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# PARTIAL PURIFICATION AND PROPERTIES OF INVERTASE FROM *CARICA PAPAYA* FRUITS

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**Key Word Index**–*Carica papaya*; Caricaceae; invertase; inhibition by fructose; inhibition by glucose; protein activation.

**Abstract**—The invertase (EC 3.2.1.26) from *Carica papaya* fruits was isolated and partially purified. The enzyme exhibits simple Michaelis–Menten kinetics and the $K_m$ is $4.2 \pm 0.02 \times 10^{-3}$M. The optimum pH is 4.5. The enzyme is inhibited by fructose but not by glucose. The inhibition by fructose fits well with a case of inhibition through two interacting sites. Inhibition by fructose is not suppressed by proteins as occurs with other plant invertases. According to these results fructose appears to be an important effector of this enzyme.

## INTRODUCTION

Higher plant invertases may be classified according to their localization and pH optimum. Thus exocellular enzymes have been generally considered as cell wall enzymes [1–6] involved in sucrose translocation. Intracellular invertases may be classified according to their pH optima into neutral (sometimes alkaline) and acid invertases. Neutral invertases are thought [7, 8], but not yet shown, to be cytoplasmic enzymes. Acid invertases appear to be present at different sites depending on the development of the tissue. Thus acid invertases of differentiated tissues appear to be vacuolar enzymes [9–12], although a similar invertase from cell cultures was found not to be in the vacuole [13]. An additional difference between acid invertases found in our laboratory, is that some of these invertases are inhibited by fructose through a complex mechanism [14] whereas others are not [15]. This paper reports a plant invertase whose action appears to be modulated by the competitive inhibitory action of fructose.

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## RESULTS

Invertase extraction was performed by two methods (Table 1). Procedure I is closer to most of the relatively low ionic strength extraction procedures described in the literature, whereas procedure II uses a buffer saturated

Table 1. Extraction and purification of the invertase from *C. papaya*.

| Step | Protein (mg) | Sp. activity (Units/mg protein) | Yield (%) |
|---|---|---|---|
| Procedure I | | | |
| Homogenate | 565 | 0.2 | 100 |
| Procedure II | | | |
| Homogenate* | 726.0 | 6.2 | 100 |
| Centrifugation at 19400 *g* | 494.0 | 7.0 | 76.8 |
| Ammonium sulphate precipitation | 131.0 | 18.7 | 54.4 |
| Centrifugation at 19 400 *g* | 109.0 | 15.7 | 37.9 |
| Sephadex G-200 filtration | 24.3 | 28.9 | 15.5 |

*The extraction buffer contained saturated ammonium sulphate as described in Experimental.

with solid ammonium sulphate. The presence of saturating amounts of ammonium sulphate produced a preferential extraction of invertase. The specific activity of the partially purified invertase obtained by the use of procedure II was 143 times higher than the specific activity of the homogenate of procedure I. Thus the second extraction procedure was used throughout this work unless otherwise stated.

The enzyme showed a single activity band on gel electrophoresis [16], and was active in a pH range from 3.5 to 7. The optimum pH was 4.5. The rate was linear over 30 min when 15 to 60 mM sucrose was used. However, 2.5 to 10 mM sucrose reduced the linear period to 5 min. Plots of the reaction rate against enzyme concentration were linear when 5 to 100 $\mu$l of the enzyme were used. The same results were found with crude extracts obtained by the two extraction methods. The Arrhenius plot for temperatures between 10 and 45° was a straight line. This differed from that of leaf sheath invertase from sugar cane which shows an inflection point at 30° [14]. The energy of activation was 8 700 cal/mol. Lineweaver–Burk plots were straight lines. Thus the enzyme shows simple kinetics. The $K_m$ was $4.2 \pm 0.02 \times 10^{3-}$ M ($N = 5$). However, with a substrate concentration higher than 100 mM sucrose, substrate inhibition occurs.

Fructose was a competitive inhibitor of the *C. papaya* invertase (Fig. 1). Replots of $K_{sapp}$ against fructose concentration give a curve, and consequently fructose inhibition is not a classical competitive inhibition as that of *Ricinus communis* invertase [15]. A replot of $1/K_{islope}$ against fructose concentration is a straight line (Fig. 2). The differences in the values of $K_i$ calculated from the intersection of the curve on the axis $1/K_{islope}$ and from the intersection on the axis of fructose concentrations indicate that a second modified $\alpha K_i$ is involved [17]. Thus the inhibitory action of fructose may be ascribed to a case of inhibition through two interacting sites on the enzyme. In agreement with this interpretation the curve of saturation by fructose was sigmoidal (Fig. 3). Glucose (0.1 to 720 mM) was not an inhibitor of the enzyme. Chemicals used as glucose analogues such as $\alpha$- and $\beta$-methyl glucosides (0.1 to 200 mM) were not inhibitors.

The action of various chemicals on the enzyme are shown in Table 2. Melezitose, $\beta$-methyl fructoside and

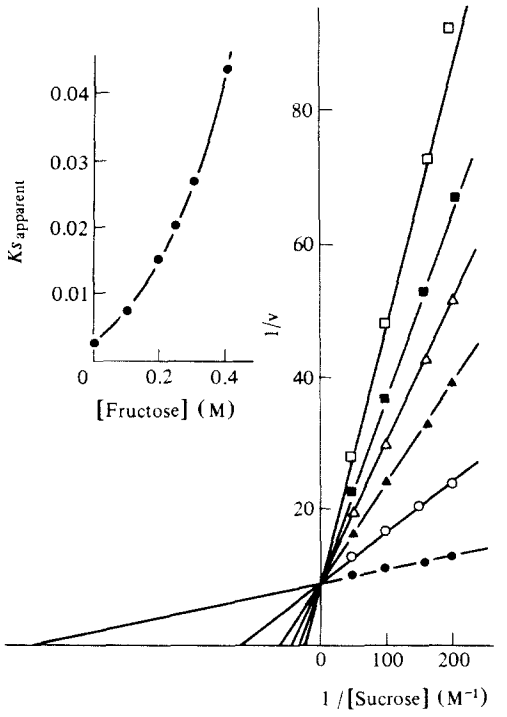


Fig. 1. Competitive inhibition of soluble acid invertase by fructose, and replots of $K_{sapp}$ against fructose concentrations. Reactions were run at 37° and at pH 4.5—●—, Without inhibitor; —○—, 0.1 M fructose; —▲—, 0.2 M fructose;—Δ—, 0.25 M fructose; —■—, 0.3 M fructose; and ——□——, 0.4 M fructose.

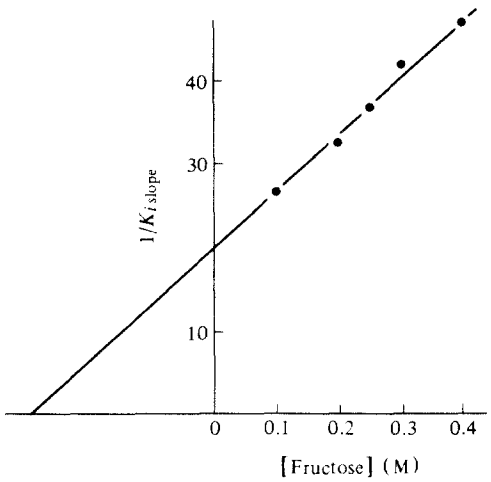


Fig. 2. Replot of $1/K_{islope}$ against fructose concentration. Values of $1/K_{islope}$ were those calculated from Fig. 1.

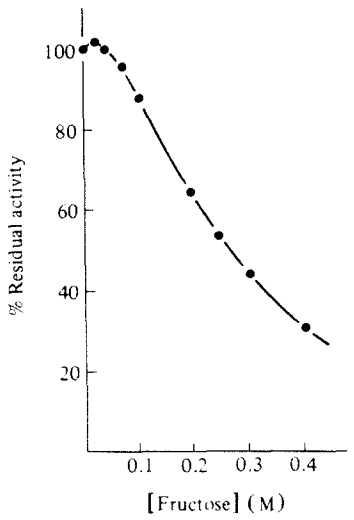


Fig. 3. Effect of fructose concentrations on the activity of the *C. papaya* invertase. A 20 mM concentration of sucrose was used. The reaction was performed as described in Experimental, except that enough fructose was added to give the concentration shown in the plot.

Table 2. Effect of chemicals on the activity of the invertase from *C. papaya*

| Chemical | Conc (mM) | Relative activity (%) | Chemical | Concentration (mM) | Relative activity (%) |
|---|---|---|---|---|---|
| Melezitose | 130 | 82 | $Hg^{2+}$ | 2.5 | 19.8 |
| β-Methyl Fructoside | 252 | 64.5 | $Hg^{2+}$ | 5.0 | 4.8 |
| Inulin | 0.6 | 84.5 | $Cu^{2+}$ | 2.5 | 87.9 |
| $K^+$ | 300 | 104 | $Co^{2+}$ | 50 | 67.3 |
| $Mg^{2+}$ | 5 | 116 | $Mn^{2+}$ | 5 | 74.8 |
| $Sr^{2+}$ | 2.5 | 104 | Tris | 50 | 74.2 |
| $Sr^{2+}$ | 7.5 | 125 | EDTA | 5 | 101 |
| $Ba^{2+}$ | 5 | 108 | $Mo_7O_{24}^{6-}$ | 5 | 2.8 |
| $Ba^{2+}$ | 7.5 | 126 | Urea | 10 | 93.2 |
| $Zn^{2+}$ | 10 | 84.2 | PCMB | 0.25 | 11.5 |
| Pyridoxal | 10 | 42.5 | Pyridoxine | 10 | 47.3 |
| Pyridoxamine | 10 | 77.8 | Pyridine | 10 | 84.8 |

inulin were inhibitors at relatively high concentrations. Some cations such as $Mg^{2+}$, $Sr^{2+}$, and $Ba^{2+}$ were activators. Others such as $Zn^{2+}$, $Cu^{2+}$, $Co^{2+}$, and $Mn^{2+}$ were inhibitors. General inhibitors of the invertases such as pyridoxal, pyridoxine, pyridoxamine, pyridine [18] and heptamolybdate [19] were also inhibitors.

The enzyme is very labile at its optimum pH, even at 0° (Fig. 4). The lability of the enzyme is not pH dependent for values between 3 and 7.

The enzyme attacks raffinose, stachyose and β-methyl fructofuranoside (Table 3). Paper chromatography of the products of raffinose hydrolysis showed galactose, sucrose, melibiose, fructose and glucose. The use of melibiose as substrate confirmed the presence of a small amount of α-galactosidase activity.

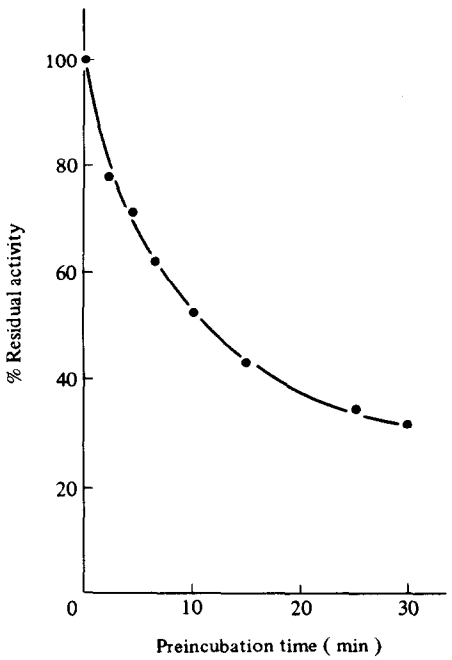


Fig. 4. Stability of the enzyme. The incubation mixtures were performed and run as described in *Enzyme assays* except that the enzyme was previously preincubated with the buffer at 0–4° for the times shown in the plot.

Table 3. Specificity of the enzyme extract

| Substrate | Concentration (mM) | Relative activity (%) |
|---|---|---|
| Sucrose | 60 | 100 |
| Raffinose | 60 | 45 |
| Stachyose | 60 | 20 |
| β-Methyl fructoside | 336 | 14.7 |
| Inulin | 0.3% | 0.0 |
| Melezitose | 60 | 0.0 |
| Trehalose | 60 | 0.0 |
| Turanose | 1.2 | 0.0 |
| α-Methyl-glucoside | 60 | 0.0 |
| β-Methyl-glucoside | 60 | 0.0 |
| Cellobiose | 1.2 | 0.0 |
| Maltose | 1.2 | 0.0 |
| Lactose | 1.2 | 0.0 |
| Melibiose | 1.2 | 3.1 |

Proteins were activators of the *C. papaya* invertase. Bovine serum albumin produced a 35% activation in a saturable fashion. A maximal activity of 0.046 invertase units was attained at $6.5 \times 10^{-4}$ mM bovine serum albumin. The synthetic polymer 2% PVP K 30, which causes activation of the *R. communis* invertase [15], is also an activator of the enzyme from *C. papaya* (22% of activation).

The $M_r$ of the invertase was found to be 52 000.

## DISCUSSION

The invertase isolated from mature-green fruits of *C. papaya* is a β-fructofuranosidase which hydrolyses sucrose as the preferential substrate. The proportionality between enzyme concentration and sucrose hydrolysis

appears to discount the presence of dissociable inhibitors or activators in the preparations.

Fructose is an inhibitor of the *C. papaya* invertase. This inhibition by fructose fits well with a case of inhibition at two interacting sites [17]. This inhibition is not suppressed by BSA, thus fructose not only shows a complex mechanism of inhibition (*in vitro*) but this mechanism probably is also working *in vivo* at difference of the inhibition of *R. communis* invertase which is suppressed by proteins [15]. Glucose, the other reaction product, is not an inhibitor of the enzyme. The same result is obtained when glucose is replaced by α-and β-methyl glucosides. In consequence this enzyme system is closer to that of the sugar cane leaf sheath whose invertase activity is modulated by fructose [14]. A difference between the *C. papaya* and sugar cane leaf sheath invertase is the inhibition of the latter enzyme by glucose. However, *C. papaya* invertase could not be purified to homogeneity, and the preparation contained contaminating proteins. Proteins were shown to be suppressors of the inhibitory effect of glucose in the case of *R. communis* invertase, and consequently the failure of the sugar to inhibit the invertase of *C. papaya* may be important.

In a previous study [20], we tentatively classified higher plant invertases into two different groups on the basis of the results obtained in our laboratory i.e. those whose activity is modulated by fructose as exemplified by the invertases from sugar cane leaf sheaths [14] and *C. papaya* fruits, and those whose activity is not inhibited by fructose in the presence of protein as expemplified by the enzymes from *R. communis* [15] and *T. majus* [20] leaves. The general lack of information about higher plant invertases does not permit further appreciation of these enzyme systems, however the regulation of the hydrolytic flux of sucrose *in vivo* may be related to the behaviour of the respective invertases towards fructose and proteins.

## EXPERIMENTAL

*Plant material.* Mature-green fruits of *Carica papaya* were selected according to the classification of Birth *et al.* [21].

*Enzyme preparation. C. papaya* fruits were peeled and cut in pieces. After removal of the seeds, 700 g of tissue were homogenized in 175 ml 50 mM Na-Pi buffer, pH 7.5, containing 5 μM $MnSO_4$, 1 mM 2-mercaptoethanol, 5% glycerol and 50 mM NaCl (buffer A). The homogenate was filtered through two layers of cheesecloth and centrifuged at 19400 *g* for 10 min. The supernatant was the 'homogenate' of Table 1 (Procedure I). An other 700 g of tissue was homogenized as described, but using buffer A saturated with $(NH_4)_2SO_4$ (Procedure II). The homogenate was filtered through two layers of cheesecloth and centrifuged. The supernatant was saturated again with solid $(NH_4)_2SO_4$ and centrifuged for 10 min at 19400 *g*. The ppt. was resuspended in 2 ml 10 mM NaOAc buffer, pH 4.5, containing 50 mM NaCl, 5% glycerol and 1 mM 2-mercaptoethanol (buffer B) and dialysed against the same buffer. The suspension was centrifuged for 10 min at 19400 *g*. Fractions of 4 ml of the supernatant were filtered through a 25 × 400 mm column of Sephadex G-75, equilibrated and eluted with buffer B. Fractions eluted between 74 and 86 ml were pooled and kept at −20°.

*Enzyme assays.* Incubation mixtures contained 20 μl enzyme, 20 μl 0.6 M sucrose, 60 μl 0.2 M NaOAc buffer, pH 4.5, and dist. $H_2O$ or effector soln in a final vol. of 200 μl. Incubations were performed at 37°, and the reactions were stopped by a Cu alkaline reagent [22]. Reducing power was measured by the method ref. [23].

*Enzyme kinetics.* Reaction mixtures contained 20 μl enzyme, 60 μl 0.2 M NaOAc buffer, pH 4.5, 20–50 μl 0.025–0.1 M sucrose and 10–40 μl 1.25–2 M fructose or 10–60 μl 2.4 M glucose, in a final vol. of 200 μl. Fructose was determined by means of the fructose dehydrogenase assay procedure [24] and glucose by the glucose oxidase assay procedure [25].

*Protein determinations.* Proteins were determined by the method of ref.[26] using BSA as standard.

$M_r$ *determinations.* These were performed by the method of ref. [27] using a 25 × 420 mm column of Sephadex G-150 equilibrated and eluted with buffer B. Trypsin ($M_r$ 23800), β-lactoglobulin (35830), BSA (67000) and aldolase (158000) were used as standards.

*Acrylamide gel electrophoresis.* Vertical gel electrophoresis was performed according to the Ornstein [28] and Davis [29] procedure. Proteins were stained with Coomassie Brilliant Blue R 250 [30], and invertase activity with 2,3,5-triphenyltetrazolium chloride [16].

*Invertase units.* One unit of the enzyme was defined as the mg of protein which yields 1 μmol of product/min at 37° and at pH 4.5.

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