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EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON INVERTASES IN CHICORY ROOT

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Abstract—The invertase present in roots of chicory (*Cichorium intybus*) has a pH optimum of 7.5 and a MW of *ca* 260 000. It requires relatively high ionic strength to remove it from DEAE cellulose. Treatment of chicory root tissue with 2,4-dichlorophenoxyacetic acid gives rise to a highly active invertase with pH optimum of 5.6 and MW of *ca* 61 000. It is more easily removed from DEAE cellulose.

INTRODUCTION

Treatment of chicory root tissue with 10^{-5} M solutions of 2,4-dichlorophenoxyacetic acid (2,4-D) causes a remarkable uptake of water [1]. The uptake is associated with extensive hydrolysis of inulin to low MW fructosans together with free reducing sugars. The enzymes which might be responsible for some of these carbohydrate changes have been examined [2, 3]. The most significant finding was that invertase activity was greatly increased by 2,4-D, whereas hydrolase activity (with an oligofructosan substrate extracted from chicory roots) was largely unaffected by 2,4-D. It was considered that the invertase produced as a result of 2,4-D treatment was a true invertase and was distinct from hydrolase. This invertase protein had different chromatographic properties to those of invertase protein preparations isolated from tissue that had been treated with either water or 3,5-dichlorophenoxyacetic acid (3,5-D) where no large uptake of water occurred [3].

Preliminary studies by Khin Lay Kyu [4] has indicated that the invertase arising from 2,4-D treated material might differ in other properties from that obtained from untreated and water or 3,5-D treated tissue. The present paper extends these studies using purer invertase preparations and presents results relating to a wider range of properties.

RESULTS AND DISCUSSION

Ammonium sulphate fractionation

In crude extracts of untreated tissue or tissue treated with water or 10^{-5} M solutions of 3,5-D, all the invertase activity was precipitated at 50% ammonium sulphate saturation (Table 1). With extracts from 2,4-D treated tissue were fractionated it was necessary to increase the ammonium sulphate concentration to 75% saturation to precipitate all the activity.

Table 1. Ammonium sulphate fractionation of crude protein extracts from untreated chicory root tissue and tissue treated with 10^{-5} M solutions of 2,4-D for 72 hr

Ammonium sulphate % saturation	Untreated		2,4-D treated	
	Protein	Invertase activity	Protein	Invertase activity
0–50	32	99	53	33
51–75	N.D.	1	33	64

Protein and invertase activity are expressed as a percentage of the total protein and invertase activity in the crude extracts.
N.D. = Not determined.

Separation of ammonium sulphate precipitated fractions on DEAE cellulose

The use of appropriate ammonium sulphate precipitated fractions gave cleaner separations on DEAE cellulose than those used earlier [3]. The fraction volume was reduced to 2.5 ml and this enabled the invertase activity (peak 2A) from 2,4-D treated tissue to be collected in a smaller volume. In other respects the results are similar to those already reported [3]. Peak 2A (Table 2) contains most (*ca* 95%) of the invertase activity associated with 2,4-D treated tissue but a smaller amount is present in peak 3. Untreated tissue had less invertase activity and this was all located in peak 3. Protein preparations from water or

Table 2. Chromatography of protein obtained by ammonium sulphate fractionation on DEAE cellulose and distribution of invertase activity*

Eluent	Eluate	Invertase activity	
		Untreated	2,4-D treated
5 mM Buffer	Peak 1	0	0
50 mM Buffer	2A	0	44·3
50 mM Buffer	2B	0	0
50 mM Buffer + 0.5 M NaCl	3	2.5	2.5

* Invertase activity expressed as nkat, assayed at 30°, and pH 5.6 for peak 2A, and pH 7.6 for peak 3.

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Table 3. pH Optima of invertase activity of protein extracts from tissue after various treatments

Treatment	Untreated	Water	10^{-5} M 3,5-D	10^{-5} 2,4-D	
Time (hr)	0	72	72	48	72
pH optimum	7–8	7–8	7–8	6–7	5–6

3,5-D treatments behaved similarly to those shown in Table 2 from untreated tissue.

Effect of pH on isolated invertase activity

Table 3 shows the pH optima for invertase activity in crude protein preparations from tissue subjected to different treatments. The significant feature is the gradual shift in pH optimum in the case of 2,4-D treated material from between 7 and 8 to between 5 and 6. Similar results were observed with invertase protein fractions eluted from DEAE cellulose columns. Peak 2A invertase from tissue treated with 2,4-D had a pH optimum of 5.6, (with half max. activity at pH 6.5 and <4.6) whereas the enzyme in peak 3 from the same treatment showed optimum activity at pH 7.6 but a minor peak of activity was noted at pH 5.6. The invertase from all other treatments had a pH optimum of 7.6 (half max. activity at pH 6.3 and 8.8). Peak 2A invertase (from 2,4-D treated tissue) retained a pH optimum of 5.6 after further purification by polyacrylamide gel electrophoresis.

The presence of two or more invertases in one plant is not uncommon and has been extensively reported [5]. In many cases both an 'acid' and 'alkaline' invertase have been described, e.g. pH optima of 5.1 and 7.3 for pea [6]. In general, the pH optima of the invertases of chicory root are in close agreement with those of other plant invertases but the 'acid' invertase from chicory root tissue occurs only after treatment with the growth regulator. This appears to have a similar pH optimum to the minor peak of activity noted in the pH profile for peak 3 protein from 2,4-D treated tissue.

Effect of temperature

At temperatures ⩾50° both peak 2A and peak 3 invertases suffered irreversible inactivation. The Arrhenius plot (logarithm of the initial rate of sucrose hydrolysis vs the reciprocal of the absolute temperature) for the invertase of peak 2A from 2,4-D treated tissue produced a straight line relationship over the temperature range tested (10–40°). The slope corresponded to an activation energy of 48.1 × 10^3 J per mol sucrose. The relationship found with the less active invertase from peak 3 differed markedly (Fig. 1). A discontinuous relationship was found giving lines with different slopes on either side of the temperature 27°. The activation energy calculated from line A was *ca* 46.9 × 10^3 J, and that from line B, *ca* 21.8 × 10^3 J per mol sucrose. (Again the similarity between peak 2A and peak 3 invertases may be noted.) The same discontinuity was found whether the peak 3 invertase arose from water or 2,4-D treated tissue.

This kind of discontinuous relationship has often been observed for enzyme catalysed reactions but few satisfactory explanations have been advanced [7]. It is unlikely that the discontinuity is a reflection of two independent active sites, since if this were so the slope at higher

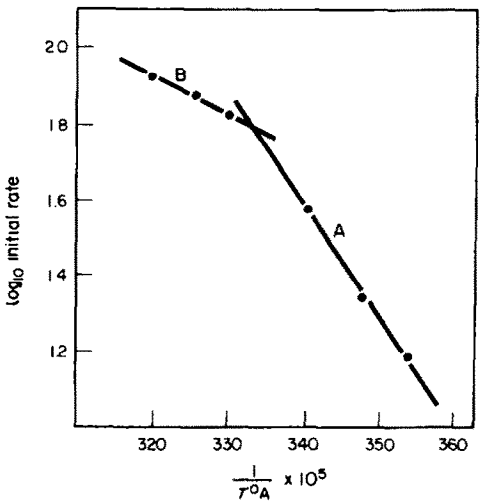

Fig. 1. The relationship between the logarithm of the initial rate of sucrose hydrolysis and the reciprocal of the absolute temperature for peak 3 invertase.

temperatures should be greater than that at lower temperatures whereas the reverse is true [7]. The relationship of the two lines might be explained by assuming that two successive reactions were involved, each with a different temperature coefficient and that at a given temperature, the step with the lower coefficient would limit the rate of the overall process. However, the hydrolysis of sucrose catalysed by invertase is generally assumed to be a single step reaction. A temperature-dependent dissociation of a large molecule into sub-units is also unlikely since although evidence based on pH profiles (see above), and gel filtration (see below) of peak 3 protein from 2,4-D treated tissue suggests the presence of two invertases, the same kind of evidence indicates only one invertase for peak 3 protein obtained from water-treated tissue, but this invertase still shows a discontinuous Arrhenius plot. Thus the interpretation of the effect of temperture is difficult and no firm conclusions can be drawn. A tentative explanation might be that a change in temperature causes a change in conformation of peak 3 invertase such that the activity is altered. The few published activation energies for invertases from other organisms are similar to those reported here (e.g. 46.0 × 10^3 J/mol for yeast invertase [8]).

The MW of peak 2A invertase protein from tissue treated with 10^{-5} M solutions of 2,4-D

An electrophoretic method was used in the first place. A freeze-dried preparation of peak 2A protein was further purified by electrophoresis on a polyacrylamide gel slab followed by electrophoresis on a cellulose acetate membrane. The protein band showing invertase activity was removed and treated with sodium dodecyl sulphate (SDS) and β-mercaptoethanol before electrophoresis on polyacrylamide gel containing SDS.

Three major bands were present in the invertase preparation. By comparison with the mobilities of standard proteins the estimated MWs were 71000 (A), 59000 (B) and 46000 (C), respectively. The amount of protein present in each band was too small for invertase activity to be assessed so it was not certain that any of these bands represented an invertase.

An alternative approach to MW determination (gel filtration) was therefore used where sufficient amounts of protein could be obtained for their invertase activity to be assessed. A freeze-dried sample of peak 2A invertase from 2,4-D treated tissue was applied to a calibrated column [9] of Sephadex G-100. A single peak of activity emerged with an estimated MW of 61 800, which corresponds fairly well with that of band B (MW 59 000) found with the electrophoretic method. It is therefore possible that band B represents the invertase protein.

The same calibrated column was used to analyse peak 3 invertase protein preparations from 2,4-D and water-treated tissue. In both cases, invertase activity appeared in the void volume although with preparations from 2,4-D treated tissue there was a trace of activity corresponding to a MW of 65 000. This protein may be related to the peak 2A invertase protein from 2,4-D treated tissue (MW 61 800). However, the major part of the invertase must have a MW in excess of 150 000 (the exclusion limit of the gel).

The analysis of these preparations containing high MW invertase was repeated on a calibrated column of Sephadex G-200 (50 × 2.5 cm dia). The peak 3 invertase from water-treated material eluted in a position corresponding to a MW of 260 000 and that from 2,4-D treated tissue with a MW of 270 000. In the latter case there was a trace of activity at an elution volume corresponding to a MW of 82 000.

Combining the circumstantial evidence of similarity between the highly active peak 2A invertase from 2,4-D treated tissue and the invertase present in peak 3 (pH profile, activation energy and MW), it is possible that the former may be a sub-unit of the latter. The relatively low activity of the large molecule may be due to conformational restraints imposed by binding the sub-units together or to an inhibitor such as a small protein necessary for binding the sub-units. Proteinaceous invertase inhibitors have been found in a number of plant tissues, e.g. potato [10].

Work in this laboratory (unpublished) using radioactive tracers has given no indication of *de novo* synthesis of a highly active invertase protein following growth regulator treatment, nor does 2,4-D have any affect on the activity of the extracted enzyme. The effect of 2,4-D may therefore be indirectly linked with the dissociation of a largely inactive enzyme complex into highly active enzyme sub-units.

EXPERIMENTAL

Chicory roots (*Cichorium intybus* L.) cv Magdeburg were obtained from F. G. Harrison, Bury St. Edmunds, Suffolk in November. Selected roots (excluding the smallest or damaged roots) were stored at 3 ± 1° in moist peat for at least 4 weeks before use. Tissue disks were prepared from the roots and incubated for up to 72 hr with either H_2O or 10^{-5} M solns of 2,4-D or 3,5-D as described previously [11].

Extraction of protein. Following treatment with H_2O, 3,5-D or 2,4-D, crude protein extracts were prepared as described in ref. [12] except that $(NH_4)_2SO_4$ was added to give a final concn of 75% instead of 95%.

Purification of invertase. (a) *Separation on DEAE cellulose.* The crude extract (1–2 ml, 15–30 mg protein) was fractionated on a column of Whatman DE52 cellulose (20 × 1 cm dia). Protein was eluted with (1) 5 mM Pi buffer pH 7.6 (providing peak 1), (2) 50 mM Pi buffer pH 7.6 (providing peaks 2A and B) and (3) 50 mM Pi buffer pH 7.6 containing 0.5 M NaCl (providing peak 3). (b) *Electrophoresis on polyacrylamide gel slabs.* Horizontal polyacrylamide gels (7.5%) were prepared as described elsewhere [5, 13]. The protein soln, absorbed in glass fibre paper, was inserted in the sample slot and a potential of 260–300 V applied for *ca* 15 hr at 5°. The position of invertase after electrophoresis was determined by removing a narrow strip from the edge of the gel and incubating this in a test-tube with buffered substrate (0.5 M acetate buffer, pH 5 and 1.5 M sucrose in the ratio 4:1). After at least 90 min, the incubation mixture was removed, the gel washed with H_2O and 2,3,5-triphenyltetrazolium chloride (TTC) (0.1% in M NaOH) added. After the reaction between TTC and liberated fructose had developed in the dark, the TTC soln was discarded and the gel washed with 2% HOAc. The position of the invertase was indicated by a red stain. Protein bands were located by further staining with Coomassie brilliant blue R 250 (0.1% in MeOH–HOAc–H_2O, 5:1:5). This procedure revealed the position of invertase (stained red with TTC) superimposed on the blue protein bands. Invertase was eluted from the unstained gel with H_2O after macerating with a glass rod. After washing twice with H_2O, no invertase activity could be detected in the remaining gel. Electrophoresis caused a considerable loss of invertase activity and so was only used with protein fractions derived from 2,4-D treated plant material. (c) *Electrophoresis on cellulose acetate membranes.* Protein (≤ 500 mg in 0.5 ml buffer was streaked on cellulose acetate membrane 3 cm from the cathodic end. The membrane had been pre-soaked in 50 mM Pi buffer, pH 8 containing 20% glycerol to prevent loss of invertase activity [5]. The electrophoresis buffer (50 mM Pi, pH 8) was cooled by circulating ice-cold H_2O around its container and 200 V (constant) applied across the membrane for 30 min. Protein bands on marker strips were detected, after fixing in 5% TCA, using 0.005% nigrosine in 2% HOAc. Using the marker strips as guides, protein bands were cut from the unstained region of the membrane and eluted with H_2O. (d) *Electrophoresis on polyacrylamide gels containing SDS.* The procedure followed that described in ref. [14] using a Quickfit apparatus. After electrophoresis of standard proteins, test samples and bromophenol blue, at 8 mA per tube for $3\frac{1}{4}$–$3\frac{1}{2}$ hr, the mobility of the standard proteins was plotted against the common logarithms of their MW. The MWs of the protein bands in the test samples were then obtained by comparison. (e) *Gel filtration on Sephadex G-100 and G-200.* The procedure of ref. [9] was followed, using a glass column 50 × 2.5 cm (dia.). The column was calibrated using the standard proteins described below. The effluent was continuously monitored by measuring the *A* of the eluate at 280 nm and the position of invertase established by assay of the collected fractions. Its MW was estimated by reference to the linear relationship between the elution vols. of the standard proteins and the common logarithm of their MWs.

Invertase activity in protein fractions was assayed by observing the hydrolysis of sucrose (0.3 M) at the appropriate pH (using 0.1 M Na, Pi or NaOAc) at 30°. Reducing sugar was estimated by the method of ref. [15].

3,5-Dinitrosalicylate reagent [15]. 3,5-Dinitrosalicylic acid (5 g) was dissolved by warming in 2 N NaOH (100 ml). NaK tartrate (150 g) was dissolved, also by warming, in H_2O (250 ml). The two solns were mixed and made to 500 ml with H_2O.

Protein determination. The method of ref. [16] was generally used and calibrated with BSA. When samples contained very small amounts of protein and also interfering compounds (e.g. eluates from polyacrylamide gels) the method of ref. [17] was used. This was *ca* 8 times more sensitive than the Lowry method.

The protein standards of known MW were as follows and were obtained from Boehringer Mannheim; cytochrome *ca* 12 500, chymotrypsinogen A 25 000, albumin (hen egg) 45 000, albumin (bovine) 67 000, aldolase 158 000, catalase 240 000, ferritin 540 000.

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