Phytochemistry, 1973, Vol. 12 ,pp. 67 to 71. Pergamon Press. Printed in England.

SOLUBILITY OF PLANT INVERTASES*

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(*Received* 29 *June* 1972. *Accepted* 4 *September* 1972)

Key Word Index—*Helianthus tuberosus*; Compositae; Jerusalem artichoke; invertase; β-fructofuranosidase; cell wall; enzyme solubilization; aged tissue slices.

Abstract—Solubilization of acid invertase associated with cell wall preparations from aged slices of Jerusalem artichoke tuber tissue was achieved at high ionic strengths. It is proposed that the soluble enzyme is bound predominantly by salt linkages to some component of the cell wall preparation.

INTRODUCTION

FREQUENT references have appeared in the literature to enzyme activities associated with residual fractions prepared by centrifugation of higher plant tissue homogenates.[1,2] The plant invertases are a case in point; Straus[3] was able to show that crude fractionation of homogenates of tissue from 10 out of 11 species of higher plants yielded residues containing most of the measurable invertase activity, and similar observations have been reported since.[4–8] The commonly assumed location of the enzyme is the cell wall. This is in line with the well documented case of yeast cell wall invertases,[9–11] and appears to account neatly for the free space invertase activity of aged tissue slices.[4,5]

Attention, of late, has focused on the physiological role of plant invertases, but progress is hampered by uncertainties as to the locations of these enzymes *in vivo*. It is by no means certain that invertase is bound to the wall in living tissue or, indeed, whether its apparent (entire or partial) insolubility is an artefact. The limited state of knowledge here is emphasized by the results of Ricardo and ap Rees.[7] They show not only that the proportions of acid invertase activity in supernatant and residual fractions of carrot tissue depend upon the pH of the extraction medium, an increase in pH increasing the proportion of soluble enzyme, but that this effect is reversible. Nakagawa *et al.*[8] have reported a similar pH effect on reversible binding of tomato pericarp invertase to a cell wall fraction prepared by sucrose density gradient centrifugation.

* E.C. 3.2.1.26.

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It seems that an enzyme present in a residual fraction must fall into one of three categories; (1) enzymes covalently bound to a component of the residue; (2) enzymes non-covalently bound, by for example H-bonding or salt linkages, to a component of the residue; and (3) enzymes occluded inside a component of the residue.[9,11]

These categories are not necessarily related to the location of the enzyme *in vivo*, and it can be predicted that category (2) in particular will contain a number of artefacts of extraction: for example, Hawker[6] has shown that grape berry invertase appears in the residual fraction unless precautions are taken during extraction against protein-tannin interaction.

The apparently anomalous results obtained by Ricardo and ap Rees[7] with borate buffers suggest that their results might be explained in terms of an ionic strength (I) effect or, at least, of the combined effects of pH and I. This is entirely reasonable for electrostatic interactions.

The work with aged Jerusalem artichoke tuber tissue described in this paper shows that ionic strength is indeed an important parameter of the extraction procedure, and may reconcile some inconsistencies existing in the literature.

RESULTS AND DISCUSSION

Figure 1 presents the results of extraction of a cell wall preparation (CW1) over the pH range 4–9, and at I values 0·2, 0·5 and 1·0. In Fig. 2 the results of extractions of CW1 at 5 values of I at pH 5 and 10 are shown. Although no attempt was made to standardize the time available for equilibration within the ground mixture, both repeated extraction and

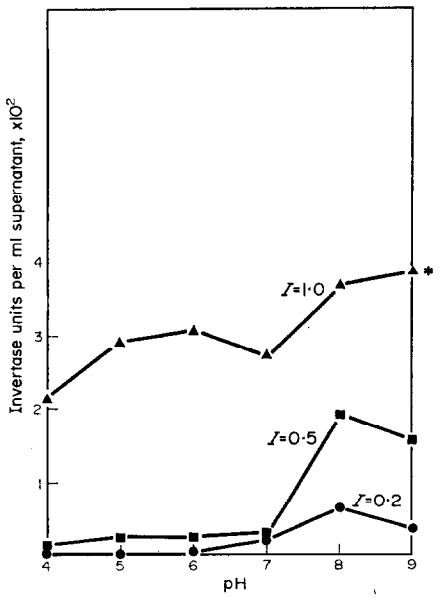

FIG. 1. EFFECT OF pH AND IONIC STRENGTH OF EXTRACTION BUFFER ON INVERTASE ACTIVITY EXTRACTED FROM A CELL WALL PREPARATION (CW1) FROM AGED JERUSALEM ARTICHOKE TUBER TISSUE SLICES (1 ml SUPERNATANT ≡ 10 mg CW ≡ *ca.* 0·5 g FRESH TISSUE SLICES).

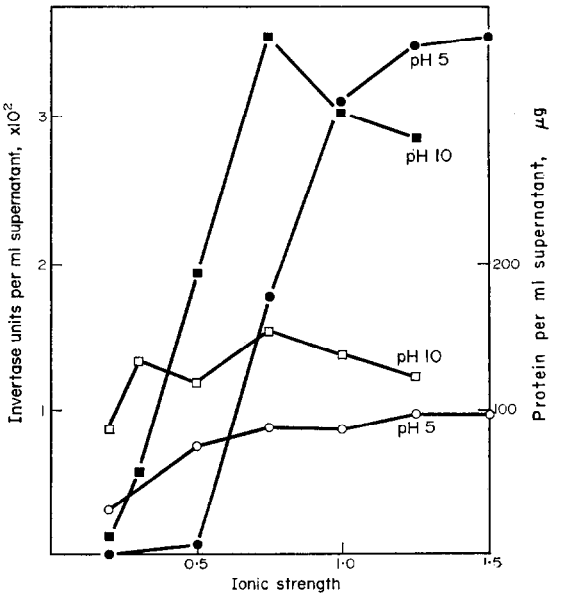

FIG. 2. EFFECT OF pH AND IONIC STRENGTH OF EXTRACTION BUFFER ON INVERTASE ACTIVITY (closed symbols) AND PROTEIN (open symbols) EXTRACTED FROM A CELL WALL PREPARATION (CW1) FROM AGED JERUSALEM ARTICHOKE TUBER TISSUE SLICES (1 ml SUPERNATANT ≡ 10 mg CW ≡ *ca.* 0·5 g FRESH TISSUE SLICES).

* This point represents solubilization of about 50% of the activity measurable after fractionation.

stirring the ground mixture for 30 min before centrifugation failed to extract significantly more enzyme than the standard procedure.

The effects of pH and I on the quantity of invertase removed from CW1 is strongly reminiscent of the situation encountered in the elution of poly-electrolytes from ion exchangers. Indeed, purification of the enzyme was achieved by judicious choice of extraction procedure. Successive extraction steps on CW1 at pH 5, $I = 0{\cdot}5$ followed by $I = 1{\cdot}4$, yielded preparations of specific activities 0·045 and 1·2 μmol RS min^{-1} mg^{-1} respectively, whereas extraction at pH 5, $I = 1{\cdot}5$ alone gave a value of 0·37 μmol RS min^{-1} mg^{-1}. This is confirmed by the results obtained by salt gradient elution of protein from a similar preparation (CW2) packed in a column (Fig. 3). At pH 5, $I = 0{\cdot}1$ the soluble enzyme was found to rebind totally to the CW1 from which it had been extracted.

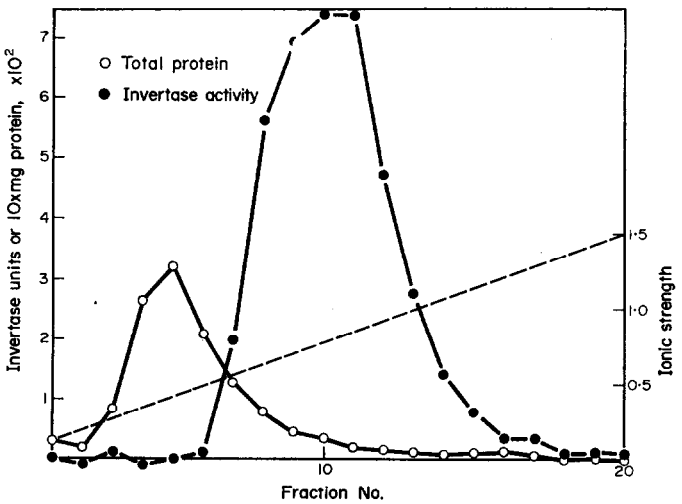

FIG. 3. ELUTION OF PROTEIN FROM A CELL WALL PREPARATION (CW2) FROM AGED JERUSALEM ARTICHOKE TUBER TISSUE SLICES. THE ELUTING SYSTEM WAS A LINEAR IONIC STRENGTH GRADIENT ($I = 0{\cdot}1 \rightarrow 1{\cdot}5$) IN ACETATE BUFFER pH 5 AT ROOM TEMP. TOTAL PROTEIN AND INVERTASE ACTIVITY PER FRACTION ARE SHOWN.

Complete removal of invertase activity from either CW1 or CW2 was not achieved by the method described using acetate pH 5, $I = 1{\cdot}5$. Of the total activity measured after fractionation about 50 and 80% appeared in the soluble fractions from CW1 and CW2 respectively. We infer that the soluble and insoluble fractions of enzyme are bound to CW in different ways.

In view of these results it is perhaps not surprising that Straus,[3] using water as an extraction medium, obtained such compelling evidence for the ubiquity of bound higher plant invertases. They also offer a reconciliation of work with Jerusalem artichoke tuber tissue which has led, on the one hand, to preparation of a soluble acid invertase,[12–14] and on the other, to solely bound acid invertase resistant to all attempts at solubilization.[4] The latter work is notable for the low Is and, as Ricardo and ap Rees observed, acid pHs of the aqueous extraction media.

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Our findings may also illuminate the anomalies obtained by Ricardo and ap Rees[7] with borate buffers. Table 1 presents data from Table 2 in their paper, but includes estimates of the I values of their extraction media.

TABLE 1. EFFECT OF pH AND IONIC STRENGTH OF EXTRACTION MEDIUM ON DISTRIBUTION OF ACID INVERTASE ACTIVITY IN HOMOGENATES OF 48 hr AGED DISCS OF MATURE CARROT ROOT (Data taken from Ricardo and ap Rees[7])

Expt.	Extraction medium pH	I	% activity in supernatant*	Expt.	Extraction medium pH	I	% activity in supernatant*
1	5·8	0·10	4	3	7·5	0·004	2
	7·0	0·18	10		8·0	0·012	1
					8·5	0·033	1
2	4·5	0·17	3	4	5·0	0·22	5
	7·0	0·54	40		6·0	0·3	10
	?	0	4		7·0	0·4	30
					8·0	0·56	40

* Calculated by taking activity in the supernatant as a percentage of total (supernatant and cell wall) activity at the lowest ionic strength within that experiment.

We propose that the soluble fraction of invertase associated with CW is bound to some charged component of CW by salt linkages.

While we cannot say that our results necessarily represent the condition of the enzyme *in vivo*, Klis[15] has recently obtained strong circumstantial evidence for the location of an ionically bound hydrolase (α-glucosidase) at the cell surface of living *Convolvulus arvensis* callus. Ionic binding of plant enzymes to residual fractions has been proposed on a number of occasions,[2,8,15,16] but this paper reports the first systematic study known to us of I as an extraction parameter. In view of our findings, we suggest that in studies involving higher plant tissue homogenization and subsequent fractionation, recognition of I as an important operational parameter is advisable.

Work in this laboratory by K. Moran with potato tuber tissue and D. Parr with carrot callus tissue has shown that a relationship between invertase solubility and ionic strength of extraction medium, similar to that described in this paper, also applies to these systems.

EXPERIMENTAL

Preparation of CW 1. 0·5 mm slices of Jerusalem artichoke tuber tissue prepared from cold-stored (4° for several months) tubers, peeled before slicing, were aged for 3 days in dist. H_2O at 25° in the dark. A modification of Hall's[17] method was used for the preparation of CW: 100 g washed aged slices were thoroughly homogenized with 25 ml phosphate buffer pH 7, $I = 0{\cdot}05$, and squeezed through 4 thicknesses of muslin. The extraction was repeated twice and the residue suspended in sufficient dist. H_2O to render it fluid, transferred to a large round-bottomed flask, shelled in a dry ice–acetone mixture, and freeze-dried. The pale brown preparation (about 2 g) was kept at −15° in a screw-topped jar where it suffered negligible loss of invertase activity over a period of 6 months.

*Preparation of CW*2. This differed from the preparation of CW1 in the following respects: (1) the tubers had been cold-stored for only a few weeks; (2) the residue was washed repeatedly with large vol. (> 100 ml) of phosphate buffer pH 7, $I = 0{\cdot}05$, and distilled H_2O prior to freeze-drying, and the freeze-dried product was pure white.

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Extraction of invertase from CW. Typically, 50 mg CW were ground with 1 g acid-washed sand and 2 ml extraction buffer (0°) in a pre-chilled pestle and mortar for 30 sec. A further 3 ml of buffer was used to wash the ground mixture into a 10-ml plastic centrifuge tube. After centrifugation at 35 000 *g* for 30 min at 4°, the supernatant was measured, and dialysed against a 100 × vol. of acetate buffer pH 5, $I = 0{\cdot}1$ in two lots overnight. 0·4 ml of re-measured dialysed supernatant was assayed for protein, and the remainder for invertase activity.

Salt gradient elution of protein from CW2. On adding 200 mg CW2 to acetate buffer pH 5, $I = 0{\cdot}1$, (starting buffer), trapped air was removed by suction and the suspension left overnight at 4°. The column was packed, equilibrated and eluted at room temp. 2 ml samples of fractions were dialysed against the starting buffer overnight at 4° and then equal portions were assayed for protein and invertase activity.

Binding of soluble invertase to CW. Aliquots of dialysed supernatant from extraction at pH 5, $I = 1{\cdot}5$, were stirred with samples of CW on ice for 15 min. After centrifugation or filtration the supernatant or filtrate was assayed for protein and invertase activity.

Protein assay. Protein was estimated according to the method of Lowry *et al.*[18] Bovine serum albumin was used to prepare standards.

Invertase assay. Assay of activities presented in Figs. 1 and 2 was performed by first diluting the remaining dialysed supernatant (usually about 3–3·5 ml) to 5·8 ml with dialysis buffer and adding 0·2 ml 10^{-1} M EDTA in dialysis buffer. After preincubation at 25° for 15 min, 0·12 ml 1·25 M sucrose in dialysis buffer was added and mixed. 4 × 1 ml aliquots were removed at 15 min intervals for reducing sugar assay, which was performed by the method of Somogyi[19] using glucose standards. 1 ml portions of dialysed fractions from salt gradient elution of CW2 were assayed by addition of 0·05 ml 10^{-1} M EDTA and 0·05 ml 1·25 M sucrose, both in dialysis buffer. After incubation at 25° for 1 hr the mixtures were assayed for reducing sugar. One enzyme unit is taken as that quantity of enzyme which catalyses the production of 1 μmol reducing sugar per min under the reaction conditions.

Buffers. Buffers were taken from the *Biochemists' Handbook*, E. & F. N. Spon, London (1961). The pH range 5–9 was covered by acetate (4–5), phosphate (6–7), Tris (8–9), and glycine (10). Extraction buffers of ionic strength > 0·2 were prepared by including KCl, up to the required ionic strength, in the buffers of 0·2 ionic strength.

Acknowledgement—This work was done under the terms of the S.R.C. Grant No. B/SR/7036.

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