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PURIFICATION AND PROPERTIES OF THE SOLUBLE ACID INVERTASE FROM *ORYZA SATIVA*

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Key Word Index—*Oryza sativa*; Gramineae; rice; invertase; fructose inhibition; protein activation.

Abstract—An invertase (β-fructofuranosidase, EC 3.2.1.26) from aerial parts of 17-day-old *Oryza sativa* plants has been purified to homogeneity by gel filtration on Sephadex G-150 and adsorption on brushite. The invertase is a dimeric glycoprotein (M_r 98 000) composed of two identical subunits (M_r 46 000). The pI is *ca* 6 and the activation energy is 8250 cal mol^{-1} over 30° and 24 000 cal mol^{-1} below this temperature. The enzyme shows a high specificity for sucrose (K_m 6.6×10^{-3}M) and its activity is modulated by fructose. Glucose is a classical non-competitive inhibitor and fructose produces a complex non-linear competitive inhibition. Proteins are activators of the invertase, but they do not suppress the inhibitory action of the reaction products. The *in vitro* spontaneous complex formation among invertases and proteins in higher plants suggests that invertases are functioning complexed with some protein *in vivo*.

INTRODUCTION

Higher plant invertases may be separated into two groups. In one group invertases are inhibited in a simple competitive fashion by fructose [1, 2] and in the other group the enzyme exhibits a complex competitive inhibition by fructose [3, 4]. Invertases from several higher plants, independently from the proposed enzyme group they belong to [1–4], are activated by proteins. However, protein activation of the group of invertases that shows simple inhibition kinetics by products produces a suppression of this inhibition. This is not the case for the complex inhibition kinetics group.

In this paper, we began the study of the invertase from *Oryza sativa*. The enzyme was purified to homogeneity for the first time and characterized. This invertase is classified into the group of complex inhibition kinetics by fructose as was described for another gramineous plant, sugar cane [3]. It was activated by proteins, differing from the fungi invertases [5] where proteins have no effect. Invertase activity was studied in relation to the binding properties to proteins and glycoproteins (lectins).

RESULTS AND DISCUSSION

Invertase purification

A soluble invertase from the aerial parts of 17-day-old plants of *O. sativa* was purified to homogeneity by homogenization, saline precipitation, gel filtration through Sephadex G-150 and adsorption chromatography on a brushite column (Table 1). Further purification by gel filtration (Sephadex G-150 and Sepharose 4B) and ion exchange chromatography (DEAE-Sepharose CL-6B), produced a single protein peak with invertase activity (not shown). The invertase gives a single protein band with invertase activity after polyacrylamide gel electrophoresis that was coincident with the protein band. Proteins were revealed with the silver nitrate method.

M_r *determinations*

Purified native enzyme gave an apparent M_r of 98 000 by gel filtration [6] (Fig. 1A). SDS–PAGE in the presence of 2-mercaptoethanol [7] showed only one protein band with an M_r of 46 000 (Fig. 1B). According to these results the invertase molecule is a dimer consisting of two similar subunits. Determination by the method of POA [8] gave an M_r of 45 900. Consequently, in this method the enzyme is dissociated into subunits.

Structural sugars

A sugar content of 15.3% (w/w) was determined in the invertase from *O. sativa* by the phenol–sulphuric acid method [9] (not shown). Thus, the invertase from *O. sativa* is a glycoprotein, like the invertases from other organisms [1, 5].

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Table 1. Purification of the invertase from *Oryza sativa*

Fraction	Total E.U.	Total protein (mg)	Specific activity	Purification	Yield %
Homogenate	600	7000	0.086	1	100
Centrifugation	480	6150	0.078	0.91	80
$(NH_4)_2SO_4$ ppt.	280	1800	0.156	1.8	47
Sephadex G-150 filtration	210	130	1.61	18.8	35
Brushite chromatography	120	9.37	12.8	149	20

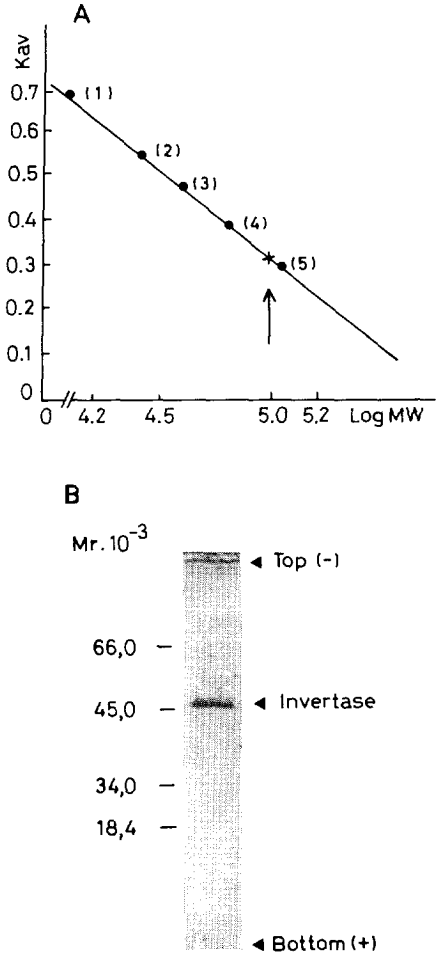

Fig. 1. (A) Gel filtration of soluble invertase from *O. sativa* on Sephadex G-150. The M_r markers used were: 1. ribonuclease A (13700), 2. chymotrypsin (25000), 3. ovalbumin (45000), 4. BSA (66000), 5. alkaline phosphatase (100000). (B) Subunit M_r determination on SDS–PAGE. Experimental conditions are described in the text.

Isoelectric point determinations

The purified invertase yielded a single protein band with an isoelectric point near 6.0 ± 1.0 ($n = 5$). This band was coincident with the activity band revealed as described in the Experimental.

Effect of pH

An optimum pH of *ca* 4.5 was determined. The enzyme shows activity in a range of pH from 3 to 7.5.

Effect of substrate concentration

The enzyme hydrolyses sucrose (K_m 6.6×10^{-3} M), raffinose (K_m 2×10^{-2} M) and stachyose (K_m 6.6×10^{-2} M) (not shown). The preferred substrate is sucrose.

Invertase specificity

The invertase was not able to hydrolyse 10–60 mM α-methylglucoside, trehalose, maltose, cellobiose, turanose and melibiose, 10–120 mM β-methylfructoside, 3% (w/v) inulin or 1% (w/v) levan (not shown).

Effect of the reaction products

The invertases from higher plants can be ascribed to two types of inhibitory mechanism by fructose: a simple competitive inhibition and a competitive non-linear inhibition. In the present case fructose was a competitive inhibitor of the enzyme activity (Fig. 2). Replots of $K_{s\ app}$ against fructose concentration gave a curve showing that fructose is not a simple competitive inhibitor. However, a replot of $1/K_{i\ slope}$ against fructose concentration is a straight line (Fig. 3). The differences in the values of K_i (57 mM) calculated from the intersection of the curve on the $1/K_{i\ slope}$ axis and from the intersection on the fructose concentration axis indicate that a second modified αK_i (65 mM) is involved. The inhibition by fructose may be ascribed to the case of inhibition through two interacting sites on the enzyme [10]. The plot of per cent activity against fructose concentration in these cases is sigmoidal (Fig. 3). Glucose was a classical non-competitive inhibitor, K_i 78 mM (Fig. 4). These results indicate that the invertase from *O. sativa* belongs to the second group of higher plant invertases.

Protein and lectin effect

A conspicuous effect of proteins on higher plant invertases is the activation of these enzymes. Table 2 shows the activating effect of various proteins on the *O. sativa* invertase. The amount of protein necessary for saturating

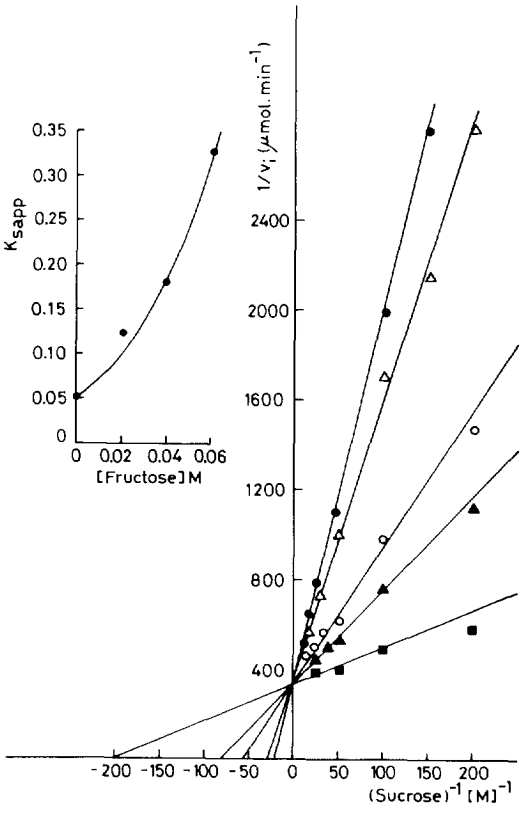

Fig. 2. Double reciprocal plot of initial velocity against sucrose concentration in the presence of various concentrations of fructose. Reactions were run at 37° and pH 4.5. —■— without fructose; —▲— 20 mM fructose; —○— 40 mM fructose; —△— 60 mM fructose and —●— 80 mM fructose. Insert: plot of $K_{s\ app}$ vs fructose concentration.

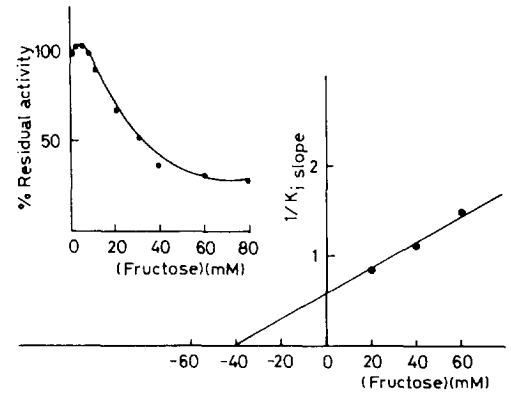

Fig. 3. Replot of $1/K_{i\ slope}$ calculated from the double reciprocal plot of initial velocity against sucrose concentration. Insert: per cent invertase activity vs fructose concentration.

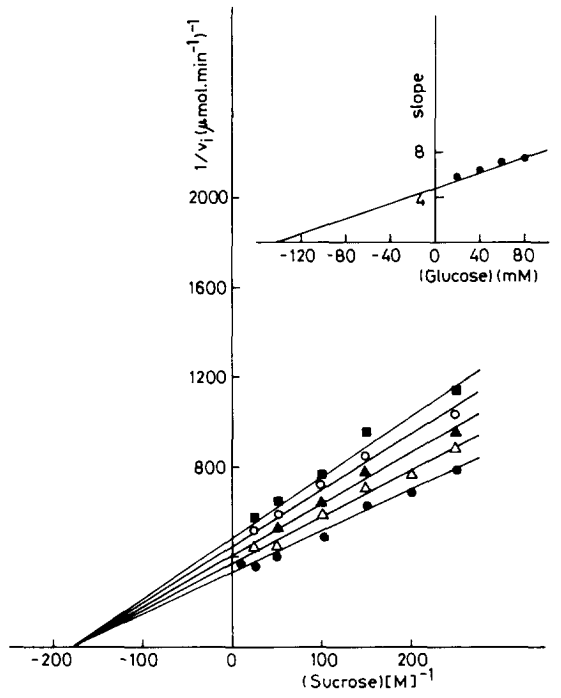

Fig. 4. Double reciprocal plot of initial velocity against sucrose concentration in the presence of various concentrations of glucose. Reactions were run at 37° and pH 4.5. —●— without glucose; —△— 20 mM glucose; —▲— 40 mM glucose; —○— 60 mM glucose and —■— 80 mM glucose. Insert: plot of slope vs glucose concentration.

Table 2. Protein and lectin effect on the *O. sativa* invertase

Protein	POA* (M)	Invertase units	% Activation
Control	—	0.0072	—
BSA	3×10^{-6}	0.0090	25
Alkaline phosphatase	4×10^{-6}	0.0097	35
Urease	1.2×10^{-7}	0.0101	41
Concanavaline A	1.0×10^{-7}	0.0144	100
Lens culinaris agg.	3.1×10^{-7}	0.0183	154
Ricin	2.1×10^{-7}	0.0190	163

*Point of optimal activation (POA): minimal concentration of effector that produces the maximal enzyme activation.

Assay mixtures were not preincubated.

the enzyme depends on the nature of each protein. This behaviour is common to most of the plant invertases [2–4]. Though this effect was found *in vitro*, it seems to be important for *in vivo* invertase activity. In fact, proteins suppress the inhibitory effect of the reaction products in plants belonging to the first group while they have no action on the invertase inhibitory effect of glucose and fructose in the second group of plants e.g. *O. sativa*. Many proteins, including soluble enzymes are present in vacuoles [11–13], the localization site of invertases

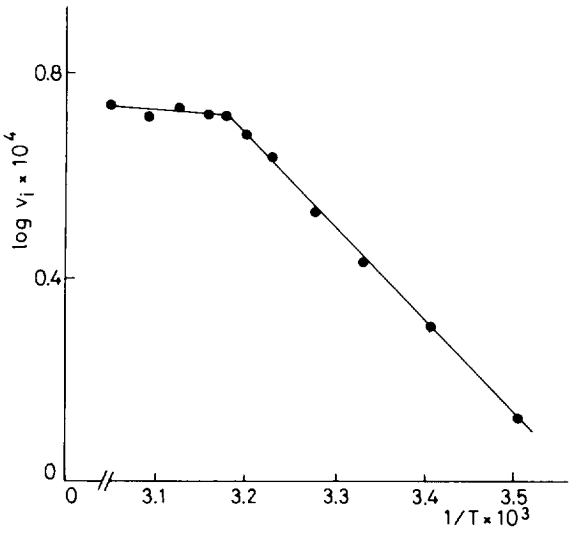

Fig. 5. Arrhenius plot for the invertase reaction. An inflection point was observed at 30°. The activation energy, E_a, was calculated from the slope of the plot of log v_i as a function of 1/T of the Arrhenius equation (log $v_i = C - 1/T \log E_a/R$).

[14–16], and protein–invertase interaction can therefore take place *in vivo*. Lectins were found to be better activators of invertase activity than other proteins, as occurs with *R. communis* leaf invertase [17]. It was suggested that this activation is a consequence of protein–protein and protein–carbohydrate interactions between the lectin and the invertase [8], taking into account the glycoprotein nature of invertases. However, lectins may be inhibitors of some higher plant invertases e.g. those from *Solanum tuberosum* tubers [18]. Fungal invertases appear not to be activated by proteins. Moreover, the invertase product inhibitions are not suppressed by proteins, nor were protein–invertase complexes detected as is the case of *Schizophylum communis* and *Pycnoporus sanguineous* invertases [5, Quiroga, E. N., Vattuone, M. A. and Sampietro, A. R., personal communication]. Consequently, protein activation appears to be a typical property of higher plant invertases. Higher plant acid invertases bind spontaneously to proteins, *in vitro*, suggesting that a protein-bound enzyme is present under physiological conditions.

Energy of activation

The temperature effect on the invertase reaction rate (Arrhenius plot) shows an inflection point at 30° (Fig. 5). The activation energy was calculated to be 8250 cal mol^{-1} over 30° and 24 000 cal mol^{-1} below this temperature. This behaviour has also been observed for sugar cane leaf sheath invertase [3].

EXPERIMENTAL

Plant material. Seeds of *O. sativa* var. Blue Bell were sown in vermiculite irrigated with 1/8 (v/v) diluted Hoagland's soln. They were submitted to 12 hr light-dark periods. After 17 days the aerial parts were gathered and frozen in liquid N_2.

Invertase preparation. The frozen plants (without roots, 200 g) were homogenized in 300 ml of 50 mM NaPi buffer, pH 7.5, containing 1 mM 2-mercaptoethanol, 5 μM $MnSO_4$ and 0.5 M NaCl. The homogenate was filtered through 2 layers of gauze and centrifuged at 27 000 *g* for 15 min. The supernatant was satd with solid $(NH_4)_2SO_4$ and after 30 min the prepn was centrifuged at 27 000 *g* for 15 min. The ppt. was resuspended in 10 ml of 10 mM NaOAc buffer, pH 4.5, containing 50 mM NaCl and 1 mM 2-mercaptoethanol (buffer B), and was dialysed against the same buffer for 2 hr. The prepn was centrifuged at 27 000 *g* for 15 min. Frs of 4 ml of this were filtered through a 20 × 400 mm column of Sephadex G-150 equilibrated and eluted with buffer B. Frs eluted between 125 and 150 ml were pooled and kept at −20°. The invertase was adsorbed in a 25 × 100 mm brushite column and eluted with 100 ml of a linear gradient of 10–500 mM of NaPi, pH 5.5. Frs of 2.5 ml were collected and those with invertase activity were pooled. The prepn was filtered through a 12 × 50 mm Bio-Gel P-6 column equilibrated and eluted with buffer B.

Protein determinations. Proteins were determined by the method of ref. [19] using BSA as standard.

Acrylamide gel electrophoresis. Vertical gel electrophoresis was performed according to refs. [20, 21]. Proteins were stained with Coomassie Brilliant Blue R-250 and with the $AgNO_3$ method. Invertase activity was revealed with 2,3,5-triphenyltetrazolium chloride [22].

M_r *determination.* The M_r of the native invertase was determined by the method of ref. [6] using a 25 × 450 mm column of Sephadex G-150 equilibrated and eluted with buffer B.

SDS–PAGE. Samples (2 μg) of invertase were treated and electrophoresed as described in ref. [7]. BSA (66 000), ovalbumin (45 000), pepsin (34 000) and β-lactoglobulin (18 400) were used as standards.

Enzyme assays. Incubation mixts contained 20 μl of enzyme, 20 μl 0.6 M sucrose, 40 μl 0.2 M NaOAc buffer, pH 4.5 and distilled H_2O or effector soln in a final vol. of 100 μl. Incubations were performed at 37°, and the reactions were stopped by the Cu alkaline reagent [23]. Reducing power was measured by the method of ref. [24].

Product inhibition kinetics. Reactions mixts contained 20 μl of enzyme, 10 μl of 0.33–1.2 M sucrose, 10 μl of 0.1–0.6 M fructose or 0.05–1 M glucose, 40 μl of NaOAc buffer, pH 4.5 and distilled H_2O in a final vol. of 100 μl. Incubations were performed at 37° by the desired time. Fructose was determined by the fructose dehydrogenase assay procedure [25] and glucose by the glucose oxidase method [26].

Invertase units. One enzyme unit was defined as the amount of enzyme which yields 1 μmol of product min^{-1} at 37° and at pH 4.5.

Isoelectric focusing. Polyacrylamide gel (5%) containing Pharmacia Fine Chemicals ampholytes in the range of pH 3–9 and pI marker proteins from Bio-Rad in the range 4.6–9 were used. A sample containing 30 μg of protein in 25 μl was charged and the focusing was done at

400 V for 1 hr. The gels were stained with Coomassie Brilliant Blue R-250. Activity was revealed with 2,3,5-triphenyltetrazolium chloride [22].

Determination of total sugars. Total sugars were determined by the method of ref. [9].

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