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ISOLATION OF INVERTASE FROM BANANA FRUIT (*MUSA CAVENDISHII*)

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Abstract—A soluble form of invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) has been purified from ripe banana fruit (*Musa cavendishii*). The enzyme has a high specific activity and an apparent MW of 220 000 daltons; it appears to be glycoprotein containing 12.5% mannose and 12% glucosamine.

INTRODUCTION

Invertase activity has long been known to be associated with the fruit of the banana but attempts to isolate the enzyme in a reasonably pure state have not been sucessful [1–3]. One of the reasons for this may be the chemical complexity of the banana fruit and the difficulty of avoiding the spurious effects of oxidation during enzyme isolation [4]. Recent reports with plant systems, and in particular with the potato tuber enzyme have shown that a naturally occurring invertase inhibitor may be associated with the enzyme *in situ* [5–7]. Provided steps are taken to selectively denature the protein inhibitor, comparatively high levels of invertase activity can be observed [6, 7]. It occurred to us that an analogous situation might occur in the banana fruit, particularly as preliminary studies had shown that the invertase activity in crude extracts of banana pulp varied widely from one preparation to the next. An exhaustive mechanical blending technique, similar to that used during the isolation of invertase from potato tubers [6], has been adopted to enhance the extraction of invertase into aqueous buffer. In this communication we describe the results obtained using this procedure and the subsequent purification and basic characteristics of invertase from Cavendish bananas.

RESULTS AND DISCUSSION

Release of invertase by mechanical blending

In line with the reports of earlier workers [1–4] our preliminary experiments showed that invertase activity was very low in aqueous extracts of banana fruit after short periods of homogenization in a Waring blender. Initially short blending times, between 30 and 60 sec were used. Enzyme analysis of ammonium sulphate fractions prepared from these extracts found that enzyme activity when it could be detected was very low. The activity was not enhanced by raising the ionic strength or by the addition of detergents such as Triton X-100 or bile salts, over a wide range of detergent : protein ratios during the initial extraction. However if the period of homogenization was extended the enzyme activity in the initial aqueous extract was considerably enhanced, and significant activity was detected after ammonium sulphate fractionation in the 45–95% ammonium sulphate cut. Maximum enzyme activity was detected after 7 min homogenization. Microscopic examination of the homogenates after different periods of blending revealed that cell disruption was essentially complete after homogenization for 1 min. For this reason it seems unlikely that the increase in the observed yield of invertase with prolonged blending is due to more complete homogenization of the tissue. The underlying chemical rationale for this result is unclear although it does seem analogous to the preferential denaturation of the potato invertase inhibitor by mechanical agitation/foaming, as first reported by Pressey [5].

The 45–95% ammonium sulphate fraction was relatively viscous, and attempts at this stage to perform molecular sieve chromatography using either Sephadex or Biogel matrices were not successful. However a significant fractionation of the crude sample was achieved by DEAE-cellulose chromatography. A discrete peak of enzyme activity was obtained, which overlapped one of the peaks detected by A at 280 nm. Polyacrylamide gel electrophoresis (PAGE) under dissociating conditions showed that the sample contained a number of proteins, but that the major band had an apparent MW of 200 000.

Further purification of the enzyme was achieved by chromatography on hydroxylapatite. The invertase was eluted from the column with *ca* 0.3 M phosphate buffer, and the invertase peak, which appeared symmetrical, overlapped one of the peaks in the 280 nm A profile. SDS–PAGE showed the presence of only one band, of MW 200 000.

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Table 1. Steps in the purification of banana invertase

Fraction	Specific enzyme activity (units/mg protein)	Purification factor	Enzyme recovery
(i) Crude isolate after 7 min homogenization	0.07	1	*—
(ii) After ammonium sulphate fractionation, and dialysis	1.6	22.9	*100
(iii) After DEAE-cellulose chromatography	21	300	44
(iv) After hydroxylapatite chromatography	74–90	1060–1300	8.8

* Enzyme recoveries have been based on enzyme activity recovered after ammonium sulphate fractionation (20–95%) and dialysis against water.

PAGE on cylindrical gels under non-dissociating conditions also displayed only one band with a MW of 200 000.

The MW of the enzymatically active unit was determined by exclusion chromatography on Biogel P-300, in the presence of high salt (0.4 M NaCl) and detergent (0.1% Triton). The invertase activity profile was symmetrical, and by comparison with proteins of known MW an apparent MW of 220 000 was determined.

In Table 1, a summary of the purification data is presented. The enzyme activity in the crude isolate after homogenization was very low, but increased over 1000-fold after hydroxylapatite-chromatography. The sp. act. at this stage was between 74 and 90 which is significantly greater than that reported for any other invertase isolated from a plant source. No further increase in sp. act. was observed after molecular sieve chromatography on Biogel. Invertase activity resides in the recognition of the fructofuranose moiety of the substrate molecule, and the capacity to cleave the glycosidic linkage between the bridge oxygen and the fructose residue. Methyl-β-D-fructofuranoside may be used in place of sucrose to confirm that the enzyme is not an α-glucosidase. When this substrate was used with the banana enzyme, reducing sugar was released at a rate of 20 μmol per min per mg protein, indicating that the enzyme is in fact a β-fructofuranosidase. The optimum pH for enzyme activity was 3.5. Alkaline invertase has been reported to be present in banana fruit pulp by Madhulika *et al.* [9] but no alkaline invertase activity was detected in our enzyme preparations. The high sp. act. promoted a comparison of the energy of activation of the banana enzyme with that of the enzyme purified from baker's yeast, *Saccharomyces cerevisiae.* From a plot of log (reaction velocity) vs (temperature)$^{-1}$ the free energy of activation of the banana enzyme was estimated to be 10.8 kcal per mol. This value is comparable to the value of 8–10, reported for the hydrolysis of sucrose catalysed by yeast invertase [10]. At pH 3.5 and with sucrose as the substrate, the optimum temperature for banana invertase activity was 40°. The enzyme displayed simple Michaelis–Menten kinetics. The Michaelis constant, was found to be 2.7 mM, and the phenomenon of fall-off in reaction velocity at high substrate concentration, as reported for grape invertase [11] was not observed.

Yeast [12], Neurospora [13] and potato tuber invertases [14] have all been shown to be glycoproteins. External yeast invertase was reported to consist of mannose, glucose and glucosamine to the extent of 40, 4 and 3%, respectively [15]. Banana invertase also appears to be a glycoprotein for the following reasons: firstly the enzyme could be retained on a Concanavalin A–Sepharose column, and subsequently specifically eluted by methyl α-D-mannoside in the presence of high ionic strength buffer (0.9 M NaCl). Secondly, carbohydrate was detected in the final enzyme preparation, which means that carbohydrate and protein had co-fractionated through a variety of separating procedures, including adsorption chromatography, ammonium sulphate fractionation and molecular sieve chromatography. Thirdly a constant ratio of protein to carbohydrate was observed for the purified enzyme fraction for each of the large number of preparations examined (>10). The total neutral sugar content of the enzyme determined by the phenol–sulphuric acid method [16] was 12.5%. Free hexosamine was estimated to be 12%. TLC on cellulose and on Si gel indicated that the constitutive sugars are mannose and glucosamine respectively.

Invertase is present in a wide range of biological systems, but although it was one of the first substances to be recognized as an enzyme, no systematic study of its mode of action has been made. The known facts are that the yeast enzyme hydrolyses sucrose, its well known substrate, and alkyl β-D-fructofuranosides [21–23]. We are studying the action of invertase on a wide range of sugar derivatives [24] in order to determine the mode of action of the yeast enzyme (*Candida utilis*), and parallel studies with the banana enzyme, should prove valuable in establishing such structure–function relationships.

EXPERIMENTAL

Invertase purification. Fruits of *Musa cavendishii* were purchased from the Queensland fruit market. Firm, yellow bananas were chosen for enzyme purification. About 1.2 kg ripe banana pulp was cut into thin slices and homogenized with an equal vol. of H_2O (0°) in a Waring blender (30 sec).

Homogenate was placed on ice for 30 sec and then homogenization was repeated for a further 30 sec. After allowing the blender to cool 1 min by immersion in ice water, homogenization was repeated (3 min), and then cooled again for 5 min. A final 3 min homogenization was carried out. Slurry was centrifuged at 35 000 g for 20 min, and resulting supernatant removed and adjusted to 45% (w/v) $(NH_4)_2SO_4$ by the addition of the solid salt. The ppt. was removed by centrifuging at 48 000 g for 20 min. The $(NH_4)_2SO_4$ concentration in the resulting supernatant was increased to 95% and then centrifuged at 48 000 g for 20 min. The pellet was recovered and resuspended in 200 ml cold H_2O. The turbid soln was dialysed 18 hr against H_2O (4°) containing 0.04% 2-mercaptoethanol (2-ME). The dialysate was then applied to a DEAE-cellulose column (Whatman). The column (20 g; 50 × 90 mm) was freshly poured from DEAE-cellulose that had previously been equilibrated with 50 mM Pi buffer, pH 7, containing 0.04% (v/v) 2-ME. The crude extract (100 ml) was applied to the column which was subsequently washed with 80 ml of 50 mM Pi buffer, pH 7. A linear salt gradient of 0–0.3 M NaCl was then applied. Fractions of the eluate (3 ml) were collected and assayed for invertase activity. Active fractions were pooled and dialysed for 18 hr as described above. Further purification was achieved by adsorption chromatography on hydroxylapatite. Hydroxylapatite powder (BioRad) (15 g) was equilibrated against 10 mM Pi buffer pH 6.8 (CO_2 free). A column (9 mm × 15 cm) was poured and eluted with 50 ml of equilibrating buffer. Dialysate (30 ml) was applied to the column, washed with 60 ml of Pi buffer pH 6.8, and finally eluted with a linear gradient from 10 mM–0.5 M phosphate buffer. Fractions (1 ml) were collected and monitored for A at 280 nm and for enzyme activity.

Invertase assay. Invertase activity was assayed by the method of ref. [17]. Enzyme activity has been reported in terms of units per mg protein, where 1 unit represents the hydrolysis of 1 μmol of sucrose/min.

Protein assay. Protein was determined by the method of ref. [18] using crystalline BSA (Sigma Chemicals) as the standard.

Carbohydrate analysis. Total sugar content was estimated by the PhOH—H_2SO_4 method. [16] Total hexosamine was determined by the Elson–Morgan method as described in ref. [25]. Identification of sugars was carried out by TLC on cellulose and Si gel with the respective solvent systems, BuOH–EtOH–H_2O (4:1:1) and EtOAc–Py–H_2O (8:2:1). Alkaline $AgNO_3$ and ninhydrin were used to locate neutral sugars and hexosamines respectively.

Gel electrophoresis. Non-dissociating PAGE was performed on 4% acrylamide gels as described in ref. [19]. Dissociating gel electrophoresis was carried out using 7.5% polyacrylamide gels in the presence of SDS as described in ref. [20]. To dissociate samples prior to electrophoresis, SDS and 2-ME were added to final concns of 2% and 0.25% respectively and the samples were dialysed 18 hr against 10 mM Pi buffer, pH 7, containing 0.1% SDS and 0.25% 2-ME.

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