Phytochemistry, 1974, Vol. 13, pp. 389 to 395. Pergamon Press. Printed in England.

ACID INVERTASE IN GERMINATING *LACTUCA SATIVA* SEEDS: EVIDENCE FOR *DE NOVO* SYNTHESIS

M. ELDAN and ALFRED M. MAYER

Botany Department, The Hebrew University, Jerusalem, Israel

(*Revised received* 16 *July* 1973. *Accepted* 11 *September* 1973)

Key Word Index—*Lactuca sativa*; Compositae; lettuce; seed germination; acid invertase; enzyme synthesis; isoelectric focussing.

Abstract—Acid invertase activity in germinating lettuce seeds is first observed after 15 hr germination, from when it rises steadily at least till 30 hr of germination. The enzyme was purified about 500-fold using ammonium sulphate fractionation followed by isoelectric focussing. Labelling the enzyme with $^{35}SO_4$ or leucine-^{14}C during development of its activity, followed by purification suggests that acid invertase is synthesized *de novo* during germination. The possible significance of acid invertase in the metabolism of the seed is discussed.

INTRODUCTION

THE LEVEL of sucrose, which is a reserve material in lettuce seeds, declines very rapidly after the onset of germination.[1] This led us to investigate invertase, and the way in which its activity arises during germination. Invertase has an important role in the metabolism of carbohydrates in plant tissues.[2–9] It has been suggested that some enzymes arise by activation from inactive forms during germination.[1] Evidence for this view has been obtained in a number of cases: acid phosphatase, a trypsin-like enzyme and NADH-cytochrome-*c* reductase in lettuce seeds;[10–12] acid phosphatase and isocitrate lyase in several seeds;[13] lipase and phytase in wheat grains[14] and the oxidases of malate and succinate and amylopectin-1,6-glucosidase in peas.[15,16,24] In the present paper the mode of formation of acid invertase in lettuce has been investigated, in order to determine whether it is synthesized *de novo* during germination, or arises from an inactive form.

[1] MAYER, A. M. and POLJAKOFF-MAYBER, A. (1967) in *Physiology, Ecology and Biochemistry of Germination* (BORRISS, H., ed.), Vol. aVIIa2, p. 483, Greifswald, Ernst-Moritz-Arndt Universität.
[2] GARDNER, D. C. J. and PEEL, A. J. (1971) *Phytochemistry* **10**, 2621.
[3] HATCH, M. D. and GLASZIOU, K. T. (1963) *Plant Physiol.* **38**, 344.
[4] HAWKER, J. S. and HATCH, M. D. (1965) *Physiol. Plant.* **18**, 444.
[5] JAISWAL, S. P. and VERMA, K. A. (1971) *Indian J. Agr. Sci.* **41**, 350.
[6] LYNE, R. L. and AP REES, T. (1971) *Phytochemistry* **10**, 2593.
[7] MCLACHLAN, G. A., DATKO, A. H., ROLLIT, J. and STOKES, E. (1970) *Phytochemistry* **9**, 1023.
[8] RICARDO, C. P. P. and AP REES, T. (1970) *Phytochemistry* **9**, 239.
[9] SEITZ, K. and LAND, A. (1968) *Plant Physiol.* **43**, 1075.
[10] ELDAN, M. and MAYER, A. M. (1972) *Physiol. Plant.* **26**, 67.
[11] MEYER, H., MAYER, A. M. and HAREL, E. (1971) *Physiol. Plant.* **24**, 95.
[12] SHAIN, Y. and MAYER, A. M. (1968) *Phytochemistry* **7**, 1491.
[13] PRESLEY, M. J. and FOWDEN, L. (1965) *Phytochemistry* **4**, 169.
[14] EASTWOOD, D., TAVENER, R. J. A. and LAIDMAN, D. L. (1969) *Biochem. J.* **113**, 32.
[15] KOLLOFEL, C. and SLUYS, J. V. (1970) *Acta Bot. Neerl.* **19**, 503.
[16] SHAIN, Y. and MAYER, A. M. (1968) *Physiol. Plant.* **21**, 765.

RESULTS

Acid invertase present in homogenates prepared from lettuce seeds was found to be soluble, after differential centrifugation. The presence of 0·4 M mannitol or 0·4 M sorbitol in the isolation medium did not affect invertase activity or its subcellular localization (Table 1). The presence of the enzyme in the supernatant was not caused by osmotic damage to subcellular particles, resulting in leakage during homogenization and centrifugation. The presence of antibiotics in the germination medium did not affect enzyme activity, indicating that bacteria were not contributing to measured invertase activity.

TABLE 1. THE EFFECT OF MANNITOL AND SORBITOL IN THE ISOLATION MEDIUM ON ACID INVERTASE ACTIVITY

Sugar added to the medium	Invertase activity in 100000 g supernatant
Control, no sugar, no antibiotics	700
Control, no sugar (seeds germinated in H_2O with 80 μg/ml chloramphenicol + 40 μg/ml streptomycin)	650
Mannitol 0·4 M	650
Sorbitol 0·4 M	650

The seeds were germinated for 21 hr. Activity of invertase is expressed as μg reducing sugar produced from sucrose during 1 hr, by enzyme from 1 g initial dry wt of seeds.

The development of acid invertase activity during germination is shown in Fig. 1. Invertase activity was first observed after 15 hr germination, and rose steadily up to at least 30 hr germination. Subsequently, activity continued to rise, but at a lower rate.

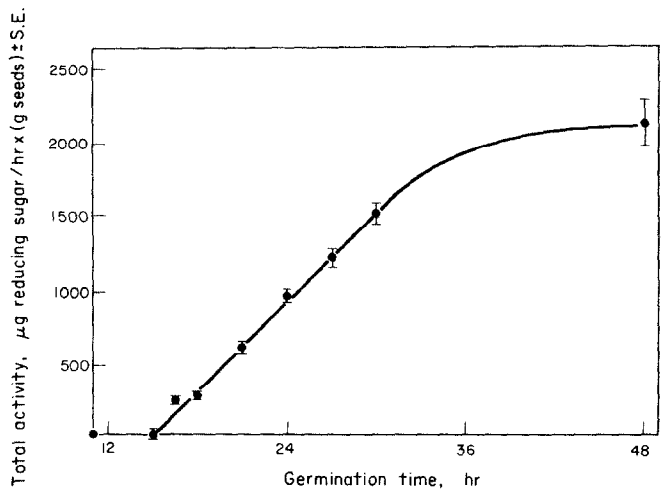

FIG. 1. The development of acid invertase activity during germination of *Lactuca sativa* seeds.

Invertase activity was not induced by its substrate, sucrose, when this was added to the germination medium, in the same concentration range used for the assay. The effect of inhibitors of protein synthesis on development of invertase activity was studied. Although results obtained by the use of inhibitors are by no means unambiguous, they may provide some indication whether the enzyme is formed by *de novo* synthesis or by activation.

Chloramphenicol and cycloheximide were introduced into the dry seeds using acetone.[17] Chloramphenicol inhibits protein synthesis mainly in subcellular particles; mitochondria and chloroplasts,[18,19] whilst cycloheximide, inhibits the synthesis of proteins synthesized in the cytoplasm.[19,20] We assumed that, because the inhibitors were introduced into the dry seeds, they would reach their site of action early and therefore act rapidly on the initial stages of protein synthesis in the germinating seeds. Thus, some insight might be obtained into whether invertase arises by *de novo* synthesis. The results are given in Tables 2 and 3.

TABLE 2. THE EFFECT OF CHLORAMPHENICOL AND CYCLOHEXIMIDE ON ACID INVERTASE ACTIVITY OF *Lactuca sativa* SEEDS

Germination (hr)	Invertase activity after treating the seeds with: Control (no treatment)	Acetone	Chloramphenicol in acetone	Cycloheximide in acetone
0	0	0	0	0
16	150	60	75	50
18	300	150	225	75
21	700	525	500	175
24	1000	800	850	400
27	1250	1050	750	825
30	1600	1050	750	950

Enzyme activity as μg of reducing sugar produced from sucrose/hr, by enzyme from 1 g initial dry wt of seed. (The seeds were pretreated for 70 hr with one of the following solns: (a) pure acetone, (b) chloramphenicol soln in acetone (5 mg/ml), (c) cycloheximide in acetone (10 μg/ml).

Acetone causes some damage to the seeds. The activity of invertase somewhat decreased in the seeds treated with acetone, as compared to the control seeds.

From the results in Table 2 it can be seen that cycloheximide markedly inhibits the appearance of acid invertase activity during the first 24 hr germination. This inhibition is overcome in the later stages of germination. Chloramphenicol has almost no effect. However, the failure of chloramphenicol to inhibit acid invertase formation might be due to the small amounts introduced into the seeds by the permeation method. Table 3 shows that when the same inhibitors were added to the germination medium, both had a considerable inhibitory effect on invertase activity. This might indicate either that invertase synthesis is not restricted to the cytoplasm, or that one of the inhibitors affects invertase activity indirectly, by inhibiting the synthesis of other proteins.

The demonstration of incorporation of radioactive label into the enzyme, during germination, would provide evidence that the enzyme is synthesized *de novo*. Sulphate-[^{35}S] and L-leucine[U-^{14}C] were used in these experiments. Sulphate was chosen because Meachum *et al.*[21] and Myrback[22] have found out that invertase contains at least two –SH groups. The sulphate supplied may be expected to be converted to SH-groups of amino acids incorporated into proteins, as has been demonstrated previously.[12] The likelihood

[17] MEYER, H. and MAYER, A. M. (1971) *Science* **171**, 583.
[18] ELLIS, R. J. (1969) *Science* **163**, 477.
[19] REGER, B. J., SMILLIE, R. M. and FULLER, R. C. (1972) *Plant Physiol.* **50**, 19.
[20] NEUPERT, W., SEBALD, W., SCHWAB, A. J., MASSINGER, P. and BUCHER, TH. (1969) *European J. Biochem.* **10**, 589.
[21] MEACHUM, Z. D., JR., COLVIN, H. R., JR. and BRAYMER, H. D. (1971) *Biochemistry* **10**, 326.
[22] MYRBACK, K. (1960) in *The Enzymes* (BOYER, P. D., LARDY, H. and MYRBACK, K., eds.), 2nd Edn, Vol. IV, p. 389, Academic Press, New York.

of exchange reactions with sulphate are also very much smaller than in the case of leucine. Neither sulphate nor leucine affected the activity of the enzyme, in the concentrations used in the incorporation experiments, during the first 24 hr germination.

TABLE 3. THE EFFECT OF CHLORAMPHENICOL AND CYCLOHEXIMIDE PERMEATED INTO DRY *Lactuca sativa* SEEDS, IN ACETONE OR ADDED TO THE GERMINATION MEDIUM, ON ACID INVERTASE ACTIVITY

Treatment before germination	Chloramphenicol in germination medium (4 mg/ml)	Cycloheximide in germination medium (10 μg/ml)	Invertase activity
No pretreatment	–	–	1000
Acetone	–	–	800
	+	–	225
	–	+	75
	+	+	50
Acetone + chloramphenicol	–	–	850
	+	–	125
	+	+	25
Acetone + cycloheximide	–	–	400
	–	+	60
	+	+	25

Enzyme activity as μg reducing sugar produced from sucrose/hr, by enzyme from 1 g initial dry wt of seeds. Seeds were germinated for 24 hr. The seeds were treated with acetone, chloramphenicol in acetone (10 mg/ml) or cycloheximide in acetone (10 μg/ml) for 70 hr prior to germination.

The results of the incorporation experiments with sulphate-[^{35}S], are given in Figs. 2 and 3. From Fig. 2 it seems that no acid invertase is present in the extract of seeds, after 10 hr germination in the presence of $^{35}SO_4^{2-}$.

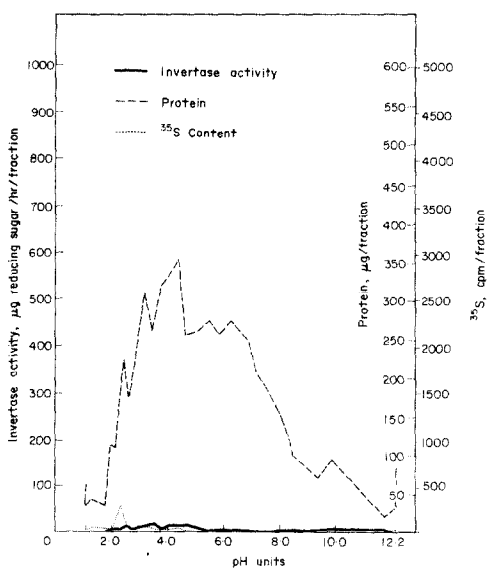

FIG. 2. ACID INVERTASE ACTIVITY, PROTEIN AND ^{35}S CONTENT OF FRACTIONS, SEPARATED BY ISOELECTRIC FOCUSSING.

The partially purified extracts were prepared from seeds germinated for 10 hr in a solution of $^{35}SO_4^{2-}$.

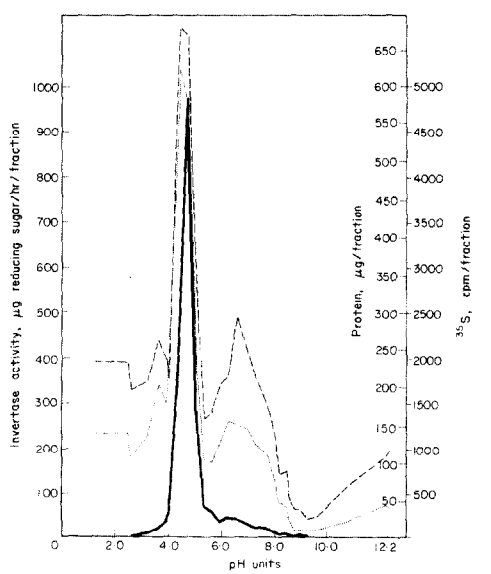

FIG. 3. ACID INVERTASE ACTIVITY, PROTEIN AND ^{35}S CONTENT OF FRACTIONS SEPARATED BY ISOELECTRIC FOCUSSING.

The partially purified extracts were prepared from seeds germinated for 24 hr in a solution of $^{35}SO_4^{2-}$. Symbols as in Fig. 2.

The partially purified preparation, prepared by ammonium sulphate fractionation, contained a single protein which seems to incorporate $^{35}SO_4^{2-}$. This protein had no invertase activity. Thus even during the early stages of germination, SO_4^{2-} is incorporated into proteins, presumably into –SH groups.

Figure 3 shows a high level of invertase activity in extracts of seeds after 24 hr germination in the presence of $^{35}SO_4^{2-}$. This activity is present in the isoelectric focussing column between pH 4·2 and 5·0. The fractions containing invertase had a specific activity 500-fold higher than the original crude extract of the seeds.

Large amounts of radioactive ^{35}S are found in the same fractions. Radioactivity was also found in additional protein fractions. Clearly a number of proteins incorporate radioactive sulfur during their synthesis during the first 24 hr germination.

Comparison of Figs. 2 and 3 shows that invertase is definitely synthesized between 10 and 24 hr germination. Experiments using radioactive leucine gave essentially the same results as those using $^{35}SO_4^{2-}$. After 10 hr germination, there were traces of invertase and some radioactivity in the same fractions. Incorporation of leucine-[^{14}C] was considerably higher than that of ^{35}S. After 24 hr germination in radioactive leucine, considerable radioactivity appears in a number of proteins. Invertase activity is again labelled.

It appears that invertase begins to be synthesized after *ca* 10 hr germination. A number of other proteins are also labelled, some of them before invertase.

DISCUSSION

Invertase might have an important role in the metabolism of carbohydrates during germination. Lettuce seeds contain large amounts of reserve fats. When these fats are metabolized, sugars are formed. These sugars serve as an energy source and as raw material for various synthetic processes.

We found an inconsistency between the time when sucrose starts to be utilized, and the time at which the activity of the enzyme can first be detected. The level of sucrose starts to decline immediately with the onset of germination, whereas acid invertase activity is found only after 10–15 hr germination. Acid invertase is apparently not the only enzyme which takes part in the hydrolysis of sucrose, at least not during the first 15 hr germination in lettuce seeds.

Invertase appears to have been little studied in relation to germination. Prentice[23] has reported on some of the properties of invertase in barley. In this tissue, both a soluble and a particulate form of the enzyme appear to be present. We were unable to detect a particulate invertase in lettuce seeds. All the activity, at various stages of germination, appeared in a soluble cell-fraction.

The enzyme is probably synthesized in the cytoplasm as indicated by the results of the experiments with inhibitors of protein synthesis. Chloramphenicol inhibited the development of the enzyme to a lesser extent than did cycloheximide, and the concentration of chloramphenicol required to cause this inhibition was very high.

The time at which acid invertase activity could first be detected was not changed by the inhibitors (Table 2). Although experiments using inhibitors of protein synthesis are always somewhat ambiguous and the specificity of the inhibitors may be questioned, the results in Tables 2 and 3 are consistent with the view that the acid invertase is formed by *de novo* synthesis.

[23] PRENTICE, N. (1972) *J. Agr. Food Chem.* **20**, 764.

The incorporation experiments using $^{35}SO_4^{2-}$ clearly showed that invertase is synthesized during germination. The synthesis seems to begin at around 10 hr after the onset of imbibition and is very marked and easily detected after 24 hr germination. Labelled protein was present in partially purified extracts of the seeds already before 10 hr germination, showing an early onset of protein synthesis. The experiments with radioactive leucine gave essentially similar results as those with $^{35}SO_4^{2-}$.

The fact that radioactivity was present in a fraction containing acid invertase with a specific activity 500 times that of the crude extract makes it very unlikely that anomalous labelling occurred. Previous work on lettuce seeds has already demonstrated that labelled precursors are incorporated into certain proteins during germination, but that such incorporation is not into all the seed proteins.[1,2]

From these experiments it also appears that during germination there is not a general resumption of synthesis of proteins. Clearly the sequence of protein synthesis is regulated.

EXPERIMENTAL

Plant material. Lettuce seeds *Lactuca sativa* L.cv. Grand Rapids, were germinated in the dark at 26° on filter paper in Petri dishes (1 g of seeds per dish), using 4·0–4·5 ml of dist. H_2O per dish, according to the duration of germination. After 90 min incubation, the seeds were illuminated for 10 min at 400 lm, and then returned to the dark.

Enzyme preparation. The dry seeds or seedlings were ground with a pestle and mortar, in 0·01 M Tris-HCl buffer, pH 7·5 containing 1 mM $MgSO_4$— using 10 ml/g initial dry wt of seeds. The crude homogenate was centrifuged for 5 min at 1000 *g*. The pellet was discarded, and the supernatant was centrifuged for 20 min at 20000 *g*. This pellet was discarded, and the supernatant was centrifuged for 1 hr, at 100000 *g*. The resulting supernatant was used as enzyme source. All operations were carried out at 2–4°.

Assay of enzymatic activity. Acid invertase activity was determined by following the formation of reducing sugars in the reaction mixture according to Somogy and Nelson.[24] The reaction mixture contained acetate buffer, pH 5·0 and sucrose to final concentrations of 30 mM, and enzyme. The final vol. of the reaction mixture was 5 ml and included 0·1 ml enzyme preparation. The Tris concentration was therefore 0·2 mM. Fractions from the isoelectric column were dialysed against 30 mM acetate buffer, pH 5·0 before assay. The activity of the enzyme is expressed as μg of reducing sugar formed/hr/g initial dry weight of seeds.

Partial purification of invertase. The enzymatic activity found in the 100000 *g* supernatant precipitated at 60% saturation with ammonium sulphate. About 90% of the original activity was recovered in the fraction precipitating between 40 and 60% saturation. The precipitate was suspended in a small volume of the same buffer used for homogenization and dialysed overnight, against the same buffer. The ammonium sulphate fractionation was repeated, resulting in total 25-fold purification of the enzyme. Further purification of the enzyme was achieved by using the isoelectric focussing technique as described by Vesterberg.[25] The column (model 8100, LKB) had a vol. of 110 ml and ampholine buffer, pH 3–10, at a concentration of 1–2% was used. Fractions of 3–4 ml were collected from the column at the end of each run. After the pH of the fractions had been measured, they were exhaustively dialysed against the buffer used for the assay of the enzyme, for several days, with several changes of buffer. The vol. of each fraction was measured and they were assayed for invertase activity and for protein according to Lowry.[26] The spc. act. of the enzyme recovered from the column was 500-fold higher than that of the original supernatant. Permeation of inhibitors of protein synthesis into the dry seeds was done according to Meyer and Mayer.[17]

Incorporation experiments. Sulphate-[^{35}-S]: the original soln was diluted with a sterile soln 5 mM K_2SO_4, containing chloramphenicol (70 μg/ml), and streptomycin (47 μg/ml). Seeds were germinated in the usual way, using the above soln, instead of H_2O. About 100 μCi of radioactive sulphate were used per g of seeds. The preparation and purification of the enzyme were done as described above. Radioactivity was determined in 1-ml aliquots of the fractions obtained from the isoelectric focussing column, in 10 ml scintillation mixture of Bray[27] using

[24] SOMOGY, M. and NELSON, N. (1955) as cited by HESTRIN, FEINGOLD and SCHRAMM, in *Methods in Enzymology* (COLOWICK, S. P. and KAPLAN, N. O., eds.), Vol. I, p. 234. Academic Press, New York.

[25] VESTERBERG, O. in *Methods in Enzymology* (COLOWICK, S. P. and KAPLAN, N. O., eds.), Vol. XXII, p. 389. Academic Press, New York.

[26] LOWRY, O. H., ROSEBROUGH, N. H., FARR, A. L. and RANDALL, R. J. (1951) *J. Biol. Chem.* **193**, 265.

[27] BRAY, G. A. (1960) *Anal. Biochem.* **1**, 279.

a Packard model Tri Carb 3002 liquid scintillation counter. L-Leucine-[U-^{14}C]: the original soln of the radioactive leucine was diluted under sterile conditions with cold 0·1 mM L-leucine containing 90 μg/ml chloramphenicol and 50 μg/ml streptomycin. *ca.* 30 μCi leucine were used per g of seeds. The preparation and assays were the same as those described for the sulphate experiments. The radioactive chemicals were purchased from The Radiochemical Center, Amersham.