Phytochemistry, 1975, Vol. 14, pp. 1259–1263. Pergamon Press. Printed in England.

EVIDENCE FOR LIPID–ENZYME INTERACTION IN STARCH SYNTHESIS IN CHILLING-SENSITIVE PLANTS

W. JOHN S. DOWNTON and JOHN S. HAWKER

C.S.I.R.O. Division of Horticultural Research, Private Mailbag, Merbein, Vic., 3505 and P.O. Box 350, Adelaide, S.A. 5001, Australia

(*Received* 8 *August* 1974)

Key Word Index—Maize kernels; avocado cotyledon; sweet potato tuber; potato tuber; starch synthesis; chilling-sensitive plants; Arrhenius plot; energies of activation; lysolecithin; *t*-butanol on starch synthetase.

Abstract—Soluble and starch grain-bound forms of ADPglucose: α-1,4-glucan α-4-glucosyltransferase (starch synthetase) from maize, avocado and sweet potato show a discontinuity (change in slope) in Arrhenius plots at about 12°. In contrast, enzymes from potato have a constant energy of activation from 23° to 3°. *t*-Butanol, but not Triton X-100 or phospholipase A, abolishes the discontinuity in the plot of grain-bound starch synthetase activity of the chilling-sensitive plants. The previously found association of starch synthetase and the lipid lysolecithin with amylose molecules suggests that the discontinuous Arrhenius plot in chilling-sensitive plants reflects a phase change in the lipid at a critical temperature as has been reported for membrane-bound enzymes in these species. *t*-Butanol in high concentration probably acts by entering the amylose helix and perturbs the usual lipid–enzyme interaction.

INTRODUCTION

Arrhenius plots for the response to temperature of photosynthetic light reactions, mitochondrial oxidation, protein synthesis and translocation in chilling-sensitive plants show a discontinuity at about 12° [1, 2]. The activation energy (E_{act}) of these processes below 12° is higher than above 12°. Chilling-resistant plants, however, do not show a change in E_{act} for these functions over a wide range of temperature. Chilling injury in plants is the subject of a recent review by Lyons [3]. ADPglucose: α-1,4-glucan α-4-glucosyl transferase (starch synthetase), an enzyme responsible for starch synthesis in plants [4], was studied from 23° down to 3°. The plants selected for study were avocado, maize and sweet potato, all chilling-sensitive plants subject to injury at temperatures below about 12°, and potato, a chilling-resistant plant. The data demonstrate that temperatures in the chilling range have quite different effects on starch synthesis in the two groups of plants. A lipid interaction for starch synthetase seems apparent.

RESULTS

Table 1 gives the distribution of starch synthetase between the starch grains and the supernatants of homogenates for each of the organs studied. The enzyme associated with starch grains could not be dissociated by freezing and thawing. The major component (75%) of the cell starch synthetase was bound to starch grains in avocado cotyledon and sweet potato tuber, but little bound synthetase (6–8%) was found in maize kernels and potato tuber.

The responses of starch synthetase to temperature in the various plants are graphed as Arrhenius plots ($\log_{10}$ glucose-[U-^{14}C] incorporated into starch vs 1/temperature). Assay duration was generally 10 min, but longer periods were used at the lower temperatures in earlier experiments. Reaction rates were linear over the time intervals used. No changes in the K_m for ADPglucose with temperature were apparent. The K_m values at 6° and 23° for both soluble and grain-bound starch synthetase in maize were 0·37 mM ADPglucose.

Table 1. The distribution of ADPglucose–starch glucosyltransferase in various plant organs

Organ	Enzyme activity (nmol/min/g fr. wt)			% Total activity grain-bound
	Soluble	Grain-bound	Total	
Avocado cotyledon	17·8	51·4	69·2	74·3
Maize kernel	100·2	5·8	106·0	5·5
Potato tuber	75·5	6·7	82·2	8·2
Sweet potato tuber	14·7	44·9	59·6	75·3

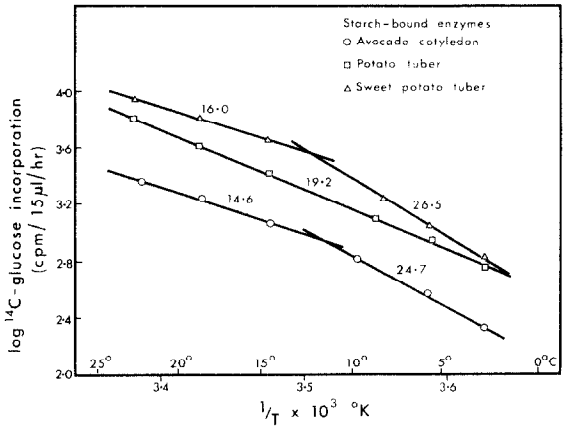

Fig. 1. Arrhenius plots of grain-bound ADPglucose–starch glucosyltransferase activity from avocado cotyledon, sweet potato tuber (both chilling-sensitive) and potato tuber (chilling-resistant). The values beside each line are the calculated activation energies in kcal/mol.

Soluble enzyme from potato tuber had a K_m of 0·43 mM ADPglucose; particulate enzyme, a K_m of 0·83 mM. Values for glucose-[U-^{14}C] incorporated into starch in Arrhenius plots are means of triplicate assays at each temperature.

Figure 1 presents typical Arrhenius plot data for starch synthetase bound to starch grains in potato tuber, sweet potato tuber and avocado cotyledon. A constant energy of activation over the temperature range of 3°–23° was found for potato. The chilling-sensitive plants avocado and sweet potato showed a discontinuity in the Arrhenius plot at about 12° with a large increase in E_{a1} below this temperature. Maize starch-bound synthetase responded similarly to avocado and sweet potato (Fig. 2).

The discontinuity in the Arrhenius plot for activity of some membrane-bound enzymes of chilling-sensitive plants can be abolished by low concentrations of detergents and phospholipase A [1, 5]. In the present work the application of Triton X-100 (0·4–0·6%) to avocado and sweet potato starch did not eliminate the discontinuity in the starch synthetase assay. Phospholipase A also had no effect on the discontinuity in the Arrhenius plot for avocado grain-bound starch synthetase. Soluble starch synthetase extracted from maize kernels also showed a discontinuous Arrhenius plot at about 12° which remained unaltered by preincubation with Triton and phospholipase A (Fig. 3).

Table 2 summarizes the E_{a1} data obtained from Arrhenius plots. Starch prepared from cotyledons of avocado fruits harvested at different times over the season showed large differences in energies of activation. Early to mid-season Fuerte fruit had an E_{a1} above the transition temperature of 8 kcal/mol compared to a value of 17 kcal/mol for late season fruit in which the seed had begun to germinate. Increases in E_{a1} also appeared below the transition temperature as the season progressed. The soluble and grain-bound forms of starch synthetase extracted from maize kernels had similar E_{a1}

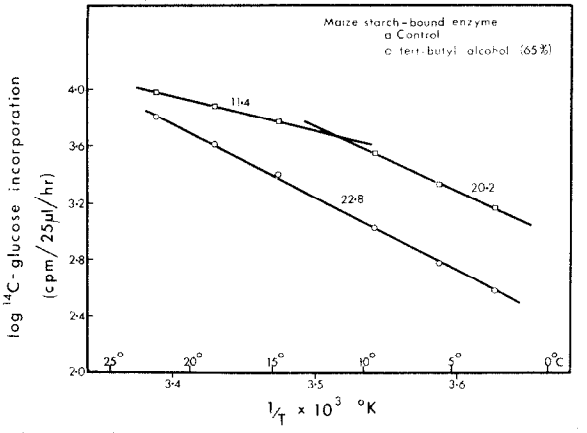

Fig. 2. Arrhenius plots of starch grain-bound ADPglucose–starch glucosyltransferase activity from maize kernels assayed with and without *t*-BuOH.

Table 2. Activation energies derived from Arrhenius plots of ADPglucose–starch glucosyltransferase activity from various plant organs

Enzyme preparation	No. expts.*	Activation energy (kcal/mol)	
		Above break	Below break
Avocado cotyledon starch			
Early July	3	8·1 ± 1·8	16·9 ± 1·7
Late August	3	17·4 ± 1·6	22·6 ± 1·2
Maize kernel starch	3	11·5 ± 0·2	23·2 ± 2·7
Maize kernel soluble	3	11·5 ± 2·5	21·0 ± 1·2
Sweet potato starch	3	16·4 ± 2·1	25·6 ± 1·6
		Over entire temperature range	
Potato tuber starch	3	17·8 ± 2·5	
Potato tuber soluble	1	14·9	

* Each at six temperatures with three replicates at each temperature.

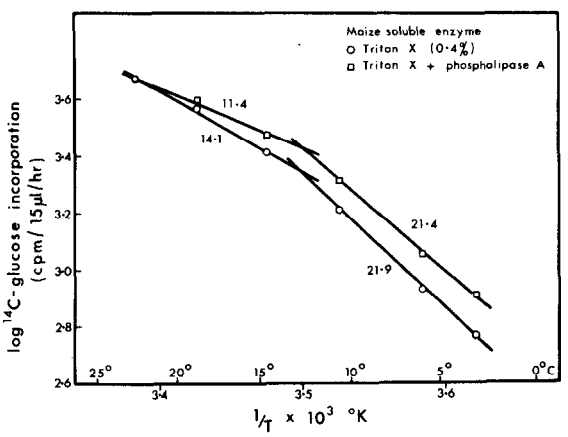

Fig. 3. Arrhenius plots of soluble ADPglucose–starch glucosyltransferase activity from maize kernels pre-treated and assayed with Triton X-100 and phospholipase A.

values both above and below the transition temperature. The E_{act} values below the transition temperature were twice as great as those above. The chilling-resistant potato showed no discontinuity in Arrhenius plots for either the soluble or grain-bound form of starch synthetase.

Figure 2 shows that when grain-bound starch synthetase from maize was assayed in the presence of high concentrations of *t*-butanol, the discontinuity in the Arrhenius plot disappeared and a constant E_{act} was obtained. This alteration in enzyme response was also found for avocado starch-bound enzyme. Addition of *t*-butanol to maize soluble enzyme strongly inactivated it. Figure 4 summarizes experiments to show that *t*-butanol can enter the amylose helix. For pure amylose (commercial preparation) plus iodine the main absorption of the blue complex occurs at about 630 nm. In the presence of 65% *t*-butanol, the iodine is displaced and the complex (max. absorption *ca* 530 nm) is red brown in color. With a soluble starch fraction of avocado (not pure amylose) a similar response to *t*-butanol is seen and the 600 nm peak is replaced by a 530 nm peak. The nature of the absorption maximum around 450 nm in the avocado preparation is unknown. Addition of water to these preparations, so that *t*-butanol is diluted out of the amylose helices, gives a return to the blue amylose–iodine complex at about 33% *t*-butanol

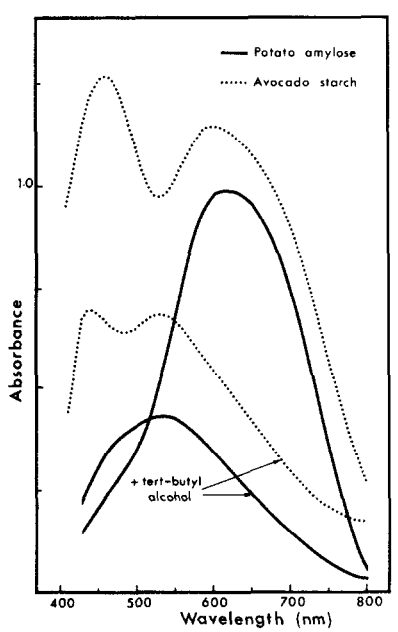

Fig. 4. Iodine absorption spectra for potato amylose and a soluble starch fraction from avocado cotyledon in the absence and presence of 65% *t*-BuOH.

concentration. X-ray diffraction data of an amylose–*t*-butanol complex support this interpretation [6].

DISCUSSION

Since starch synthetase in the chilling-sensitive plants maize, avocado and sweet potato showed a break in the Arrhenius plot at the same temperature that discontinuities have been reported for membrane-associated enzymes of these species [1, 3], we believe that a lipid interaction is responsible for the behaviour of starch synthetase at chilling temperatures.

X-ray diffraction investigations have established that amylose exists in a helical configuration and that the characteristic blue color of the amylose–iodine complex results from the arrangement of iodine molecules within the helix [7]. A number of other compounds can also complex with and selectively precipitate amylose, e.g. *n*-butanol, *t*-butanol [6], fatty acids [8] and lysolecithin [9]. Lysolecithin is of interest in view of recent reports that of the small lipid component in starch (about 1% of dry weight) up to 85% of this can be lysolecithin [10–12].

Lysolecithin is specifically associated with the amylose component of starch as evidenced by its proportionality to amylose content in kernels of maize mutants [11], its absence in waxy varieties of maize and rice [13] where the starch consists almost entirely of amylopectin and its increase in growing barley grains which runs parallel to the increase in amylose content [14]. Furthermore starch synthetase is considered to be bound to the amylose in starch grains. In developing waxy grains of rice, the enzyme is located almost exclusively in the soluble fraction and can be selectively bound to starch grains from non-waxy sources and to amylose [15, 16].

The observations that amylose is associated with both starch synthetase and lysolecithin provide the necessary elements for proposing a lipid–protein interaction in starch grains. Membrane-bound enzymes in chilling-sensitive plants show a discontinuity in the Arrhenius plot at about 12° which is attributed to a phase change in the lipid component of the membrane inducing comformational changes in associated enzymes [1]. For grain-bound starch synthetase it is proposed that a phase change in a lipid (possibly lysolecithin) at about 12° is responsible for the discontinuity in the Arrhenius plot. The stimulatory effects of *iso*propanol and cetyltrimethyl-ammonium bromide on particulate starch synthetase may reflect the presence of lipid [17, 18]. The recent suggestion that small glucoamylase resistant primers are attached to soluble starch synthetases [19, 20] to which lysolecithin could be bound, may explain the break in the Arrhenius plot for maize soluble enzyme. The straight-line relationship found for potato starch synthetase could result either from the documented absence of lysolecithin from potato starch [11] or from a lowered transition temperature of a more unsaturated lipid [1].

Triton X does not affect starch synthetase response to temperature, but detergents have been reported ineffective in eliminating the low temperature break in the Arrhenius plot for another enzyme, succinate oxidase of apple mitochondria [5]. Phospholipase A can remove the discontinuity in the Arrhenius plot of certain enzymes associated with membranes [1, 5]. Its lack of effect in the starch grain system is possibly a consequence of phospholipase being unable to penetrate the amylose helix or lack of effect of phospholipase A on certain lipids [10].

The elimination of the discontinuity by *t*-butanol in the grain-bound enzyme system suggests a perturbation of the synthetase–lipid interaction. Amylose, although blue colored when complexed with iodine, stains red when experimentally complexed to lysolecithin [9]. X-ray analyses add further support to the idea that lysolecithin occupies the helicoidal structure of amylose. From the results shown in Fig. 4 it is concluded that *t*-butanol can enter the amylose helix and could disturb an interaction between starch synthetase and lipid. Possibly *t*-butanol removes the lipid from the grains.

These studies add another dimension to the concept of enzyme control by lipid interaction and suggest that non-membrane lipids associated with enzymes can influence activity as a function of temperature. This work further suggests a unique role for a lipid, probably lysolecithin, in starch grain biogenesis since "no other native biological material is known in which the proportion of lysolecithin in the total lipid is so high" [12]. The presence of lysolecithin within some amylose molecules might inhibit the enzymatic transformation

of amylose to amylopectin by branching enzyme and permit the deposition of both unbranched polyglucan and amylopectin within the same grain. In this regard it seems significant that lysolecithin is not found in waxy starches.

EXPERIMENTAL

Materials. Cotyledons of avocado (*Persea americana* Mill. cv. Fuerte), 22-day-old self-pollinated kernels of maize (*Zea mays* L. cv. NES 1002), sweet potato tubers (*Impomoea batatas* Lam.) and potato tubers (*Solanum tuberosum* L.) were studied. Phospholipase A from *Crotalus terr. terr.* was purchased.

Extraction procedures. 2 g Sweet potato tubers were ground in a glass piston-type homogenizer with 10 ml 50 mM Tris–HCl buffer, pH 8·5 containing 10 mM DTT and 10 mM EDTA. In some experiments the tissue was ground in 50 mM Tris-acetate buffer at pH 8·5 with 2 mM $Na_2S_2O_5$. The homogenate was filtered through Miracloth and centrifuged at 30000 *g* for 15 min. The supernatant was assayed for soluble starch synthetase. The starch pellet was separated from other debris, resuspended in grinding buffer and centrifuged for 5 min at 10000 *g*. The starch pellet was repeatedly washed and centrifuged (from 3 to 10 times) until white. The final pellet was resuspended in *ca* 2 ml grinding buffer (omitting EDTA in experiments with phospholipase A) and used to assay starch grain-bound synthetase.

5 g Potato tuber were homogenized and treated as for sweet potato except that 10 ml of 50 mM Tris–acetate buffer, pH 8·5 containing 2 mM $Na_2S_2O_5$ was consistently used throughout the extraction procedure.

2 g Thinly sliced (0·5 mm) avocado cotyledon were ground in a mortar with 10 ml 350 mM Tris–acetate buffer, pH 8·5 containing 20 mM EDTA, 11 mM diethyldithiocarbamate, 15 mM cysteine-HCl and 6% Carbowax 4000. The starch pellet was purified with the above buffer mixture except that the Tris–acetate concn was decreased to 20 mM. The avocado homogenate was processed as for sweet potato.

6 g Maize kernels were ground in a mortar with 10 ml 50 mM Tris–acetate buffer, pH 8·5 containing 10 mM EDTA and 1 mM DTT and handled as for sweet potato. EDTA was omitted from preparations where soluble enzyme was to be assayed in the presence of phospholipase A.

Assay of ADPglucose: α-1,4-glucan α-4-glucosyltransferase. Transfer of glucose-[U-^{14}C] to a primer was determined as previously described [21]. Reaction mixtures contained 140 nmol of ADPglucose-[U-^{14}C] (600–700 cpm/nmol), 20 μmol Bicine buffer pH 8·5, 5 μmol potassium acetate, 2 μmol DTT, 1 μmol EDTA (omitted in phospholipase A treatments and replaced by 5 μmol $CaCl_2$), 1 mg rabbit liver glycogen as primer for soluble starch synthetase assays only, and enzyme in a final vol. of 0·2 ml. For assays of grain-bound starch synthetase, the starch grains themselves acted as primer for the reaction. Reactions were terminated by the addition of 2 ml of 1% KCl in 75% aq. MeOH [22]. For expts in which *t*-butanol was present (see below) or when large quantities of starch were used in the grain-bound system, the pellet was twice washed with MeOH–KCl, boiled with 0·5 ml H_2O, cooled and re-precipitated with 5 ml MeOH–KCl to further reduce the counts in controls containing boiled enzyme. The final pellet was boiled in 1 ml H_2O and 0·5 ml counted by liquid scintillation spectrometry.

Enzyme preparations treated with Triton X-100 were preincubated with 0·4–0·6% of the detergent on ice for 30 min prior to assay. Triton was also present during the subsequent assay.

Experiments with phospholipase A were done by preincubating 30 μl of the starch synthetase preparation with 2·4 i.u. of commerical phospholipase (with $CaCl_2$ and in some cases Triton X-100) for 15 min at 23° prior to assay. Phospholipase remained during the assay. Reaction mixtures with *t*-BuOH contained either 65 or 75% final concn of *t*-BuOH, 140 nmol ADPglucose-[U-^{14}C] and enzyme in a final vol. of 0·2 ml. H_2O was substituted for *t*-BuOH to provide control data. Assays with boiled enzyme provided additional controls for monitoring the removal of unreacted ADPglucose-[U-^{14}C] from starch in terminated reactions. Assays were conducted at six temps between 3 and 23°, a digital thermometer being used to ensure that the temps remained within ±0·1°.

Absorption spectra of α-glucan–I_2 complex. Avocado cotyledon starch was purified as described above, swirled in H_2O at about 70° for 10 min, and filtered through Whatman No. 1 paper to give a "soluble starch" fraction. A 0·2% soln of commercial potato amylose was also made in hot H_2O. Absorption spectra (430–800 nm) of the α-glucan–I_2 complex were recorded on a double beam spectrophotometer with the reference cuvette containing I_2 soln and in some instances *t*-butanol at the same concn as used in the sample cuvette. Final concns for the iodine soln were 0·2% KI + 0·02% I_2, and for *t*-BuOH, 65%.

Acknowledgement—Technical assistance by Vivienne Richards is gratefully acknowledged.

REFERENCES

1. Raison, J. K. (1973) *Bioenergetics* **4,** 285.
2. Giaquinta, R. T. and Geiger, D. R. (1973) *Plant Physiol.* **51,** 373.
3. Lyons, J. M. (1973). *Ann. Rev. Plant Physiol.* **24,** 445.
4. Ozbun, J. L., Hawker, J. S., Greenberg, E., Lammel, C., Preiss, J. and Lee, E. Y. C. (1973) *Plant Physiol.* **51,** 1.
5. McGlasson, W. B. and Raison, J. K. (1973). *Plant Physiol.* **52,** 390.
6. Bear, R. S. (1944) *J. Am. Chem. Soc.* **66,** 2122.
7. Rundle, R. E., Foster, J. F. and Baldwin, R. R. (1944) *J. Am. Chem. Soc.* **66,** 2116.
8. Schoch, T. J. and Williams, C. B. (1944) *J. Am. Chem. Soc.* **66,** 1232.
9. Nakamura, A., Shimizu, R., Kôno, T. and Funahashi, S. (1958) *Bull. Agr. Chem. Soc. Japan* **22,** 324.
10. Acker, L. and Schmitz, H. J. (1967) *Die Stärke* **19,** 233.
11. Acker, L. and Schmitz, H. J. (1967) *Die Stärke* **19,** 275.
12. Wren, J. J. and Merryfield, D. S. (1970) *J. Sci. Food Agr.* **21,** 254.
13. Nakamura, A., Kôno, T. and Funahashi, S. (1958) *Bull. Agr. Chem. Soc. Japan* **22,** 320.
14. Becker, G. and Acker, L. (1972) *Fette Seifen Anstrichmittel* **74,** 324.
15. Akazawa, T. and Murata, T. (1965). *Biochem. Biophys. Res. Commun.* **19,** 21.
16. Akazawa, T. (1965) in *Plant Biochemistry* (Bonner, J. and Varner, J. E., eds.), p. 258, Academic Press, New York.
17. Judewicz, N. D., Lavintman, N. and Cardini, C. E. (1972) *Phytochemistry* **11,** 2213.
18. Lavintman, N. and Cardini, C. E. (1972) *Plant Physiol.* **50,** 205.
19. Schiefer, S. (1973) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32,** 603.
20. Hawker, J. S., Ozbun, J. L., Ozaki, H., Greenberg, E. and Preiss, J. (1974) *Arch. Biochem. Biophys.* **160,** 530.
21. Ozbun, J. L., Hawker, J. S. and Preiss, J. (1971) *Biochem. Biophys. Res. Commun.* **43,** 631.
22. Ghosh, H. P. and Preiss, J. (1965) *Biochemistry* **4,** 1354.