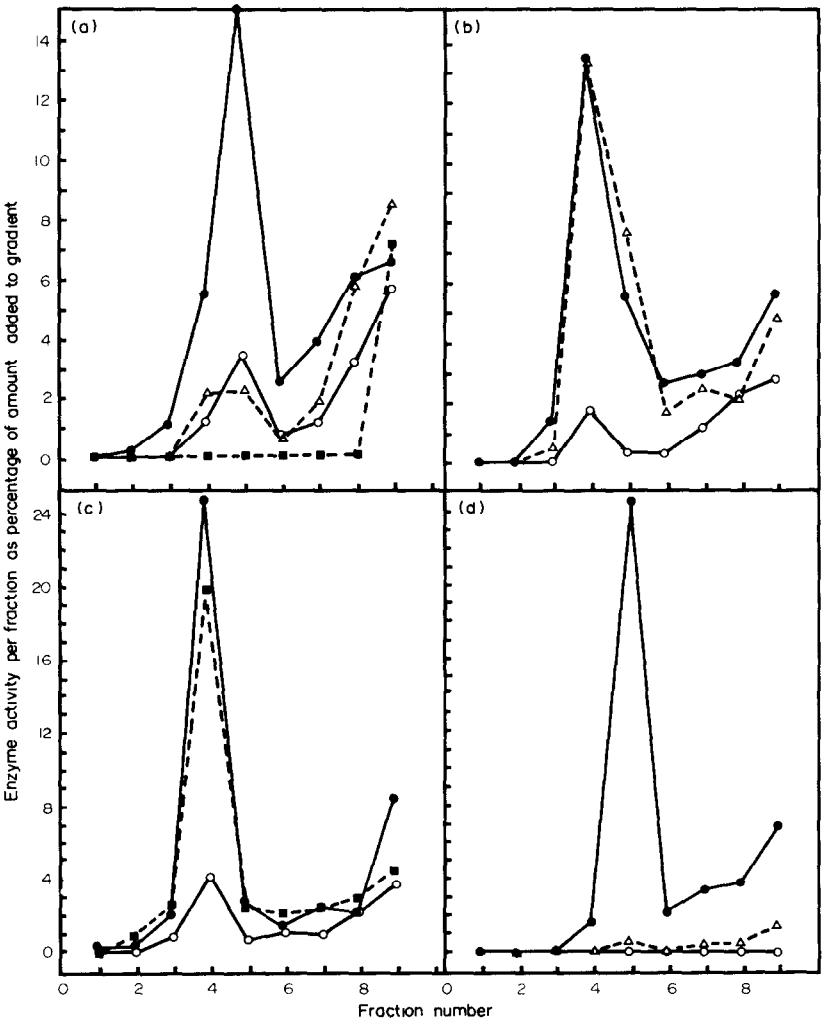

Fig 1 Purification of amyloplasts on continuous gradients of Nycodenz As it was not practicable to assay every enzyme on one gradient, four gradients are shown to reveal the distribution of marker enzymes Fractions were of 2 ml and no 1 is the bottom of the gradient In order to reveal the characterisation of the amyloplast fraction the scale on the vertical axis has been made too great to allow the activities found in the top fraction of the gradient to be shown These are given below (as % of activity added to gradient) in parenthesis after the name of each enzyme

A, ●——●, PPase (55), Δ---Δ, PFK [PP_i] (76), ○——○, citrate synthase (73), ■---■, cytochrome *c* reductase (82) B, ●——●, PPase (55); Δ---Δ, ADPglucose pyrophosphorylase (58), ○——○, PFK [PP_i] (82) C, ●——●, PPase (60); ■---■, starch synthase (20), ○——○ PFK [PP_i] (89), D, ●——●, PPase (57), Δ---Δ, UDPglucose pyrophosphorylase (97), ○——○, alcohol dehydrogenase (107)

Nycodenz Solutions of 60% Nycodenz were prepared by dissolving solid Nycodenz in 50 mM Hepes–NaOH, pH 7.5; solns of 40, 20 and 10% Nycodenz were obtained by diluting 60% Nycodenz with 0.5 M sucrose–50 mM Hepes, pH 7 5, so that they contained 0 17, 0 33 and 0 42 M sucrose, respectively The 0.5 M sucrose–50 mM Hepes and the 60% Nycodenz have similar osmolarities (580 and 610 mosM, respectively), thus the gradient was made of solns that were roughly iso-osmotic. The gradients were centrifuged at 70 g_{max}(540 rev/min MSE Mistral) for 5 min at 4° protoplasts were collected in 1 0 ml from the 40–20% boundary

Fractionation To each 1-ml sample of purified protoplasts we added 0 6 ml DEAE–dextran to give a final concn of 6 5 mg/ml 3 min later 0 4 ml dextran sulphate was added so that the final concn was 6 5 mg/ml The DEAE-dextran and the dextran sulphate were dissolved in sucrose–Hepes After each addition to the protoplasts the suspension was mixed gently The protoplasts were kept in ice during the above additions and were then incubated for 90 min in ice before being filtered through nylon mesh (70 μm) The above procedure produced 4 filtrates from the original 8 samples These filtrates were combined to give a vol of *ca* 8 ml, which was then put on a Nycodenz gradient The latter was made by carefully layering, in a 43 ml glass centrifuge tube, 7-ml portions of 60, 40 and 20% (w/v) Nycodenz (diluted with sucrose–Hepes as described above) The tube was stoppered and put on its side for 1 hr at 20° to allow a continuous gradient to form [9] The tube was stood vertically and cooled in ice before adding the protoplast lysate Next the

Table 1 Activities of marker enzymes in preparations of amyloplasts

Enzyme	No of lysates assayed	Activity in unfractionated lysate (nmol/min per ml lysate)	Percentage of activity in unfractionated lysate that was recovered in Amyloplasts	Sum of all gradient fractions
Alkaline pyrophosphatase	13	177±24	17.8±1.2	94±7
ADPglucose pyrophosphorylase	3	32±9	18.4±6.9	72±10
Starch synthase	3	3±0.2	25.6±9.7	65±7
UDPglucose pyrophosphorylase	7	490±94	1.8±0.4	105±13
PFK (PP_i)	7	74±16	2.5±0.7	99±6
Alcohol dehydrogenase	1	7	none found	111
Citrate synthase	4	6±2	4.7±1.6	91±9
Cytochrome *c* oxidase	2	194,675	none found	83,115
Cytochrome *c* reductase	2	82,67	none found	67,89

Values are means ± s.e.

gradient was stood in ice for 4–6 hr, and then fractionated from the top. The fractions were of 2 ml except that the final one was of 5 ml, and the first fraction corresponded to the volume of lysate added to the gradient (4.8–7.0 ml).

Assays. Except for the points noted, the following enzymes were assayed as in the references quoted: alkaline PPase [4], for control samples the TCA was added to the extract before the PP_i; cytochrome *c* oxidase, alcohol dehydrogenase, UDPglucose pyrophosphorylase, ADPglucose pyrophosphorylase [1], cytochrome *c* reductase [13], starch synthase [14], 0.61 μmol ADP[U-^{14}C]glucose (0.25 μCi/μmol), no GSH, in 200 μl at 30°, citrate synthase [6], PFK(PP_i) [15]. ADPglucose pyrophosphorylase was also assayed spectrophotometrically at 25° [16] in a reaction mixture that contained in 1 ml: 80 mM Hepes, pH 8.0, 1 mM $MgCl_2$, 1 mM $Na_4P_2O_7$, 0.4 mM NAD, 0.8 mM ADPglucose, 4 units phosphoglucomutase and 1.4 unit glucose-6-phosphate dehydrogenase (NAD-specific from *Leuconostoc mesenteroides*). A similar assay was used for UDPglucose pyrophosphorylase [17] except that the ADPglucose was replaced with UDPglucose.

Protection was measured as in ref. [1]. Latency was measured by comparing activities in samples in which organelles and protoplasts were kept intact, by including 0.5 M sucrose in the assay mediums, with activities found in samples in which membranes had been ruptured either by adding Triton X-100 at 0.1% (v/v) or by 3 cycles of freezing and thawing (PPase). The difference between the activity in the intact and ruptured samples is expressed as a % of that in the ruptured sample to give latency. Otherwise all measurements of enzymes were made on samples that had been ruptured as described above. [^{14}C]Glucose was fed to endosperm by incubating 20 endosperm halves in 3 ml 20 mM [U-^{14}C]glucose (0.025 Ci/mol), 310 mM sorbitol, 60 mM KCl, 10 mM Mes–NaOH, pH 6.5, 0.1% (w/v) bovine serum albumin at 25°. For protoplasts the medium was 20 mM [U-^{14}C]glucose (0.05 Ci/mol), 40% (w/v) Nycodenz, 200 mM mannitol, 60 mM KCl, 10 mM Mes–NaOH, pH 6.4, 1 mM $CaCl_2$, 0.1% (w/v) bovine serum albumin. ^{14}C in starch was determined as the ^{14}C released after the starch had been digested as in ref. [18].

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