β -Fructofuranosidases from Roots of Dandelion (*Taraxacum officinale* Weber)

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(Received 10 September 1971)

1. Three β -fructofuranosidases were separated by chromatography on a DEAE-cellulose column from the soluble protein extracted from dandelion (*Taraxacum officinale* Weber) roots. 2. One enzyme, which acted on sucrose, was characterized as an invertase, with a K_m of 2.00×10^{-2} M and pH optimum of 7.5. 3. The other two enzymes are hydrolases (A and B), which act on the inulin series of oligosaccharides [general formula glucose-fructose-(fructose) $_n$]. They both have a pH optimum of 4.0 and K_m of 1.54×10^{-2} M but differ in their chromatographic behaviour on DEAE-cellulose. Neither of the hydrolases is inhibited by sucrose. 4. The physiological role of these three hydrolytic enzymes is discussed.

The dandelion (*Taraxacum officinale* Weber), in common with a considerable number of the Compositae, contains in its roots a series of β -D-fructofuranosides (Bacon & Edelman, 1951), each member of which has the general formula Glc-Fru-Fru $_n$, where Glc-Fru represents a sucrose group and n the number of additional fructosyl residues; n may be any value from zero (sucrose) to about 35 (inulin). The fructose residues appear to be (2 $\rightarrow$ 1')-linked, giving linear chains (Percival, 1962).

It has been shown by Rutherford & Weston (1968) that cold storage of roots from chicory and dandelion and tubers from Jerusalem artichoke brings about a breakdown of inulin and high-molecular-weight oligosaccharides (degree of polymerization > 10) to oligosaccharides of lower degree of polymerization. The hydrolytic enzymes responsible for this depolymerization of polysaccharides to simple sugars have been characterized from artichoke (Edelman & Jefford, 1964) and their presence has been demonstrated in chicory (Flood *et al.*, 1967); it seems likely that similar enzymes may be present in dandelion roots.

This paper describes the quantitative separation of soluble protein from dandelion roots and the fractionation of these extracts into enzymes possessing a number of hydrolytic activities. The properties and possible physiological roles of these enzymes are considered.

In view of the unsatisfactory classification of these enzymes, the nomenclature used by Edelman & Jefford (1964) is adhered to throughout this paper.

Materials and Methods

Plant material

Dandelions were dug up from the University site in April, the leaves trimmed and the roots stored in

moist peat at $5^\circ \pm 2^\circ\text{C}$ until required. The content of the enzymes examined (expressed as units/g fresh wt.) did not alter significantly over the period of our investigation (April to July 1971).

Substrates

Raffinose, melibiose and melezitose were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Inulin was supplied by Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. Oligosaccharide substrate M, consisting of a mixture of oligosaccharides with the general formula Glc-Fru-Fru $_n$, where n is between 5 and 12, was prepared by a method similar to that described by Edelman & Jefford (1964) for artichoke. Unless stated otherwise this substrate was prepared from dandelion roots.

Buffers

Phosphate-citrate buffer was prepared by adding 0.1 M-citric acid to 0.2 M- Na_2HPO_4 until the desired pH was reached. Phosphate buffers were prepared by mixing Na_2HPO_4 and KH_2PO_4 solutions of the required strength to give the desired pH.

Soluble protein extracts

These were obtained by the quantitative method described by Rutherford *et al.* (1969), a procedure modified from that first described by Edelman & Jefford (1964) for artichoke. Dandelion roots were washed, peeled, cut into approx. 1 cm cubes and extracted in a juice extractor (Moulinex) that incorporated an automatic grater and basket-type centrifuge. Extraction was made in the presence of 100 mM-phosphate buffer, pH 7.6, containing

100mM-cysteine and 100mM-sodium diethyldithiocarbamate. This and all subsequent operations were carried out at 5°C. After extraction the residue was removed from the centrifuge, ground in a mortar and transferred to a small filter press. A little of the phosphate buffer was added and the fluid squeezed out in the press. This operation was repeated twice more. Altogether 25ml of buffer was used to extract 50g of tissue. The total extract was dialysed overnight against 20mM-phosphate buffer, pH 7.6, containing 5mM-cysteine and 5mM-sodium diethyldithiocarbamate (three changes) and the non-diffusible material centrifuged at 25000g for 20min to remove an insoluble residue. Protein was precipitated from the supernatant by the careful addition of $(\text{NH}_4)_2\text{SO}_4$ to 95% saturation, the pH being maintained at 7.6. After standing overnight the precipitated protein was separated by centrifugation at 15000g for 30min and then dissolved in 5mM-phosphate buffer, pH 7.6. The solution was then dialysed overnight against 5mM-phosphate buffer, pH 7.6 (three changes), and the non-diffusible material centrifuged at 25000g for 20min. Samples (0.5ml) of the clear supernatant were used directly for assay of the hydrolytic enzymes present, part was used for fractionation and the remainder freeze-dried for further use.

Fractionation of protein preparation

A column (5g; 10mm×100mm) was prepared from DEAE-cellulose (Whatman DE52, microgranular form) that had previously been equilibrated with 50mM-phosphate buffer, pH 7.5. Protein preparation (4ml, containing 20–40mg of protein) was applied to the column and eluted first with 50mM-phosphate buffer alone, and then with phosphate buffer solutions containing 0.1M-, 0.2M-, 0.3M- and finally 0.6M-NaCl, all at pH 7.5. The fractionation was performed at $2 \pm 1^\circ\text{C}$ with a flow rate of 20ml/h.

Assay procedure

Invertase was assayed by incubating 0.5ml of the enzyme with 0.5ml of phosphate-citrate buffer, pH 7.5, containing 0.2M-sucrose. Hydrolase assays were performed by adding 0.5ml of enzyme to 0.5ml of phosphate-citrate buffer, pH 4.0, containing 40mg of oligosaccharide substrate M. All incubations were carried out at 25°C and controls containing substrate alone and substrate plus boiled enzyme were included. Samples were removed at 1h intervals for determination of reducing sugar (addition of the reagents was sufficient to stop the reaction). The initial rate of release of reducing sugar was calculated from the first three samples. The reducing sugar found in the control containing boiled enzyme and substrate was never significantly greater than that found in the

control containing substrate alone. The progress curves were always linear for the first 3h and the enzyme activity was proportional to its concentration.

Enzyme units

Units of hydrolase activity are expressed as μmol of hexose liberated from 40mg of oligosaccharide substrate M/min at 25°C and pH 4.0, and units of invertase activity as the number of $2\mu\text{mol}$ of hexose liberated from 0.1M-sucrose/min at 25°C and pH 7.5.

Sugar determinations

Reducing sugar was determined by the cuprimetric method of Nelson (1944) as modified by Asatoor & King (1954). Glucose was determined by the glucose oxidase method by using a Biochemica Test Combination supplied by Boehringer Corp. (London) Ltd., London W.5, U.K.

Protein estimations

Measurement of the soluble protein in the unfractionated extracts was by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The eluate from columns was monitored continuously (by measuring the percentage transmission) at 280nm (Warburg & Christian, 1941).

Results

Protein extracts

Extracts of soluble protein prepared from dandelion roots were capable of hydrolysing sucrose, inulin and oligosaccharide substrate M to reducing sugars, and therefore contain invertase and hydrolase activity (Edelman & Jefford, 1964).

Fractionation

Chromatography of this protein preparation was carried out on DEAE-cellulose columns and the fractions were assayed for activity against both sucrose and oligosaccharide substrate M. Fig. 1 shows that stepwise increments in the ionic strength of the eluent effect a separation of invertase and hydrolase activities. The hydrolase activity was resolved into two fractions; 60–70% of the activity occurred in the first peak (hydrolase A) and 30–40% in the second peak (hydrolase B). The fractions from each hydrolase peak were combined and separately rechromatographed on a similar column. Both hydrolases were eluted completely separate from each other and in similar positions to those shown in the original elution diagram.

Fig. 1. Chromatography of soluble protein preparation on DEAE-cellulose

Sample applied contained 31mg of protein, 0.20 unit of invertase and 1.45 units of hydrolase. Elution was effected with 50mM-phosphate buffer, pH7.5, changed stepwise as indicated by arrows to 0.1M-, 0.2M-, 0.3M- and finally 0.6M-NaCl, all contained in 50mM-phosphate buffer, pH7.5. The volume of each fraction was 2ml. Δ , Hydrolase activity (substrate M); $\blacktriangle$, invertase activity (sucrose as substrate); --, % transmission at 280nm (monitored continuously in a 3 mm flow cell).

Substrate specificity

Hydrolases A and B were both inactive against sucrose but hydrolysed both inulin and oligosaccharide substrate M. Oligosaccharide substrates (40mg/ml of digest) prepared from artichoke and chicory were hydrolysed by hydrolase A at rates of 129 and 81% respectively, by hydrolase B at 132 and 88% (rates expressed as a percentage of the rate at which dandelion substrate was hydrolysed by that particular enzyme).

The invertase peak possessed no activity against either inulin or oligosaccharide substrate M. Invertase acted on sucrose (0.1M) to release reducing sugar and glucose at rates of 1.54 and 0.74 respectively (units: μmol released/h per ml of digest). Thus a half of the reducing sugar produced by the action of invertase on sucrose is glucose. Invertase also acted on raffinose to liberate reducing sugar at the rate of $0.38\mu\text{mol/h}$ per ml of digest without the formation

of any free glucose, but was inactive against both melezitose and melibiose.

Effect of pH

The effect of varying the pH on the activity of all three enzymes is shown in Fig. 2. Both hydrolases A and B have a broad optimum at pH4.0 and invertase a sharper optimum at pH7.5.

Effect of substrate concentration

With sucrose as substrate, invertase followed Michaelis-Menten kinetics with respect to substrate concentration. The Michaelis constant was $2.00 \times 10^{-2}\text{M}$, as determined by the double-reciprocal plot method of Lineweaver & Burk (1934).

Although it was difficult to study the kinetics of the hydrolases owing to the low solubility of inulin at

Fig. 2. Effect of pH on the activity of the fractionated β -fructofuranosidases

0.1 M-Sucrose was used as substrate for invertase and 40 mg of oligosaccharide substrate M for both hydrolases. Phosphate-citrate buffer was used throughout. $\circ$, Hydrolase A; $\bullet$, hydrolase B; $\blacktriangle$, invertase. Results are expressed for each enzyme as % of the activity at the pH optimum of that enzyme.

25°C, both hydrolases followed Michaelis-Menten kinetics over the concentration range investigated (0.44–8.9 mM). Assuming a molecular weight of 5600 for inulin (Phelps, 1965), the Michaelis constant for both hydrolases A and B was estimated as 1.54×10^{-2} M from double-reciprocal plots (Fig. 3).

Effect of sucrose on hydrolases A and B

In view of the fact that artichoke hydrolase B is inhibited by sucrose (Edelman & Jefford, 1964) the effect of sucrose on both dandelion hydrolases was investigated. Hydrolases A and B were each incubated with inulin (4 mM) in the presence and absence of sucrose (10 mM). The rates of hydrolysis of inulin (expressed as μmol of hexose released/h per ml of digest) in the absence and presence of sucrose were 0.26 and 0.23 respectively for hydrolase A and 0.13 and 0.14 for hydrolase B. It was concluded that sucrose did not significantly alter the rate of hydrolysis of inulin by either hydrolase A or B.

Discussion

The above results show that in common with artichoke (Edelman & Jefford, 1964) and chicory (Flood *et al.*, 1970), dandelion root extracts contain three β -D-fructofuranosidases. All three enzymes were completely separated from each other by chromatography on DEAE-cellulose.

Two of these enzymes (hydrolases A and B) are

Fig. 3. Double-reciprocal plots to determine the K_m values of hydrolases A and B

The substrate was inulin. $\circ$, Hydrolase A; $\bullet$, hydrolase B. pH was 4.0. Units: substrate concentrations [S] are molar, velocities (v) are expressed as μmol of hexose liberated/min per ml of incubation mixture.

completely inactive against sucrose but attack both inulin and a standard substrate consisting of a mixture of oligosaccharides from the inulin series with a degree of polymerization of between 5 and 12 (Edelman & Jefford, 1964). Any variation observed in the rate at which oligosaccharide substrate mixtures prepared from different sources are hydrolysed can be explained by differences in the distribution of the various members of the inulin series of sugars found in different plants (Rutherford & Weston, 1968), since each member is attacked at a different rate (Edelman & Jefford, 1964). The hydrolases of artichoke, chicory and dandelion therefore are of a similar nature, a situation that may well exist in all inulin-storing plants.

Hydrolases A and B differed only in their chromatographic behaviour on DEAE-cellulose, their pH optima and Michaelis constants being identical. This situation could be due either to the existence of different enzymes, e.g. isoenzymes, or to a single enzyme binding to other proteins present in the extract, which are themselves resolved by the chromatographic system employed. If the latter explanation is correct then the enzyme protein aggregates do not readily dissociate, since both hydrolases remain homogeneous on rechromatography.

The invertase activity in the soluble protein extract

is undoubtedly due to a different enzyme from that responsible for the hydrolase activity, since the two activities are chromatographically separable, have different pH optima and substrate specificities (invertase attacking fructosyl moieties attached to glucosyl residues, hydrolases attacking fructosyl moieties attached to other fructosyl residues). The invertase is a β -fructofuranosidase and not an α -glucopyranosidase, since it attacks raffinose but not melezitose; the hydrolysis of raffinose was not due to α -galactosidase activity, since melibiose was not attacked by the enzyme. The invertase peak was also free of significant sucrose-sucrose 1-fructosyltransferase activity, since one-half of the reducing sugar liberated from sucrose by this enzyme was glucose.

Edelman & Jefford (1968) have proposed a mechanism for fructosan metabolism in the artichoke involving the integrated action of fructosyltransferases and hydrolases; they suggest that their hypothesis is also applicable to other fructosan-containing plants. We have shown that hydrolases (approx. 50 munits/g fresh wt.) are also present in the dandelion and could well be responsible for the depolymerization of polysaccharides to simple sugars, which occurs during the cold storage of this plant (Rutherford & Weston, 1968). It is of interest that sucrose does not inhibit either of the dandelion hydrolases and so, unlike the findings with the artichoke (Edelman & Jefford, 1964), cannot directly control the breakdown of fructosan by hydrolase B.

We have also found significant amounts (approx. 7 m-units/g fresh wt.) of invertase in the soluble protein extract. Since the pH optimum of this enzyme is 7.5, it can be classified as a neutral or alkaline invertase (Ricardo & ap Rees, 1970; Lyne & ap Rees, 1971). The relative activity of invertase, and hence its

physiological function, will depend very much upon the pH at its location inside the cell. If invertase acts at its optimum pH *in vivo*, then it may considerably affect the availability of sucrose for fructosan synthesis. This situation will remain obscure until more information is available about the relationship of invertase and other enzymes with sucrose metabolism in the dandelion root.

A. C. D. thanks the Science Research Council for a research studentship.

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