Some Properties of β -Fructofuranosidases Partially Purified from *Phaseolus vulgaris* and *Solanum tuberosum*

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1. Soluble β -fructofuranosidases were purified 16-fold from French-bean-pod extracts and 35-fold from potato-tuber extracts. 2. The two enzymes had similar overall properties but differed from each other quantitatively in lability and reaction kinetics. 3. A non-diffusible inhibitor of β -fructofuranosidase was present in the potato-tuber extracts but was absent from the bean extracts. This non-competitive inhibitor was acid-labile. 4. The possible roles of imidazole, carboxyl and thiol groups in β -fructofuranosidase action are discussed, and the properties of the bean and potato enzymes are compared with published data for yeast, mould and grape-berry enzymes.

Extensive studies of β -D-fructofuranoside fructo-hydrolase (EC 3.2.1.26) (trivial names β -fructo-furanosidase, invertase and sucrase) from yeast have been reviewed by Myrbäck (1960). Yeast β -fructo-furanosidase has proved difficult to purify, but relatively pure preparations have been reported (Neumann & Lampen, 1966, 1967; Fischer & Kohtès, 1951). Homogeneous preparations of β -fructo-furanosidases from *Bacillus subtilis* (Negoro, 1957) and *Neurospora crassa* (Metzenberg, 1963) have also been reported. In higher plants, enzymes catalysing the hydrolysis of sucrose have β -fructosidase specificity and hydrolyse saccharides with a β -fructofuranoside group, with slightly acid pH optima (Myrbäck, 1960). They share with the yeast and mould β -fructofuranosidases the ability to transfer fructosyl radicals to other primary alcohol groups.

Cooper & Greenshields (1961*a,b*, 1964) detected two sucrose-hydrolysing enzymes in *Phaseolus vulgaris*. One was purified 100-fold, representing the first substantial purification of a β -fructo-furanosidase from a higher-plant source. The second enzyme, alkaline sucrase, appeared to be specific for sucrose and had a pH optimum at pH 7.7. Schwimmer, Makower & Rorem (1961) detected an invertase (a β -fructofuranosidase; Rorem & Schwimmer, 1963) and a non-diffusible

endogenous inhibitor in potato-tuber extracts, and suggested that a study of the enzyme and the inhibitor might be valuable in determining the metabolic role of the enzyme.

Studies of β -fructofuranosidases from different higher-plant sources have revealed considerable variation in kinetic properties, e.g. in pH optima (pH 2.0–7.8) and K_m values (0.5–25 mM) (Arnold, 1965; Pridham & Walter, 1964; Henderson, Morton & Rawlinson, 1959; Hatch, Sacher & Glasziou, 1963). Evaluation of these differences is complicated by the use of different experimental conditions by the various workers. In the present work the properties of two β -fructofuranosidases, partially purified from *Phaseolus vulgaris* and *Solanum tuberosum*, have been directly compared.

MATERIALS AND METHODS

Plant material. Seeds of the French bean (*Phaseolus vulgaris* L. var. Improved Canadian Wonder) were obtained commercially. Potato tubers (*Solanum tuberosum*, var. Dr Mackintosh, unless otherwise stated) were obtained from the University of Birmingham Botanic Gardens. Etiolated *Phaseolus vulgaris* seedlings were germinated as described by Cooper & Greenshields (1964). To obtain mature *Phaseolus vulgaris* plants, seeds were planted in the field at a depth of 25 cm. after previous treatment of the ground with superphosphate. The plants used in studies of subcellular distribution of the sucraes were grown in 3½ in. pots in the greenhouse under artificial illumination at a temperature of 55–65°F.

Yeast β -fructofuranosidase. Invertase concentrate (Honeywill and Stein Ltd., London, W. 1) was diluted 1000- to 10000-fold before use.

Determination of protein. Total nitrogen was determined

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by the micro-Kjeldahl method after thorough dialysis. For clear solutions the extinction in 1 cm. cells at 280 m μ was used as an index of protein content.

Determination of saccharides. Reducing sugars were estimated colorimetrically by using the reagents of Somogyi (1945) and Nelson (1944). Glucose was determined with a glucose oxidase-peroxidase reagent (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) modified by the addition of tris at a final concentration of 0.3 M to inhibit interference by maltase (Dahlqvist, 1961; White & Subers, 1961).

Paper chromatography. One-dimensional descending paper chromatography on Whatman no. 3MM paper was employed, with butan-1-ol-acetic acid-water (12:3:5, by vol.) or butan-1-ol-pyridine-water (6:4:3, by vol.) as solvent.

Saccharides were detected with benzidine-trichloroacetic acid reagent (Bacon & Edelman, 1951), ketose sugars with phloroglucinol-trichloroacetic acid reagent and reducing sugars with 3,5-dinitrosalicylic acid-NaOH reagent (Smith, 1960).

Cell fractionation of bean hypocotyl tissue. Hypocotyls (30 g.) were cut into 5 mm. sections and homogenized below 5° with 20 ml. of medium (see the Results section) for two periods of 20–30 sec. in a Potter-type homogenizer with a smooth loose-fitting pestle. The homogenate was diluted with 1 vol. of medium and centrifuged for 8 min. at 200g to obtain the cell-wall fraction. When mannitol solutions were used, the cell walls were filtered off on four layers of muslin. The cell walls were washed five times by being resuspended in the homogenizing medium. Subcellular particles were sedimented by centrifugation at 1000g for 10 min., 14000g for 20 min. and 100000g for 30 min. The precipitates were washed three times.

Preparation of juice extracts. Bean pods washed externally in water, or potato tubers peeled and cut into small pieces, were fed into a Multipress juice extractor (A. Braun, Frankfurt/Main, Germany) at room temperature, and the collected juice was cooled in ice-water. Only traces of β -fructofuranosidase activity were detected in the resuspended residues from the juice extractor.

Preparation of Sephadex columns. Before being packed into columns, Sephadex (grades G-100 and G-200; A.B. Pharmacia, Uppsala, Sweden) was suspended in buffer, allowed to swell for 1–3 days, and washed by decantation. The columns were washed with three to five times the bed volume of buffer and eluted with the starting buffer, after application of the samples.

Assay of enzyme activities. Except where otherwise stated, incubation mixtures contained 58.4 mM-sucrose, and acetate buffer, pH 4.9, at a concentration of 0.1 M for crude extracts and 0.05 M for purified enzyme preparations. Each assay tube contained the equivalent of 0.03–0.05 unit of β -fructofuranosidase activity determined under optimum conditions, in a final volume of 0.2 or 0.4 ml. The mixtures were incubated for 5–20 min. at 25°. For non-reducing and reducing substrates the reaction was stopped by addition of the reagents.

β -Fructofuranosidase activity in potato extracts containing the endogenous inhibitor was determined at two sample concentrations differing fivefold.

Eqn. (1) (Schwimmer *et al.* 1961) was used to calculate activity in the absence of the inhibition:

$$E/k_{\text{obs.}} = 1/k_0 + CE \quad (1)$$

where $k_{\text{obs.}}$ = activity (units/ml.) of enzyme at relative concentration E , k_0 = activity (units/ml.) at infinite dilution, and C is a constant. By plotting E against $E/k_{\text{obs.}}$, the reciprocal of k_0 was obtained by extrapolating to zero enzyme concentration. The extent of endogenous inhibition was expressed as the percentage difference between the observed activity in a mixture of equal volumes of enzyme extract and buffered substrate and the calculated activity at infinite dilution k_0 (eqn. 2):

$$\text{Inhibition (\%)} = \frac{k_0 - k_{50\%}}{k_0} \times 100 \quad (2)$$

where $k_{50\%}$ = activity (units/ml.) at 50% of the original sample concentration.

Unit of enzyme activity. One unit is defined as the amount of enzyme that catalyses the transformation of 1 μ mole of substrate/min. at 25°.

Determination of K_m values. Enzyme activity was determined at six or seven concentrations of substrate: for sucrose, 4.87–29.2 mM (except at pH 7.0, where concentrations of 1.17–29.2 mM were employed); for raffinose, 4.97–79.3 mM. Phosphate buffers were used for determinations at pH 5.0, 6.0 and 7.0 and acetate buffers at other pH values. In some cases, the extent of hydrolysis exceeded 5% and a correction for substrate consumed was applied to the value of $1/s$. The method of least squares was used to determine the slope and intercept of the reciprocal plot (Lineweaver & Burk, 1934), from which the values for K_m and V were calculated. The s.d. of the points from the line of best fit was also calculated. The enzyme preparations were stable for the duration of the incubations in control mixtures at the pH and ionic strength of the test mixtures except for the bean preparation at pH 3.5; for this the reaction was started by adding a portion of the enzyme preparation to the buffered substrate solution, and incubation was continued for 4 min. Under these conditions loss of activity due to denaturation was found to be negligible.

Preparation of bean β -fructofuranosidase. With the exception of two experiments on inactivation indicated below, the kinetic properties of the bean enzyme were determined on a single preparation obtained from 1.4 kg. of bean pods. All preparative steps except the initial extraction were performed at 0–4°. The scheme of purification is shown in Table 1. At the last stage the supernatants from step III were combined and applied to a Sephadex G-200 column (3.8 cm. $\times$ 55 cm., void vol. 100 ml.). The eluent was 0.01 M-phosphate buffer, pH 6.0. Invertase activity was detected as a single symmetrical peak in fractions eluted between 230 and 350 ml. eluate volume. The most active fractions (69% of the activity applied) were combined and freeze-dried. The dry preparation (162 mg.) contained 85 mg. of potassium phosphate and 10.3 mg. of nitrogen.

Preparation of potato β -fructofuranosidase. The properties of two preparations of the potato enzyme were determined. Preparation A was made from 3.3 l. of extract obtained from 5.6 kg. of freshly harvested tubers; preparation B was made from 400 ml. of extract obtained from 585 g. of tubers that had been stored for 6 months in a dark cool cellar and had developed sprouts. The scheme of purification is shown in Table 2. The supernatant from step III was applied to a Sephadex G-200 column (3.7 cm. $\times$ 38 cm., void vol. 70 ml.) and eluted with 0.02 M-phosphate buffer, pH 6.5. Invertase activity was detected as a single symmetrical peak in

Table 1. *Summary of purification of bean β -fructofuranosidase*

Step	Volume (ml.)	Total enzyme activity (units)	Sp. activity $\left(\frac{\text{units/ml.}}{E_{280}}\right)$
Crude extract	904	1060	0.23
I Extract adjusted to pH 5.0 with 2.0 M-acetate buffer, pH 4.2, then centrifuged at 16000g for 15 min. Precipitate discarded. Supernatant taken	936	1050	1.15
II Supernatant from I treated with solid $(\text{NH}_4)_2\text{SO}_4$ and centrifuged at 16000g for 15 min. after standing for 16 hr. Precipitates resuspended in 0.01 M-phosphate buffer, pH 6.0			
0-40%-saturated $(\text{NH}_4)_2\text{SO}_4$ fraction	24	362	—
40-60%-saturated $(\text{NH}_4)_2\text{SO}_4$ fraction	32	600	—
III Resuspended precipitates dialysed against 0.1 M-phosphate buffer, pH 6.0, for 38 hr. After centrifugation, residue discarded			
Supernatant from 0-40%-saturated $(\text{NH}_4)_2\text{SO}_4$ fraction	26	320	1.9
Supernatant from 40-60%-saturated $(\text{NH}_4)_2\text{SO}_4$ fraction	37	580	1.5
IV Sephadex G-200 eluate (see the Materials and Methods section) of combined fractions from III	62	616	3.8

Table 2. *Summary of purification of potato β -fructofuranosidase*

Preparation	Step	Volume (ml.)	Total enzyme activity (units)	Sp. activity $\left(\frac{\text{units/ml.}}{E_{280}}\right)$	
				$\left(\frac{\text{units/ml.}}{\text{mg. of N}}\right)$	$\left(\frac{\text{units/ml.}}{E_{280}}\right)$
A	Crude extract	3300	350	0.08	—
	I Extract adjusted to pH 4.0 with 2N-acetic acid, then centrifuged at 16000g for 10 min. Precipitate discarded. Supernatant taken	3525	328	—	—
	II Supernatant from I treated with solid $(\text{NH}_4)_2\text{SO}_4$ and centrifuged at 16000g for 15 min. after standing for 16 hr. Precipitates resuspended in 0.02 M-phosphate, pH 6.5				
	0-30%-saturated $(\text{NH}_4)_2\text{SO}_4$ fraction	505	294	0.26	—
	III Resuspended precipitates dialysed against 0.1 M-phosphate, pH 6.0, for 36 hr. Residue discarded after centrifugation				
	Supernatant from 0-30%-saturated $(\text{NH}_4)_2\text{SO}_4$ fraction	160	168	0.71	0.06
	IV Sephadex G-200 eluate (see the Materials and Methods section)	248	120	3.0	0.22
B	Crude extract	400	134	0.26	—
	II Procedure as in AII above				
	0-30%-saturated $(\text{NH}_4)_2\text{SO}_4$ fraction	23	108	—	—
	III Procedure as in AIII above				
	Supernatant from 0-30%-saturated $(\text{NH}_4)_2\text{SO}_4$ fraction	23	86	5.6	—
	IV Sephadex G-200 eluate (see the Materials and Methods section)	78	63	9.0	0.50

fractions eluted between 150 and 240 ml. The most active fractions were combined and freeze-dried.

RESULTS

Location of the enzyme in bean-seedling hypocotyls

The cell-fractionation procedure (see the Materials and Methods section) was applied to homogenates

of 10-12-day-old seedlings in the following media: (a) 0.18 M-potassium chloride, (b) 0.18 M-potassium chloride containing 1 mM-di-isopropyl phosphorofluoridate, (c) 0.42 M-mannitol and (d) water. In all cases most of the enzyme activity was recovered in the supernatant and washings. An increase in specific activity of the supernatant was observed after the removal of each precipitate and activity

Table 3. β -Fructofuranosidase activity of cell walls prepared in glycerol

Sections of hypocotyls (5 mm.) in 4 vol. of cold glycerol were homogenized for two periods of 30 sec. in an MSE top-drive homogenizer at 14000 rev./min. The homogenate was filtered with suction through a 1 cm. layer of 200–300 μ glass beads on a porosity 1 sintered-glass filter. The upper part of the glass-bead layer containing the cell walls was washed twice by being suspended in 80% (v/v) glycerol and filtering. The cell walls were resuspended a third time in 80% (v/v) glycerol and allowed to stand for 30 min. to allow most of the beads to settle. The cell walls were collected by centrifuging at 35000g for 30 min. and resuspended in 0.02 M-phosphate buffer, pH 6.5.

Sample	Total enzyme (units)	Activity of enzyme (units/mg. of N)
Whole suspension	4.9	0.58
Supernatant solution	3.8	1.12
Washings	0.5	0.34
Cell-wall suspension	0.7	0.20

detected in the resuspended precipitates never exceeded 2% of that of the whole homogenate. The technique of Kivilaan, Beamann & Bandurski (1961) for the preparation of enzymically active cell walls was also used (Table 3); microscopic examination of the cell walls revealed no intact cells or smaller subcellular particles. A sample of the dialysed cell-wall suspension was stored at 4° for a further 48 hr. and centrifuged at 35000g for 30 min. Of the initial activity of the cell-wall preparation, the supernatant contained 18% and the resuspended cell walls 82%. Hence the bulk of the activity was not eluted from the cell walls by prolonged standing in aqueous solution. The supernatant from the glycerol homogenate hydrolysed sucrose at five times the rate for raffinose, and the same ratio was shown by the supernatant of the large-bean-pod preparation, whereas the corresponding ratio for the cell-wall suspension was three. Paper chromatograms of the products of hydrolysis of raffinose by cell-wall suspensions showed the presence of glucose, fructose, galactose, sucrose and melibiose, indicating the presence of an α -galactosidase in addition to the β -fructofuranosidase. In contrast, chromatograms of the product of hydrolysis of raffinose by the glycerol supernatant solution showed the presence of fructose and melibiose, confirming β -fructofuranosidase activity alone.

Occurrence of β -fructofuranosidase and an endogenous inhibitor in potato-tuber extracts

An extract (75 ml.) from 117 g. of potato tubers (var. Dr Mackintosh) had 0.22 unit of enzyme

Fig. 1. Effect of dilution on β -fructofuranosidase activity of a concentrated potato-tuber extract. E, Relative concentration of enzyme; $k_{obs.}$, activity (units/ml.).

activity/ml., and 9% inhibition was indicated by the dilution technique described above (see the Materials and Methods section). To confirm the presence of this endogenous inhibitor, the extract was concentrated by dialysis against saturated ammonium sulphate. The resulting precipitate was removed by centrifugation at 35000g for 15 min., redissolved in 12.5 ml. of 0.02 M-phosphate buffer, pH 6.5, and dialysed against the same buffer. Enzyme activity was determined at five dilutions of the concentrated extract. By applying eqn. (1), k_0 was found to be 1.2 units/ml. (see Fig. 1). Since k_{50} was measured as 0.8 unit/ml., the most concentrated extract assayed had 33% inhibition.

Extracts from tubers of five other varieties were prepared and assayed for β -fructofuranosidase; the results are compared with those for the extracts used in preparations A and B in Table 4.

On purification of extracts A and B from Dr Mackintosh tubers, no endogenous inhibition was detected after the pH 4 precipitation stage. Addition of the resuspended pH 4 precipitate to portions of the supernatant caused no inhibition of β -fructofuranosidase.

An extract from 220 g. of Dr Mackintosh tubers was adjusted to pH 5.0 and centrifuged; a 30–60% saturated ammonium sulphate fraction was then prepared from the supernatant. No inhibition was detected at pH 3.0–6.0.

Properties of the partially purified β -fructofuranosidases

Behaviour on Sephadex columns. Samples (7.0 units, 5 ml.) of a mixture containing equal amounts of the bean and potato (preparation A) enzymes were applied to a Sephadex G-200 column (39 cm. x

Table 4. *β -Fructofuranosidase and inhibitor activity in potato-tuber extracts*

Extracts were prepared from tubers 4 weeks after they were harvested, except for preparation B (see the Materials and Methods section). Values of $k_{50\%}$ and $k_{10\%}$ were calculated by dividing the relative concentration of enzyme by the measured activity of 0.1 ml. of enzyme extract in a final volume of 0.2 ml. and 1.0 ml. respectively. Values of k_0 were found by extrapolating E/k_{obs} to infinite dilution.

Variety	Wt. of tissue (g.)	Juice yield (ml.)	$k_{50\%}$	$k_{10\%}$	k_0	Inhibition (%)
King Edward	60	40	0.031	0.034	0.035	12
Arran Pilot	146	88	0.058	0.073	0.078	26
Arran Victory	150	90	0.000	0.000	0.000	?
Duke of York	216	120	0.032	0.049	0.057	44
Golden Wonder	189	90	0.009	0.021	0.031	71
Dr Mackintosh (prep. A)	5600	3300	0.106	0.116	0.118	10
Dr Mackintosh (prep. B, tubers)	585	400	0.335	0.358	0.364	8
Dr Mackintosh (prep. B, sprouts*)	18	15	0.202	0.216	0.220	8

* Extract from sprouts present on the tubers were used to obtain preparation B.

Fig. 2. Second elution of a mixture of bean and potato β -fructofuranosidases from a column of Sephadex G-200. ●, E_{280} ; ○, activity (unit/ml.).

2 cm., void vol. 20 ml.). β -Fructofuranosidase was eluted as a single symmetrical peak, with 97% recovery. The four most active fractions were combined and freeze-dried. A redissolved sample (21 units, 2.0 ml.) was applied to a similar column and activity was again eluted as a single symmetrical peak (see Fig. 2).

Specificity. The rates of hydrolysis of five disaccharides and trisaccharides by the bean and potato enzymes were compared. No significant hydrolysis occurred with melibiose, melezitose, maltose and turanose. The raffinose digests examined by paper chromatography showed that

the only detectable products of the bean-enzyme action were melibiose and fructose, whereas the potato enzyme gave, in addition, galactose in small amounts; but no sucrose was detected. Since the limit for the detection of galactose by the spray reagent employed is much lower than that for fructose (Greenshields, 1960), these results indicate that a relatively high β -fructofuranosidase activity and a low α -galactosidase activity were present in the potato preparation.

The oligosaccharides turanose and melezitose (58.4 mm) had no effect on the rates of hydrolysis of 29.2 mm- or 2.43 mm-sucrose.

Fig. 3. Stability of bean and potato β -fructofuranosidases at 37° as a function of pH. The bean preparation was purified 12-fold as a supernatant from a crude hypocotyl extract by dialysing exhaustively against cold water and centrifuging at 35 000g for 15 min. E_{280} values in the incubation mixtures were 0.63 (bean) and 0.33 (potato); final buffer concentration was 0.025 M. $\circ$, Δ , $\square$, Bean enzyme; $\bullet$, $\blacktriangle$, potato enzyme. Buffers: $\circ$, $\bullet$, citrate; Δ , $\blacktriangle$, phosphate; $\square$, acetate.

Transfructosylase activity. The courses of digestion of 0.58M-sucrose by the bean and potato (preparation A) enzymes were compared with a parallel digestion with yeast β -fructofuranosidase. Production of reducing sugars by the bean and potato enzymes was found to be linear up to at least 60% hydrolysis. Chromatograms of the transfer products formed by the bean and potato enzymes and yeast invertase were identical except for the presence of a fourth, slower-moving, component formed by the yeast enzyme. The bean and potato enzymes appeared to produce smaller amounts of transfer products than the yeast enzyme (cf. Allen & Bacon, 1956). The action on 58.4mM-sucrose of the bean and potato enzymes yielded no transfer products after 50% hydrolysis, whereas the yeast enzyme gave three.

Stability of the β -fructofuranosidases. Portions of an extract from bean seedlings and of potato preparation B were incubated at 37° for 20 min. at several pH values, cooled to 0°, brought to pH 4.9 by addition of acetate buffer, and assayed for enzyme activity (Fig. 3). In a further experiment the inactivation of a mixture of the bean-pod enzyme and the potato enzyme preparation B at pH 3.5 in 0.1M-acetate at 25° was measured and compared with the inactivation of the separate enzymes under similar conditions. The results in Fig. 4 show that inactivation of the two enzymes in the mixture proceeded at essentially the same rate as in the separate incubations, indicating that the greater stability of the potato enzyme was not due to stabilization by a component of the potato-enzyme preparation.

The effects of the presence of several substances

Fig. 4. Comparison of the time-course of inactivation of the bean enzyme and potato enzyme (preparation B) with that of a mixture of equal amounts of the two enzymes. At the end of the incubation period at 25° in 0.1M-acetate buffer, pH 3.5, an equal volume of 0.117M-sucrose in 0.2M-acetate buffer, pH 4.9, was added to yield an assay mixture at pH 4.6. $\bullet$, Bean; Δ , potato; $\circ$, mixture.

on the rate of inactivation of bean β -fructofuranosidases at pH 4.9 were studied by the procedure outlined in the previous stability experiments. Cysteine, EDTA and melezitose had no significant effect on the rate of inactivation. Sucrose had a protective effect at concentrations greater than the experimentally determined K_m for the bean enzyme (see Table 6). Sodium sulphate (0.5M) accelerated the inactivation; the half-life of the enzyme activity under the conditions employed was 1 min. in the presence of 0.5M-sodium sulphate and 8 min. in the control incubation. Since melezitose has no effect, the protective action of sucrose is probably a specific stabilization through binding at the active site.

Effects of pH on hydrolysis of 29.2mM substrates. For sucrose, the bean β -fructofuranosidase had an optimum at pH 4.9; the optimum for the potato enzyme (preparation A) was at pH 4.0. The pH curve for the bean enzyme fell away steeply on the acid side (Fig. 5). For raffinose, the bean enzyme had an optimum at pH 5.5, and the potato enzyme (preparation A) had an optimum at pH 4.8. The pH-activity curves for the bean β -fructofuranosidase were essentially similar to those reported previously for bean-seedling preparations acting on 58.4mM-sucrose (Cooper & Greenshields, 1964). The relative rates of hydrolysis of sucrose and raffinose are given in Table 5. Except for the bean enzyme at pH 3.5–4.0, the rate of sucrose hydrolysis

Fig. 5. pH-activity curves for the β -fructofuranosidases acting on 29.2mM-sucrose. ●, Bean enzyme; ○, potato enzyme. The enzymes were incubated for 7.5 and 15 min.; no differences in rates between the two incubation periods were detected. Acetate buffers were used for pH values 3.8–5.6; phosphate buffers were used for the other pH values. The activity for the potato enzyme at pH 2.0 was corrected for some non-enzymic hydrolysis; non-enzymic hydrolysis was insignificant at pH 2.5 and above. The pH values are those of the actual incubation mixtures.

Table 5. Effect of pH on the ratio of the rates of hydrolysis of sucrose and raffinose

These values were calculated from the data used for the pH-activity curves (Figs. 5 and 6).

pH	Rate of hydrolysis of sucrose/rate of hydrolysis of raffinose	
	Bean enzyme	Potato enzyme
3.0	—	6.4
3.5	4.7	4.6
4.0	7.1	4.2
4.4	5.7	3.9
4.9	5.4	3.4
6.0	4.6	2.3
6.5	3.8	2.0
7.0	2.5	1.3

relative to raffinose hydrolysis decreased with increasing pH over the range of values studied.

Effects of pH on K_m values. K_m values for the bean β -fructofuranosidase at pH values between 3.5 and 7.0 and for the potato (preparation A) enzyme at pH values between 3.0 and 7.0 were determined as described above (Table 6). The variation of pK_m ($-\log K_m$) with pH is shown in Fig. 6.

Lineweaver-Burk plots for all the determinations of K_m gave straight lines. The K_m values for the bean enzyme were higher than those for the potato

enzyme under all conditions. For both enzymes K_m values for raffinose were higher than those for sucrose. At all the pH values investigated, the ratio K_m (sucrose)/ K_m (raffinose) was lower for the bean enzyme than for the potato enzyme. The K_m for the bean enzyme with sucrose as substrate at pH 6.0 was not significantly different in acetate or phosphate buffers. The presence of 0.25M-sodium sulphate in the reaction mixture with sucrose at pH 4.0 did not significantly affect the value of K_m for the potato enzyme.

The K_m varied with pH for the β -fructofuranosidases and the pH optima of the enzymes were dependent on substrate concentration. By using the relative activities in Figs. 5 and 6 for 29.2mM substrate, the relative values of V were calculated for each value of K_m by substitution in the Michaelis-Menten equation. Values of V cannot be measured directly for the bean and potato enzymes since, like the yeast enzyme (Nelson & Schubert, 1928), they exhibit an optimum substrate concentration. The relative values of v_0 , the activity at infinite dilution of substrate (Laidler, 1958), were calculated from the same data. From the values of V and v_0 the approximate limits of the pH optima as a function of substrate concentration were determined and are given in Table 7. Both enzymes show a variation of 1–2 pH units between the optima at very high and those at very low substrate concentrations. The pH optima calculated for the potato β -fructofuranosidase were all more acid than the corresponding values for the bean enzyme.

Effect of high sucrose concentration. The activities of the bean and potato (preparation B) β -fructofuranosidases were determined in mixtures containing up to 63% (w/v) sucrose (1.84M). The bean enzyme retained 50% and the potato enzyme 30% of its maximum activity (Fig. 7). The decrease in activity of yeast β -fructofuranosidase with increasing sucrose concentration was slightly greater than that observed for the potato enzyme (Nelson & Schubert, 1928).

Effects of specific group reagents on the activity of the β -fructofuranosidases

p-Chloromercuribenzoate. PCMB* was an inhibitor of both β -fructofuranosidases (see Table 8). Incubation of the enzyme preparations with 1mM-sodium benzoate had no effect; thus the inhibition by PCMB was not an effect of the benzoate group. Preincubation of the potato enzyme with PCMB at pH 7.0 had a larger effect on the activity than addition of PCMB to the buffered substrate solution with subsequent addition of the enzyme. This effect may have been due to the difference in pH and ionic

* Abbreviation: PCMB, *p*-chloromercuribenzoate.

Table 6. K_m values for the bean and potato β -fructofuranosidases

For details of the procedure see the text.

pH	K_m for potato enzyme (mM)		K_m for bean enzyme (mM)	
	Sucrose	Raffinose	Sucrose	Raffinose
3.0	10.9 $\pm$ 0.4	25 $\pm$ 9	—	—
3.5	6.2 $\pm$ 0.4	12.3 $\pm$ 2.3	10.2 $\pm$ 0.4	40.6 $\pm$ 7.7
4.0	3.7 $\pm$ 0.3	11.3 $\pm$ 0.6	7.8 $\pm$ 1.2	33.8 $\pm$ 4.1
4.0	3.3 $\pm$ 0.2*	—	—	—
4.9	2.7 $\pm$ 0.2	7.3 $\pm$ 0.4	5.4 $\pm$ 0.8	24.2 $\pm$ 1.0
4.9	—	—	5.3 $\pm$ 0.7†	—
6.0	1.2 $\pm$ 0.1	4.4 $\pm$ 0.3	3.1 $\pm$ 0.3	13.8 $\pm$ 0.7
6.0	—	—	2.8 $\pm$ 0.5‡	—
7.0	0.82 $\pm$ 0.02	2.0 $\pm$ 0.5	1.16 $\pm$ 0.06	7.8 $\pm$ 0.2

* 0.25 M- Na_2SO_4 present.

† Enzyme prepared from seedlings.

‡ Acetate buffer.

Fig. 6. Effect of pH on pK_m for the β -fructofuranosidases: ●, potato, sucrose; ○, potato, raffinose; ▲, bean, sucrose; △, bean, raffinose. K_m values are given as molarities.

strength at which the enzymes were exposed to PCMB. Further incubation with reagents containing thiol groups after incubation with PCMB caused some recovery of activity. Incubation of the enzyme preparations with thiol-containing reagents alone had no effect on the activity. The β -fructofuranosidase activity of a crude extract from bean seedlings, prepared as described by Cooper & Greenshields (1964), with a specific activity about 0.67% of that of the bean-pod preparation, was completely inhibited by the addition of 0.1 mM-PCMB to the reaction mixture, whereas 0.01 mM-PCMB had little effect; hence inert material present in the crude seedling extract caused some protection.

N-Ethylmaleimide. β -Fructofuranosidase activity of the bean-pod and potato preparations (A and B) was not affected by prior incubation for 30 min. at 25° with 1 mM-*N*-ethylmaleimide in 0.02 M-phosphate buffer at pH 6.0, 7.0 and 7.5. Under these conditions the activity of the controls was un-

changed. At pH 8.0 and 25° in 0.02 M-phosphate buffer the control samples lost activity, but the inactivation rate was approximately doubled for the bean enzyme by the presence of 10 mM-*N*-ethylmaleimide; much smaller increases in inactivation rate were shown by the potato enzyme. Subsequent incubation with a tenfold molar excess of cysteine did not restore any activity.

Cations. Portions of the bean preparation and of preparation A from potato tubers were incubated for 10 min. at 25° and pH 7 with several concentrations of silver nitrate and cupric sulphate. Ag^+ ions at or above 0.01 mM completely inhibited both enzymes and 0.1 mM- Cu^{2+} ions produced the same effect. Cooper & Greenshields (1964) reported 90–95% inhibition of partly purified bean-seedling β -fructofuranosidase by 0.1 mM- Ag^+ ions.

Succinic anhydride. Samples of the bean preparation, potato preparation B and yeast β -fructofuranosidase (an 8000-fold dilution of the concentrated preparation) were incubated at 25° for 30 min. with succinic anhydride (0.5–5 mM) at pH values 4.9 and 5.6. In some experiments, enzyme was preincubated before the addition of sucrose. Succinic anhydride had no effect on the potato or yeast enzymes under the conditions employed. On prior incubation with 0.5 mM-succinic anhydride or higher concentrations, the bean enzyme lost 30% of its maximum activity, but inclusion of succinic anhydride in the buffered substrate mixture had no effect on activity; thus sucrose protects the enzyme from inactivation by succinic anhydride.

Photo-oxidation in the presence of methylene blue. The effect of illumination of the bean preparation and potato preparation B in the presence of methylene blue and thioethanol was investigated under similar conditions to those employed by

Table 7. Variation of the optimum pH of the enzymes with substrate concentrations

V and v_0 were calculated from K_m values in Table 8 and relative activities in Figs. 5 and 6. $[S]$, Substrate concentration.

Source of enzyme	Substrate	pH optima of velocity		
		$[S]=\infty; V$	$[S]=29.2\text{ mM}$	$[S]=0; v_0$
Potato	Sucrose	3.7*	4.0	5.6
Potato	Raffinose	4.4*	4.8	5.6
Bean	Sucrose	4.7	4.9	6.0
Bean	Raffinose	4.7	5.5	6.0

* V decreased above, and remained constant below, these pH values.

Fig. 7. β -Fructofuranosidase activity at high sucrose concentrations: ●, bean; ○, potato.

Table 8. Effect of p-chloromercuribenzoate on β -fructofuranosidase activity

The preparations were incubated with PCMB for 2 min. at 25° in a solution containing 0.02 M-phosphate buffer, pH 7.0. Assay of β -fructofuranosidase activity was then started by the addition of buffered sucrose solution to give final concentrations of 59.4 mM-sucrose and 0.05 M-acetate buffer, pH 4.9. Reversibility of the inhibition by PCMB was investigated by adding a 100-fold molar excess of cysteine or a 10-fold molar excess of thioethanol after 2 min. and incubating for a further 2 min. before adding the buffered sucrose. In the PCMB-sucrose incubations, the enzyme samples were added to a buffered sucrose solution containing PCMB. The PCMB concentrations given are those present in the assay incubation in each case. The control was unaffected by the thiol reagent.

Preparation	Incubation	Activity	
		Concn. of PCMB (μM)	relative to control (%)
Bean	PCMB	10	1
Bean	PCMB, cysteine	10	25
Bean	PCMB, sucrose	10	12
Bean	PCMB	1	65
Bean	PCMB, sucrose	1	64
Potato B	PCMB	10	18
Potato B	PCMB, thioethanol	10	39
Potato B	PCMB, sucrose	10	32
Potato B	PCMB	1	47
Potato B	PCMB, thioethanol	1	63
Potato B	PCMB, sucrose	1	77

Weil & Buchert (1951). In the presence of methylene blue, activity was rapidly lost under illumination, but activity was completely retained in the dark, or in the absence of methylene blue.

Di-isopropyl phosphorofluoridate. 1 mM-Di-isopropyl phosphorofluoridate had no effect on the activity of the bean and potato (preparation B) β -fructofuranosidases when samples were incubated for 10 min. at 25° in 0.02 M-phosphate buffer, pH 7.0, with the compound, or when the compound was included in the buffered substrate solution.

DISCUSSION

The specific activity of 60 units/mg. of nitrogen of the β -fructofuranosidase preparation obtained from bean pods was about half that of the earlier preparation from bean seedlings (Cooper & Greenshields, 1964) and was much lower than that of the grape β -fructofuranosidase preparation reported by Arnold (1965). The specific activities of the potato preparations were about one-eighth (preparation B) and one-twentieth (preparation A) of that of the bean preparation, for which a turnover

number of 800 moles/100 000 g. (freeze-dried wt.)/min. was calculated. This relatively low value suggests that the preparations were impure, perhaps containing less than 1% of protein as enzyme. The low concentrations of PCMB and Ag^+ ions that affected the bean and potato preparations, and the identical K_m values and pH-activity curves observed for samples of different purity (cf. Cooper, 1961), show that the impurities present probably had no effect on the kinetic properties of the enzymes.

An increase in specific activity of β -fructofuranosidase on dilution of dialysed crude extracts from potato tubers of five varieties confirmed the conclusion (Schwimmer *et al.* 1961) that an endogenous inhibitor is present. No inhibition occurred in the supernatant solution after the solution had been adjusted to pH 4.0, but the resuspended pH 4.0 precipitate was not inhibitory; thus the inhibitor appeared to be acid-labile. Rorem & Schwimmer (1963) also suggested that the differences in K_m values at pH 4.0 and pH 6.0 observed in tuber extracts reflected competitive inhibition. In the present work, however, K_m remained pH-dependent in inhibitor-free preparations of the potato enzyme, so that there was no evidence that this inhibition was competitive. It should be noted that non-diffusible inhibitors of trypsin and chymotrypsin have also been prepared from potato tubers (Ryan & Balls, 1962; Rabek & Mansfield, 1963) and it is possible that similar substances are present in dialysed tuber extracts, which act as enzyme inhibitors through non-specific binding. No physiological role could be attributed to this inhibitor.

The bean and potato β -fructofuranosidases differed somewhat in the effect of pH on activity. The calculated pH optima for very high and very low substrate concentrations were lower in all cases for the potato enzyme and, within the range of pH values studied, the ratios $K_m(\text{sucrose})/K_m(\text{raffinose})$ and rate of raffinose hydrolysis/rate of sucrose hydrolysis were all higher for the potato enzyme than for the bean enzyme. The dependence of K_m on pH for the enzymes acting on the two substrates was similar to that reported by Arnold (1965) for grape β -fructofuranosidase acting on sucrose. The pH- pK_m curves can be interpreted on the basis of ionizing groups located at the active site (Dixon, 1953). According to the rules given by Dixon for such an interpretation, the curve consists of regions of integral slope joined by short curved portions. A unit increase in slope indicates the pK of a group in the free enzyme or substrate. The observed curves for the bean enzyme are consistent with the occurrence of groups with pK values about 4.0 and 7.4, which are changed to about 3.5 and 6.5 on substrate-binding. The pK 7.4 for a group in the free enzyme was estimated by extrapolation of the data for K_m and v_0 (see below). In the potato enzyme an ionizing group with pK about 7.0 changing to about 6.2 on substrate binding is indicated, together with a group of pK 3.6 unchanged by substrate binding. This explanation of the effect of pH on K_m for the potato and bean β -fructofuranosidases would imply that substrate binding at pH values more acid than the pK of the basic group releases a proton from a basic group, whereas at pH values more acid than the pK for the acidic group substrate binding causes protonation

of this group. When the values of V and v_0 were calculated from the pH curves for 29.2 mM substrates, all were found to exhibit pH optima with the exception of V for the potato enzyme. Both acidic and basic groups in the bean enzyme therefore appear to be implicated in hydrolytic action. For the potato enzyme the acidic group is involved in cleavage but probably only the basic group takes part in substrate binding (cf. Laidler, 1955). The pH values where v_0 and V were both half-maximal were the same as those derived from the plots of pK_m against pH. All the pK values of the potato enzyme were a little lower than those of the bean enzyme. The data given by Arnold (1966) are consistent with pK values 1.8 and 6.0 in the free enzyme and 4.0 in the Michaelis complex. The pH range over which activity of the bean and potato enzymes could be studied was too limited to permit a more detailed analysis of the binding and catalytic groups (cf. Myrbäck, 1960).

K_m for yeast β -fructofuranosidase is constant between pH 4.0 and 8.0 (Josephson, 1924; Myrbäck & Bjorklund, 1952) but increases sharply below pH 4.0. A possible role in substrate binding has been attributed to a basic group ($pK \sim 3$). A group with $pK \sim 7$ affects V but not K_m and therefore is involved in the catalytic reaction but not in substrate binding; the $pK \sim 3$ and $pK \sim 7$ groups have been identified with carboxyl and imidazole groups respectively (Myrbäck, 1960), and the pK values found in the bean and potato enzymes may also implicate these groups. In contrast with the yeast enzyme, binding of either sucrose or raffinose appears to affect the ionization of the acidic ($pK \sim 7$) group. The rate of hydrolysis of sucrose by the bean and potato enzymes showed an optimum value of substrate concentration similar to those reported for the yeast enzyme (Nelson & Schubert, 1928) and the grape enzyme (Arnold, 1965). Above 5% (w/v) sucrose the inhibition was more marked in the potato enzyme. This inhibition could probably be attributed to several factors, including substrate inhibition, viscosity of the medium (Ruchti & McLaren, 1964), transferase action and water concentration (Nelson & Schubert, 1928; Bacon & Bell, 1957), but no simple dependence on any one of these factors could be demonstrated.

No differences between the bean and potato enzymes could be positively established by the group reagents employed. PCMB (1 μ M) inhibited both the bean and potato enzymes, probably through reaction with one or more thiol groups. Such thiol groups may be concerned directly in catalysis; alternatively their substitution by PCMB may lower the activity through steric effects (Boyer & Schulz, 1959). *N*-Ethylmaleimide affected both enzymes slightly at pH 8.0 and had no effect at pH 7.5, suggesting that the thiol groups con-

cerned are relatively unreactive. Both enzymes were almost completely inactivated by 0.01M- Ag^+ ions; Cu^{2+} ions inhibited less strongly. These metal ions react with carboxyl, thiol or imidazole groups (Myrbäck, 1957a,b; Jensen, 1959). This last-named group is also implicated by the rapid inactivation of the bean and potato enzymes by methylene blue, by photo-oxidation and by the effects of pH and substrate concentration on activity.

With the exception of a difference in sensitivity to succinic anhydride, the bean and potato enzymes differed little and resembled *Neurospora* and grape invertases (Metzenberg, 1963; Arnold, 1965). More fundamental differences were apparent between the plant invertases and those of yeast and certain moulds other than *Neurospora*. Thus the yeast showed no dependence of K_m on pH above pH 4.0 and produced different oligosaccharides during the transferase reaction (Myrbäck, 1960; Bacon, 1960). The mould enzymes produce tetra- and higher oligo-saccharides from sucrose, and also transfer fructose from sucrose to free glucose (Bealing & Bacon, 1953; Edelman, 1954). *Neurospora* invertase differs from those of other moulds in that it does not transfer fructose to glucose (Trevithick & Metzenberg, 1964). The bean enzyme, however, does resemble yeast and *Neurospora* invertases in the details of the acid branch of the pH-activity curve.

All the evidence available from pH and inhibition studies of yeast, higher-plant and mould enzymes is consistent with the participation of carboxyl and imidazole groups in catalysis. In yeast, potato and grape invertases a carboxyl group may also participate in substrate binding.

Imidazole and carboxyl groups have been implicated in catalytic activities of several glycosidases (Larner & Gillespie, 1955, 1956; Neely, 1958, 1959; Thoma & Koshland, 1960). Thoma, Wakim & Stewart (1963) have pointed out that α - and β -amylase probably have a common catalytic site but differ in the substrate-binding groups (cf. Koshland, Ray & Erwin, 1958). It thus appears that carboxyl and imidazole groups are the probable catalysts of glycoside bond cleavage in both hydrolysis and transglycosylation.

The absence of resolution of the bean and potato β -fructofuranosidases on Sephadex-gel columns indicates a similarity in molecular size (Neumann & Lampen, 1967). The effects of pH, substrate concentration and group inhibitors are consistent with participation of the same amino acid residues in enzyme action. The quantitative differences in the effects of these factors on activity, on stability and on the relative velocities of sucrose and raffinose hydrolysis can be explained by differences in auxiliary amino acid residues. In conclusion, it

seems probable that the enzymes have similar overall structures and probably have a common evolutionary precursor.

REFERENCES

- Allen, P. J. & Bacon, J. S. D. (1956). *Biochem. J.* **63**, 200.
 Arnold, W. N. (1965). *Biochim. biophys. Acta*, **110**, 134.
 Arnold, W. N. (1966). *Arch. Biochem. Biophys.* **113**, 451.
 Bacon, J. S. D. (1960). *Bull. Soc. Chim. biol., Paris*, **42**, 1441.
 Bacon, J. S. D. & Bell, D. J. (1957). *J. chem. Soc.* p. 3581.
 Bacon, J. S. D. & Edelman, J. (1951). *Biochem. J.* **48**, 114.
 Bealing, F. J. & Bacon, J. S. D. (1953). *Biochem. J.* **53**, 277.
 Boyer, P. D. & Schulz, A. R. (1959). In *Sulfur in Proteins*, p. 199. Ed. by Benesch, R. New York: Academic Press Inc.
 Cooper, R. A. (1961). Ph.D. Thesis: University of Birmingham.
 Cooper, R. A. & Greenshields, R. N. (1961a). *Biochem. J.* **81**, 6p.
 Cooper, R. A. & Greenshields, R. N. (1961b). *Nature, Lond.*, **191**, 601.
 Cooper, R. A. & Greenshields, R. N. (1964). *Biochem. J.* **92**, 357.
 Dahlqvist, A. (1961). *Biochem. J.* **80**, 547.
 Dixon, M. (1953). *Biochem. J.* **55**, 161.
 Edelman, J. (1954). *Biochem. J.* **57**, 22.
 Fischer, E. H. & Kohtès, L. (1951). *Helv. chim. Acta*, **34**, 1123.
 Greenshields, R. N. (1960). Ph.D. Thesis: University of Birmingham.
 Hatch, M. D., Sacher, J. A. & Glasziou, K. T. (1963). *Plant Physiol.* **38**, 338.
 Henderson, R. W., Morton, R. K. & Rawlinson, W. A. (1959). *Biochem. J.* **72**, 340.
 Jensen, E. V. (1959). In *Sulfur in Proteins*, p. 75. Ed. by Benesch, R. New York: Academic Press Inc.
 Josephson, K. (1924). *Hoppe-Seyl. Z.* **134**, 50.
 Kivilaan, A., Beamann, T. C. & Bandurski, R. S. (1961). *Plant Physiol.* **36**, 605.
 Koshland, D. E., Ray, W. J. & Erwin, M. J. (1958). *Fed. Proc.* **17**, 1145.
 Laidler, K. J. (1955). *Trans. Faraday Soc.* **51**, 550.
 Laidler, K. J. (1958). *Chemical Kinetics of Enzyme Action*, p. 63. London: Oxford University Press.
 Larner, J. & Gillespie, R. E. (1955). *Arch. Biochem. Biophys.* **58**, 252.
 Larner, J. & Gillespie, R. E. (1956). *J. biol. Chem.* **223**, 709.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **45**, 1539.
 Metzenberg, R. L. (1963). *Arch. Biochem. Biophys.* **100**, 503.
 Myrbäck, K. (1957a). *Ark. Kemi*, **11**, 47.
 Myrbäck, K. (1957b). *Ark. Kemi*, **11**, 471.
 Myrbäck, K. (1960). In *The Enzymes*, vol. 4, p. 379. Ed. by Boyer, P. D., Lardy, M. & Myrbäck, K. New York: Academic Press Inc.
 Myrbäck, K. & Bjorklund, U. (1952). *Ark. Kemi*, **4**, 567.
 Neely, W. B. (1958). *J. Amer. chem. Soc.* **80**, 2010.
 Neely, W. B. (1959). *Arch. Biochem. Biophys.* **79**, 297.

- Negoro, H. (1957). *J. agric. chem. Soc., Japan*, **31**, 253; cited in *Chem. Abstr.* **51**, 13996.
- Nelson, J. M. & Schubert, M. P. (1928). *J. Amer. chem. Soc.* **50**, 2188.
- Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
- Neumann, N. P. & Lampen, J. O. (1966). *Fed. Proc.* **25**, 588.
- Neumann, N. P. & Lampen, J. O. (1967). *Biochemistry*, **6**, 468.
- Pridham, J. B. & Walter, M. W. (1964). *Biochem. J.* **92**, 20p.
- Rabek, V. & Mansfield, V. (1963). *Experientia*, **19**, 151.
- Rorem, E. S. & Schwimmer, S. (1963). *Experientia*, **19**, 150.
- Ruchti, J. & McLaren, A. D. (1964). *Enzymologia*, **27**, 185.
- Ryan, C. A. & Balls, A. K. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1839.
- Schwimmer, S., Makower, R. U. & Rorem, E. S. (1961). *Plant Physiol.* **36**, 313.
- Smith, I. (1960). In *Chromatographic and Electrophoretic Techniques*, 2nd ed., vol. 1, p. 250. Ed. by Smith, I. London: W. Heinemann (Medical Books) Ltd.
- Somogyi, M. (1945). *J. biol. Chem.* **160**, 61.
- Thoma, J. A. & Koshland, D. E. (1960). *J. molec. Biol.* **2**, 169.
- Thoma, J. A., Wakim, J. & Stewart, L. (1963). *Biochem. biophys. Res. Commun.* **12**, 350.
- Trevithick, J. R. & Metzenberg, R. L. (1964). *Arch. Biochem. Biophys.* **107**, 260.
- Weil, L. & Buchert, A. R. (1951). *Arch. Biochem. Biophys.* **34**, 1.
- White, J. W. & Subers, M. H. (1961). *Analyt. Biochem.* **2**, 380.