Phytochemistry, 1971, Vol. 10, pp. 2593 to 2599. Pergamon Press. Printed in England.

INVERTASE AND SUGAR CONTENT DURING DIFFERENTIATION OF ROOTS OF *PISUM SATIVUM*

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(*Received* 12 *March* 1971)

Abstract—Acid and alkaline invertases (pH optima pH 5·1 and pH 7·3; K_m for sucrose 5·3 $\times$ 10^{-3} M and 13·1 $\times$ 10^{-3} M, respectively) were demonstrated in roots of pea (*Pisum sativum* L.). Their activities and the contents of sucrose, glucose, and fructose were related to protein content and cell number in successive 3 mm segments of the apical 24 mm of the root, and to protein content in stele and cortex isolated from the root 6–24 mm from the apex. Alkaline invertase varied little during differentiation but was most active in the stele where the sucrose concentration was highest. Acid invertase activity was low in the apical 3 mm, rose to a high peak 3–9 mm from the apex and then declined to a steady level 15–24 mm from the apex. Nearly all of the acid invertase 6–24 mm from the apex was in the cortex. Acid invertase activity was inversely related to sucrose content. Measurements of invertase activity in cell fractions, and of hexose formation from sucrose supplied to untreated tissues and to tissues pretreated with ethyl acetate, indicated that all the alkaline invertase and at least half of the acid invertase was intracellular.

INTRODUCTION

THERE is considerable evidence that acid invertase, optimum pH 3·8–5·5, and alkaline or neutral invertase, optimum pH 7·0–8·5, are important in the regulation of sucrose metabolism in plants. High acid invertase activity appears to be characteristic of growing cells, and in storage tissue of carrot and sugar cane the activity is inversely related to sucrose content (Ref. 1, and references therein). Ricardo and ap Rees[1] proposed that acid invertase, some of which is intracellular, possibly located at the tonoplast, directs exogenous and endogenous sucrose to hexose in cells where the demand for hexose is high. They also argued that acid invertase could determine the ability of cells to store sucrose in that appreciable storage was possible only in cells with low activity of intracellular acid invertase. Finally they suggested that the alkaline or neutral invertase was located in the cytoplasm and catalysed sucrose hydrolysis in cells that lacked acid invertase, most notably in cells that stored sucrose.

We have investigated the above views by determining whether the relationships between invertase activity and sugar content, predicted by the hypotheses, hold during the changes that occur in the differentiation of roots of pea, a plant known to translocate much of its carbon as sucrose.[2] First we determined whether pea roots contained acid and alkaline invertases. Then we measured the activities of the two enzymes, and the contents of sucrose, glucose, and fructose in root cells at different stages of differentiation. These stages were obtained by cutting the apical 24 mm of the root into eight successive 3 mm segments, and by carefully separating stele from cortex in the region 6–24 mm from the root apex. As there are reports (Ref. 1 and references therein) that an appreciable proportion of acid invertase of many plant cells is located on the cell wall, we tried to distinguish between cell-wall and intracellular acid invertase by fractionation of cell extracts and by the method of Bacon *et al.*[3]

[1] C. P. P. RICARDO and T. AP REES, *Phytochem.* **9**, 239 (1970).
[2] H. WANNER, *Ber. Schweiz. Botan. Ges.* **62**, 205 (1952).
[3] J. S. D. BACON, I. R. MACDONALD and A. H. KNIGHT, *Biochem. J.* **94**, 175 (1965).

In this latter method production of hexose from exogenous sucrose supplied to unbroken tissues is equated with cell-wall invertase, and production from sucrose supplied to unbroken tissues that have been pretreated with ethyl acetate is equated with total acid invertase. We realize that assays of invertase in root segments have been reported previously[4–6] but point out that these measurements were made before it was appreciated that plants contained alkaline as well as acid invertases.

RESULTS

Evidence for Acid and Alkaline Invertases

Invertase activity in the soluble fraction of extracts of the apical 3 cm of pea roots showed a peak at pH 5·1 with a shoulder from pH 6·0–7·0. This result, and the pH optima of known acid and alkaline invertases, prompted us to see whether invertase activity at pH 5·0 and at pH 7·0 in the above extracts could be separated by fractionation with ammonium sulphate. Activities at the two pH values separated cleanly (Fig. 1). We determined the

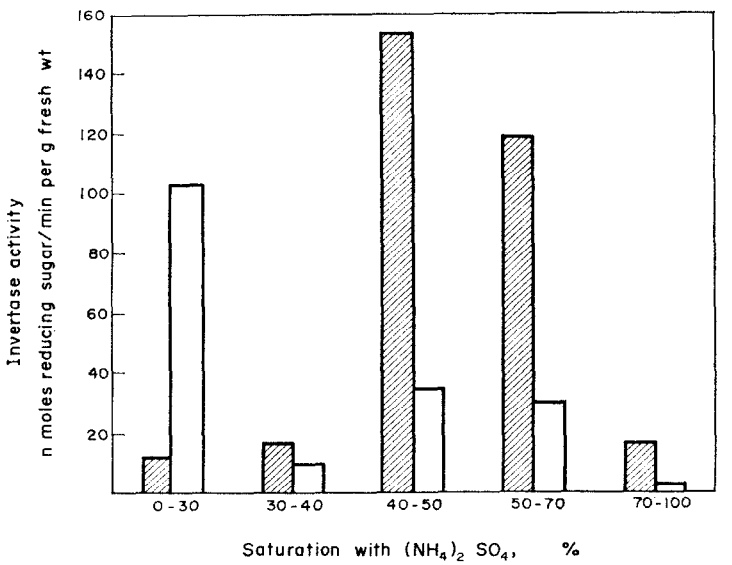

FIG. 1. PARTIAL SEPARATION OF INVERTASE ACTIVITIES AT pH 5·0 (▨) AND pH 7·0 (□) BY AMMONIUM SULPHATE FRACTIONATION OF EXTRACT OF APICAL 3 cm OF PEA ROOT.

effect of pH on the invertase activity of the fraction that precipitated between 40 and 70 per cent saturation and found a broad peak of activity from pH 4·5 to pH 6·0 with an optimum at pH 5·1. Similar experiments with the fraction that sedimented between 0 and 30 per cent saturation revealed a sharp optimum at pH 7·3 with very little activity below pH 6·5. We refer to the invertase activities with pH optima at pH 5·1 and at pH 7·3 as acid and alkaline, respectively. The above partially purified preparations were used for determining Km for sucrose. Using both the Lineweaver and Burk plot[7] and the Wolf plot,[7] we

[4] E. ROBINSON and R. BROWN, *J. Exptl. Bot.* **3**, 356 (1952).
[5] J. A. HELLEBUST and D. F. FORWARD, *Can. J. Bot.* **40**, 113 (1962).
[6] R. SEXTON and J. F. SUTCLIFFE, *Ann. Bot.* **33**, 407 (1969).
[7] M. DIXON and E. C. WEBB, *Enzymes*, p. 69, Longmans, London (1965).

obtained values of $5{\cdot}3 \times 10^{-3}$ M for the acid invertase and $13{\cdot}1 \times 10^{-3}$ M for the alkaline invertase.

Invertase Activity and Sugar Content during Differentiation

The characteristics of the segments used in our determination of the distribution of the invertases along the root are shown in Table 1. The values for invertase represent activity in the soluble fraction of the extracts. Less than 2 per cent of the total alkaline invertase activity was recovered in the cell-wall fractions. The percentages of the total acid invertase recovered in the cell-wall fractions of the different segments were, successively from the apex, 4, 5, 3 8, 10, 12, 14 and 16. As the acid invertase shows appreciable activity at pH 7·3, the soluble fractions of the extracts of segments and steles and cortices were fractionated with ammonium sulphate before assay of the enzymes. In this work we used more dilute extracts than those prepared for the separation shown in Fig. 1. Under these conditions we found

TABLE 1. INVERTASE ACTIVITY AND SUGAR CONTENT OF SEGMENTS OF PEA ROOT TIPS

Property	Root segments (mm from tip) 0–3	3–6	6–9	9–12	12–15	15–18	18–21	21–24
Cells/segment $\times 10^{-3}$	156	76	41	29	34	32	33	29
Extracted protein (μg/segment)	56	27	16	16	19	20	21	20
Sugar content (mg/g fresh wt.)								
(i) Sucrose	3·6	2·3	1·8	2·6	3·1	3·5	3·7	3·9
(ii) Glucose	2·8	7·3	7·3	7·6	9·3	9·1	9·5	9·5
(iii) Fructose	< 0·3	1·8	3·2	2·0	2·0	< 0·3	< 0·3	< 0·3
Activity of extracted invertase (nmoles reducing sugar/min/mg protein)								
(i) Acid invertase	62	335	427	272	171	125	102	101
(ii) Alkaline invertase	36	42	47	48	48	46	50	47
Reducing sugar (nmoles/min/mg protein) in medium after incubating segments in:								
(i) Sucrose	55	86	101	93	65	58	50	43
(ii) Ethyl acetate followed by sucrose	105	459	455	297	185	129	111	98

that the optimum separation of the two invertases was obtained by taking the fraction sedimenting between 39 and 95 per cent saturation as the acid invertase, and that between 0 and 39 per cent saturation as the alkaline invertase.

We stress the following from Table 1. We determined the pattern of invertase activities along the root six times. We found close agreement within the same batch of peas but the absolute values sometimes varied with different batches of peas. None the less the pattern of invertase activities and its relation to sugar content and differentiation was very similar in all peas examined. The activity of the alkaline invertase did not vary greatly along the root. The activity of the acid invertase in the soluble fraction of the homogenates always exceeded that of the alkaline invertase. Acid invertase activity varied considerably, rising sharply from the apex to a peak of very high activity 3–9 mm from the tip and then falling to a steady

value. Hexose production from sucrose supplied to segments treated with ethyl acetate corresponded to the activity of acid invertase in the cell fractions in each segment. In contrast hexose production by untreated segments was always less than half that achieved by segments that had been treated with ethyl acetate. This discrepancy was greatest 3–9 mm from the apex. Glucose was the predominant sugar and every segment had more glucose than fructose. Finally, the sucrose content of all segments was low and the lowest values coincided with the highest acid invertase activities.

TABLE 2. INVERTASE ACTIVITY AND SUGAR CONTENT OF STELE AND CORTEX 6–24 mm FROM APEX OF PEA ROOT

Property	Stele	Cortex
Fresh wt. (mg/stele or surrounding cortex)	1·7	15·5
Protein (μg/stele or surrounding cortex)	22	78
Sugar content (mg/g fresh wt.)		
(i) Sucrose	10·9	1·8
(ii) Glucose	4·7	10·6
(iii) Fructose	0·7	1·2
Activity of extracted invertase (nmoles reducing sugar/min/mg protein)*		
(i) Acid invertase	21 ± 3 (4)	181 ± 10 (5)
(ii) Alkaline invertase	53 ± 4 (4)	38 ± 3 (5)
Activity of acid invertase in isolated cell wall fraction (nmoles reducing sugar/min/mg protein†)	3	30
Reducing sugar (nmoles/min/mg protein) in medium after incubating segments in:		
(i) Sucrose	21	105
(ii) Ethyl acetate followed by sucrose	19	201

* Values are given as means ± S.E. The number of extracts assayed is given in parentheses.

† Represents protein extracted from tissue used to prepare cell-wall fractions.

The properties of isolated steles and cortices, shown in Table 2, were determined with a number of different batches of roots. There was less variation than with the root segments and the relative distribution of sugars and invertase activities was constant. Composite samples, composed of 215 mg fresh wt. of stele and 215 mg fresh wt. of cortex, yielded activities of acid and alkaline invertase that were predictable from measurements made with separate samples of stele and cortex. Thus it is unlikely that the activities of the two invertases in extracts of either stele or cortex were greatly affected by inhibitors produced or released during the preparation of the extracts. The stele was characterized by the association of very low acid invertase activity with a concentration of sucrose that was high relative to that in the cortex. Conversely, in the cortex, very high acid invertase activity was associated with low sucrose concentration. The concentrations of glucose and fructose were greater in the cortex than in the stele, and both tissues contained more glucose than fructose. Alkaline

invertase activity in the stele was consistently higher than that in the cortex. The production of hexose from sucrose given to cortical and stelar tissues that had been treated with ethyl acetate was similar to the acid invertase activities of the cell fractions. Hexose production by untreated cortices was low compared with that by treated cortices. Steles differed from cortices in that treatment with ethyl acetate did not enhance their ability to release hexose from exogenous sucrose.

DISCUSSION

We conclude that pea roots contain an acid and an alkaline invertase. Our evidence is the separation of activity at pH 5·0 from that at pH 7·0, the different pH optima and Michaelis constants of the two fractions so obtained, and the independent variation of the two activities during differentiation. These results strengthen further the view that an ability to form an acid and an alkaline or neutral invertase is a general feature of higher plants.

The activity of the alkaline invertase in relation to protein was remarkably constant throughout the root despite the appreciable changes in cell protein content that occurred during differentiation. The somewhat higher activity of the stele was an exception and this coincided with a relative lack of intracellular acid invertase and the highest concentration of sucrose found in the root. The close relationship between alkaline invertase activity and protein content, the lack of such activity in the cell-wall fractions, and an optimum at pH 7·3, all suggest that alkaline invertase is located in the cytoplasm in pea roots. Thus our results are consistent with the view that the role of alkaline invertase is sucrose hydrolysis in the cytoplasm, particularly in cells that contain a lot of sucrose and lack appreciable intracellular acid invertase activity.

In general the ability of tissues to hydrolyse sucrose after pretreatment with ethyl acetate corresponded to the activity of acid invertase found in homogenates. This correspondence indicates that both methods were adequate assays for acid invertase activity. This activity varied considerably in a manner that suggests that high activity diverted sucrose to hexose and was characteristic of tissues with a marked need of hexose. The highest activity was found 3–9 mm from the root apex, a region where large amounts of hexose are required for biosynthesis, respiration,[8] and also possibly for osmotic purposes. From the properties and relative activities of the pea root invertases, it seems likely that the high invertase activities, reported by others[4–6] for region 3–12 mm from root tips, represent acid invertase. Thus an association between cell elongation in roots and high acid invertase activity may be general.

The reversible effects of pH on the distribution of acid invertase in carrot extracts[1] make it difficult to interpret the distribution of this enzyme in pea extracts. The ability of untreated tissues to produce hexose in the presence of exogenous sucrose is evidence of a cell-wall invertase. However, the extent of this hexose production is not necessarily an accurate measure of cell-wall invertase as some of the hexose found in the external medium could have originated from intracellular hydrolysis of the supplied sucrose. Despite these limitations, both the distribution of acid invertase in pea extracts, and the effects of ethyl acetate on the ability of root segments, steles, and cortices to hydrolyse sucrose, indicate that at least half, and in many regions of the root much more, of the acid invertase is intracellular. The low pH optimum of this enzyme and our evidence for a distinct cytoplasmic alkaline invertase suggest to us that the intracellular acid invertase in pea roots may be at the tonoplast or in the vacuole. Such a location could prevent sucrose storage by hydrolysis of vacuolar sucrose. We point out that the effects of ethyl acetate on hexose production by root segments supplied

[8] M. W. Fowler and T. ap Rees, *Biochem. Biophys. Acta* **201**, 33 (1970).

with sucrose, indicate that the highest intracellular activity of acid invertase was 3–9 mm from the root apex where the concentration of sucrose was lowest. Similarly the cortex had high intracellular acid invertase activity and a low concentration of sucrose. In contrast the stele, with the highest concentration of sucrose, appeared to have the lowest activity of intracellular acid invertase. This concentration of sucrose in the stele as opposed to the cortex is not a peculiarity of pea roots as a similar situation has been demonstrated in roots of *Pandanus*.[9]

Our results indicate that some of the acid invertase in pea root cells is located outside the plasmalemma. The function of this enzyme is not yet apparent. It could regulate sucrose concentration in the free space. Such regulation may be important in morphogenesis as there is evidence that sucrose can contribute to the induction of vascular tissue[10] and of phloem[11,12] in particular. It is of interest that our results indicate that cell-wall invertase activity was highest in parenchymatous cells that do not normally develop into vascular tissue.

We think that our data for acid and alkaline invertases support the hypothesis put forward by Ricardo and ap Rees.[1] In addition our results indicate that changes in the activities of these enzymes are important aspects of the differentiation of the pea root. It appears that during rapid cell expansion alkaline invertase increases in proportion to protein, whilst acid invertase increases much more. As growth slows down the activity of acid invertase in the cortical cells drops significantly but still remains high. In contrast, during differentiation of the stele, acid invertase is either lost or not synthesized, whereas alkaline invertase increases slightly to reach a higher activity in the stele than in the cortex. These changes in invertase activity may constitute part of the mechanism whereby the developing root regulates sucrose metabolism.

EXPERIMENTAL

Material. Seeds of pea (*Pisum sativum* L. var Kelvedon Wonder) were surface sterilized, soaked in running tap H_2O for 24 hr, germinated for 48 hr between paper towels moistened with 0·2 mM $CaCl_2$, and then grown in an aerated solution of 0·2 mM $CaCl_2$ for 48 hr. Germination and growth were at 25° in the dark. Roots were excised and sectioned mechanically in an apparatus similar to that described by Brown and Broadbent[13] except that it was modified to cut 100 roots at a time. The root segments were kept in H_2O at 2° until sampled. All operations from the excision of the root to the end of the sampling were carried out at 4°. The time between the cutting of the first segment and the preparation of the extracts was 4 hr or less.

For the separation of stele from cortex the apical 6 mm of the root was excised. The root was then bent 3–4 cm from the tip so that the cortex between the stele and the upper side of the bend snapped. The piece of cortex between this break and the root tip was then gently pulled away from the remainder of the root. The other half of the cortex was then removed in the same way. This left the stele attached to the main root and gave the cortex in two roughly equal pieces. Cortex and stele from the region 6–24 mm from the original apex of the root were then excised and stored at 2° until used. Prior to sampling the tissues were rinsed 3 × H_2O at 2°. The time between the removal of the first stele and the preparation of the extracts was 3 hr or less. Sections were cut from a random selection of isolated steles and cortices, stained, and examined. These showed that we were separating the two parts of the root reproducibly and cleanly at the endodermis and that there was very little cross-contamination. In order to obtain this reproducibility we found it essential to use fully turgid roots.

Methods. The phosphate–citrate buffers were prepared from appropriate mixtures of NaH_2PO_4, Na_2HPO_4, citric acid and Na citrate and the pH was adjusted with NaOH. For the separation in Fig. 1 and the measurement of pH optima and K_m, tissue was homogenized in 1·5 vol. 0·1 M NaH_2PO_4–0·1 M Na citrate (pH 7·0).

[9] C. P. SIDERIS, B. H. KRAUSS and H. Y. YOUNG, *Plant Physiol.* **12**, 899 (1937).
[10] L. W. ROBERTS, *Bot. Rev.* **35**, 201 (1969).
[11] R. H. WETMORE and J. P. RIER, *Am. J. Bot.* **50**, 418 (1963).
[12] R. A. JEFFS and D. H. NORTHCOTE, *J. Cell Sci.* **2**, 77 (1967).
[13] R. BROWN and D. BROADBENT, *J. Exptl. Bot.* **1**, 249 (1950).

Extracts of root segments, steles, and cortices were made from samples of 400–700 mg fresh wt. in 10 vol. of the above buffer. For assay of invertase in the soluble fraction, homogenates were centrifuged at 35,000 *g* for 15 min and the resulting supernatant was fractionated at pH 7·3 by successive additions of solid $(NH_4)_2SO_4$. Alkaline invertase precipitated by $(NH_4)_2SO_4$ was dissolved in 0·1 M NaH_2PO_4–0·1 M sodium citrate (pH 7·3) and then dialysed for 18–24 hr against two successive 5 l. quantities of the same buffer at 0·1 strength. Acid invertase was treated similarly except that the buffer was 0·1 M Na_2HPO_4–0·1 M citric acid at pH 5·1. Cell-wall preparations were obtained by resuspending in extraction buffer the fraction of the crude homogenate that sedimented at 35,000 *g* and then centrifuging this suspension at 1100 *g* for 15 min. The resulting precipitate was then washed, by resuspension and sedimentation at 1100 *g*, twice in 0·1 M Na_2HPO_4–0·1 M citric acid at pH 5·1 and twice in the same buffer at 0·2 strength, to give the cell-wall fraction.

For the assay of invertase the method of Arnold[14] was modified by using the above phosphate–citrate buffers instead of acetate buffer. The final concentration of the buffer was usually 5 mM with respect to both phosphate and citrate and was always within the range 5–40 mM, a range of concentrations that we showed to have no effect on the assay. We overcame the lag in the standard curve, noted by Arnold, by including 0·75 μmoles of glucose in each assay mixture. Alkaline and acid invertase were routinely assayed at pH 7·3 and pH 5·1, respectively. Assay of acid invertase in HOAc–NaOAc buffer, and of alkaline invertase in veronal-HCl buffer, gave activities similar to those obtained in the presence of phosphate. Thus the invertase activities can not be ascribed to sucrose phosphorylase.

The ability of tissues to hydrolyse exogenous sucrose was estimated at 30° by incubating samples of 0·2–1·0 g fresh wt. in at least 25 vol. 0·1 M sucrose in 0·01 M Na_2HPO_4–0·01 M citric acid buffer (pH 5·1). Hexose production, over the period 30–90 min from the addition of sucrose, was measured as above. Pretreatment with EtOAc involved incubation in the solvent at 2° for 25 min followed by rinsing in H_2O at 2° (2 × 5 min and then 2 × 30 min). Untreated samples were also rinsed in cold H_2O (3 × 5 min) before incubation. Hexose production by untreated tissue in 0·1 M mannitol was 10% or less than that in 0·1 M sucrose. Thus we have disregarded differences of 10% or less in the ability of tissues to hydrolyse exogenous sucrose.

Sugars were extracted in boiling 80% (v/v) EtOH. Sucrose was determined from the increase in reducing sugar, measured as described above, caused by treatment with invertase. Glucose and fructose were isolated by paper chromatography in EtOAc–pyridine–H_2O (8:2:1) and then assayed by the method of Wilson.[15]

Protein was determined by the Folin[16] method and cell numbers by the method of Brown and Rickless.[17]

Acknowledgements—We are very grateful to Mrs. W. J. L. Wong for teaching us how to separate stele from cortex. R.L.L. thanks the Science Research Council for a research studentship.

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[15] C. M. Wilson, *Analyt. Chem.* **31,** 1199 (1959).
[16] E. Layne, in *Methods in Enzymology* (edited by S. P. Colowick and N. O. Kaplan), Vol. 3, p. 447, Academic Press, New York (1957).
[17] R. Brown and P. Rickless, *Proc. R. Soc.* **136B,** 110 (1949).