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Purification and characterization of an alkaline invertase from shoots of etiolated rice seedlings

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SUMMARY

One alkaline invertase and two acid invertase activities were detected in the shoots of etiolated rice (*Oryza sativa*) seedlings. The alkaline invertase (AIT) was purified to homogeneity through steps of ammonium sulphate fractionation, concanavalin A-Sepharose affinity chromatography (non-retained), DEAE-Sephacel chromatography and preparative electrophoresis. The pH optimum of AIT was 7.0 and the molecular mass, determined by gel filtration, was 240 kDa. It is apparently a homotetrameric enzyme (subunit molecular mass 60 kDa). The isoelectric point was 4.4 by isoelectric focusing. The best substrate of the enzyme was sucrose, with a K_m of 2.53 mM. The enzyme also hydrolysed raffinose, but not maltose or lactose, so it is a β-D-fructofuranosidase. It gave negative glycoprotein staining. Of the hydrolysis products, fructose was a competitive inhibitor and glucose was a non-competitive inhibitor. Treatment with an alkaline phosphatase could activate AIT, whereas other proteins such as BSA, concanavalin A and urease had no effect on the enzyme activity. The enzyme activity was inhibited by Tris, thiol reagents and heavy metal ions.

Key words: *Oryza sativa* (rice), shoots, alkaline invertase, purification, characterization.

INTRODUCTION

Sucrose is one of the predominant initial products of photosynthesis and serves as the major form of carbohydrate translocation in higher plants. Invertase (β-D-fructofuranosidase, EC 3.2.1.26) catalyses the hydrolysis of sucrose into D-glucose and D-fructose, the main forms of carbon and energy supply in plant metabolism. Plant invertases include a variety of forms that can be categorized in terms of solubility, optimum pH, isoelectric point (pI) and subcellular localization (Sturm & Chrispeels, 1990). They are classified as acid and alkaline/neutral invertases according to the optimum pH of catalysis (Copeland, 1990; Pollock & Lloyd, 1977). Soluble acid invertases have been found mainly in the vacuole and apoplast of plant tissues (Fahrendorf & Beck, 1990; Krishnan *et al.*, 1985; Masuda *et al.*, 1988). The enzyme activities seem to be correlated with the growth and differentiation of certain plant tissues, particularly with organ elongation and cell enlargement (Doehlert & Felker, 1987; Lopez *et al.*, 1988; Masuda & Sugawara, 1980; Pollock & Lloyd, 1977; Schaffer, 1986). The alkaline invertase might be present exclusively in the cytoplasm of mature tissues (Ricardo, 1974), and coexist with acid invertase in some tissues (Fay & Ghorbel, 1983; Masuda *et al.*, 1988). The enzyme might regulate hexose and sucrose concentrations in cytoplasm (Hatch & Glasziou, 1963; Masuda *et al.*, 1988; Ricardo & ap Rees, 1970). We have found one alkaline/neutral invertase and two acid invertases in the shoots of etiolated rice seedlings. Here we describe the purification to apparent homogeneity, and characterization, of the alkaline invertase.

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MATERIALS AND METHODS

Plant materials and reagents

Rice (*Oryza sativa*, cv. Tainong no. 67) was grown in the field at the experimental farm of the National

Taiwan University during the summer of 1993. Rice seeds were sterilized with a dilute solution of sodium hypochlorite and soaked in water at 30°C for 24 h. Seeds with emerged radicals 2–4 mm long were selected and placed on a bed of net in trays and incubated in darkness at 30°C. On the 12th day after germination, shoots were dissected and stored at −70°C until use. Chemicals and enzymes for the coupling assay of invertase activity were purchased from Sigma and Boehringer. Reagents for PAGE were purchased from Bio-Rad. Concanavalin A (Con A)-Sepharose and DEAE-Sephacel were obtained from Pharmacia. All chemicals were of reagent grade.

Extraction and purification of enzyme

Unless stated otherwise, all procedures were conducted at or near 4°C. Frozen shoots (150 g) were finely ground with a pestle in a mortar prechilled with liquid nitrogen. Buffer A (600 ml; 50 mM sodium phosphate containing 1 mM 2-mercaptoethanol, 1 mM EDTA, 0.5 mM phenylmethanesulphonyl fluoride and 1 mM benzamidine, pH 7.0) were added and the slurry was filtered through four layers of cheesecloth. The filtrate was centrifuged at 6000 ***g*** for 30 min. Protamine sulphate was added to the supernatant to a final concentration of 0.25 mg ml^{-1} to remove nucleic acids. After centrifugation, proteins in the supernatant were fractionated by the addition of solid ammonium sulphate. Precipitates were cut at 10% intervals from 5% to 85% of salt saturation. The bulk of enzyme activity was precipitated in between 25% and 45% saturation. The precipitate was collected by centrifugation (10000 ***g***, 15 min) and dissolved in buffer B (buffer A containing 0.5 M NaCl), then dialysed against three changes of 4 l aliquots of the same buffer every 4 h. The enzyme solution was loaded on a Con A-Sepharose column (1.6 cm × 10 cm) pre-equilibrated with buffer B. This step separated the alkaline invertase (not retained) from the acid invertases (retained). The acid invertases were eluted with a linear gradient of 0–0.3 M α-methyl-D-mannoside in buffer B in a total volume of 300 ml; 5 ml fractions were collected. The fractions containing invertase activity were pooled and concentrated by ultrafiltration with an Amicon YM-10 membrane. The alkaline invertase (non-retained fraction) was loaded on a DEAE-Sephacel column (2.6 × 14.0 cm) pre-equilibrated with buffer A. The column was washed with buffer A at a flow rate of 25 ml h^{-1} until A_{280} decreased to a steady level. The enzyme was eluted with a linear gradient of 0–0.5 M NaCl in buffer A in a total volume of 400 ml; 5 ml fractions were collected. The activity-containing fractions were pooled and concentrated by ultrafiltration (Amicon YM-10) to 1.5 ml and dialysed against buffer A. The dialysed solution was loaded on a preparative PAGE (7.5%). After electrophoresis the gel with invertase activity was sliced out and put in a ISCO Model 1750 electrophoretic concentrator to elute the enzyme.

Invertase assay

Invertases were assayed in a 0.36 ml mixture of 0.1 M sucrose in either 50 mM sodium phosphate (pH 7.0) for alkaline invertase or 100 mM sodium acetate (pH 5.0) for acid invertase. The reaction was performed at 37°C for 10 min and the amount of reducing sugar was measured by the Somogyi–Nelson method (Nelson, 1944). A standard curve was established for an equimolar mixture of glucose and fructose.

Protein determination

Protein concentration was determined by the method of Bradford (1976) with BSA as standard. The amount of protein in chromatographic fractions was measured by A_{280}.

Inhibition kinetics

In a total volume of 0.36 ml, the reaction mixture contained 60 µl of enzyme solution, 50 mM sodium phosphate (pH 7.0) and 0.025–0.20 M sucrose, together with 60 µl of 0.12–0.48 M fructose, 0.12–0.60 M glucose or 0.012–0.12 M Tris. Incubations were at 37°C. The amount of glucose released was determined by the glucose oxidase method (Ebell, 1969).

Determination of molecular mass

The molecular mass of the enzyme was estimated on a calibrated Zorbax GF-450 column, on which 20 µg of purified enzyme, together with the molecular mass standard proteins (20 µl), were loaded. Fractions (0.2 ml) were collected at a flow rate of 0.4 ml min^{-1} and the column void volume was measured with Blue dextran. The enzyme activity and A_{280} in each fraction were measured and elution volumes for marker proteins and the enzyme were calculated. For N-terminal sequencing, alkaline invertase was electroblotted on an Immobilon poly(vinylidene difluoride) membrane (Millipore IPVH 000 10) and sequenced by Edman degradation.

Gel electrophoresis and isoelectric focusing

SDS-PAGE and native PAGE were performed by the method of Laemmli (1970). For isoelectric focusing, the procedure of the instruction manual of Pharmacia Ampholine PAGplate was followed. The

Pharmacia gel plate containing Ampholine in the pH range 4.0–6.5 was used. Proteins were stained with either Coomassie Blue R-250 or silver staining kit (for protein).

Glycoprotein staining

Glycoprotein staining of SDS-PAGE gels was performed by the periodic acid-Schiff method (Segrest & Jackson, 1973). Two SDS-PAGE gels were run in parallel in a Hoefer vertical mini-apparatus. One was stained with Coomassie Blue R-250 and the other soaked overnight in 100 ml of a fixing solution (40% (v/v) ethanol and 5% (v/v) acetic acid). The fixed gel was put in a 0.7% periodic acid solution for 2–3 h, then transferred to 0.2% sodium metabisulphite. The metabisulphite treatment continued for 2–3 h with one change of the solution after 30 min. The gels were destained with 5% (v/v) methanol and 7.5% (v/v) acetic acid until the background became clear, after which they were treated with Schiff's reagent for 12–18 h at room temperature, then stored at 4°C.

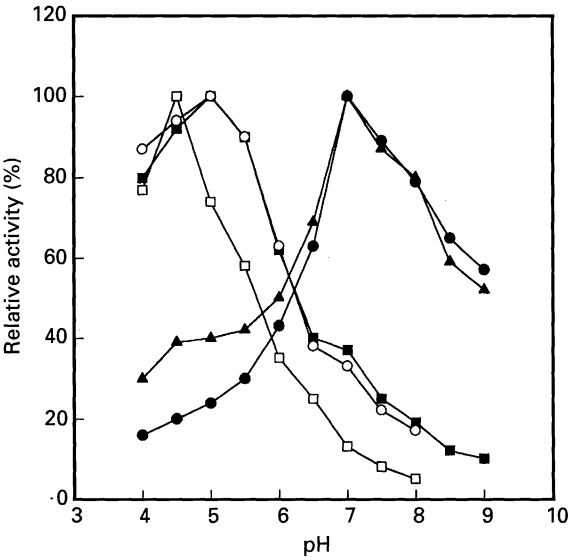

Fig. 1. The profiles of pH against activity of invertases from rice. Filled triangles, 25–45% ammonium sulphate precipitate; filled squares, 45–65% ammonium sulphate precipitate; filled circles, purified alkaline invertase; open circles, purified acid invertase II; open squares, purified acid invertase II. The buffer used included citric acid-Na_2HPO_4 (pH 4–6.5), NaH_2PO_4-Na_2HPO_4 (pH 6.5–8.0) and glycine-NaOH (pH 8.0–9.0). The reaction was performed at 37°C for 10 min.

RESULTS

Separation of alkaline invertase from acid invertases

Both alkaline and acid invertases were present in crude extracts from shoots of etiolated rice seedlings. The alkaline and acid invertase accounted for approx. 40% and 60%, respectively, of total activity in shoots 12 d old. Alkaline invertase could be separated from acid invertases by ammonium sulphate fractionation (Cooper & Greensheilds, 1964; Ricardo & ap Rees, 1970; Masuda *et al.*, 1987). The activity measured at pH 7.0 was precipitated mainly at 25–45% salt saturation, whereas the activity at pH 5.0 was concentrated in the precipitate of 45–65% saturation. Similar salting-out patterns were reported for other plant tissues such as young corn roots and soybean hypocotyls (Chen & Black, 1992). The optimum pH of the precipitate at 25–45% saturation was 7.0, whereas that from the precipitate at 45–65% saturation peaked at approx. 5.0. The final alkaline invertase preparation also had an optimum pH of 7.0 (Fig. 1).

Purification of alkaline invertase

The results of alkaline invertase purification are summarized in Table 1. The results of purification steps, ammonium sulphate fractionation, Con A-

Table 1. *The results of purification steps for alkaline invertase activity, starting from 150 g of shoots from etiolated rice seedlings*

Step	Total protein (mg)	Total activity (units)*	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	585.98	124.93	0.21	1.00	100
25–45% Ammonium sulphate fractionation	126.56	63.43	0.50	2.39	50.77
Con A–Sepharose (non-retained)	46.53	51.05	1.10	5.24	40.86
DEAE–Sephacel	5.66	39.44	6.97	33.19	31.57
Preparative electrophoresis	0.19	3.48	18.32	87.22	2.78

*One unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol of reducing sugar from sucrose per min at 37°C at pH 7.0

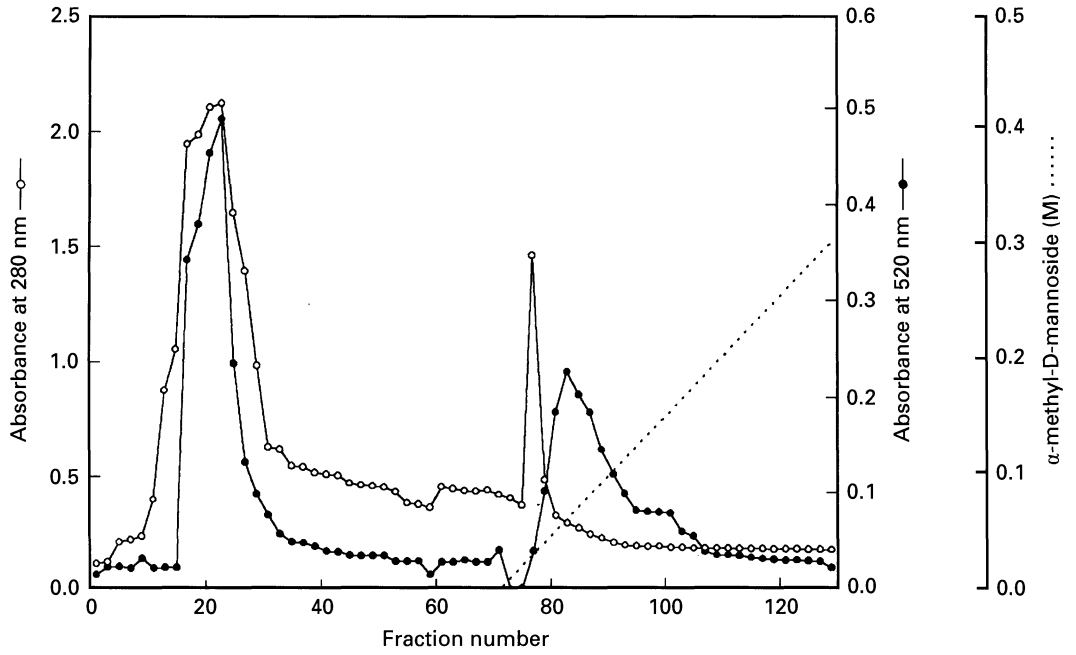

Fig. 2. Con A-Sepharose affinity chromatography of invertases from rice. The invertase activity was assayed at pH 7.0 (non-retained fraction) for alkaline invertase or at pH 5.0 (retained fraction) for acid invertase by the Somogyi–Nelson method (A_{520}). Protein content was monitored at 280 nm.

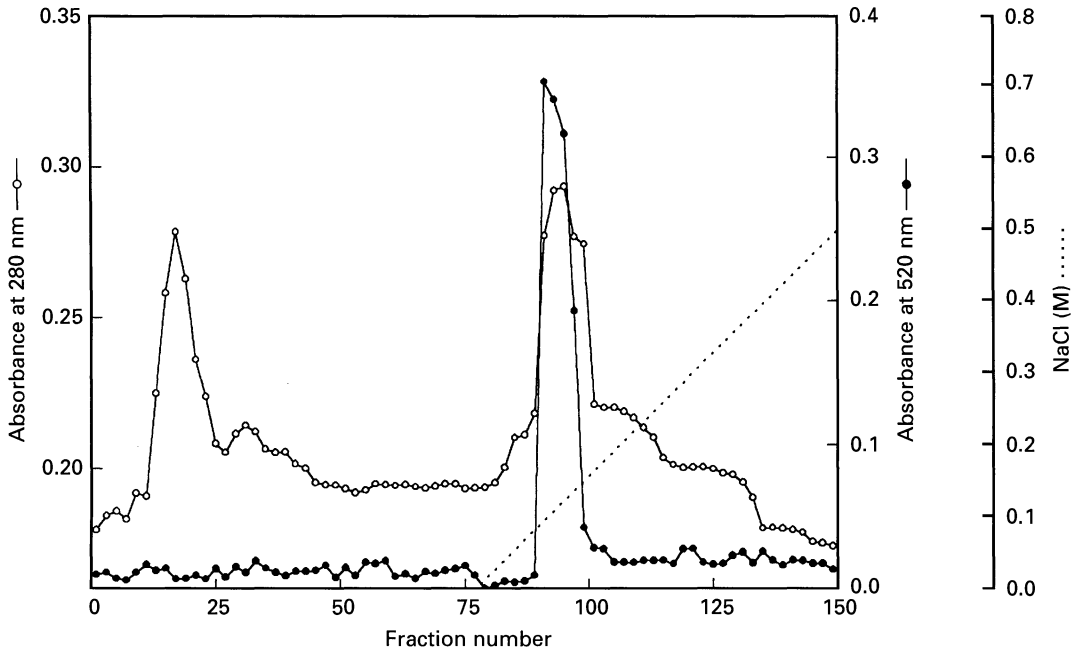

Fig. 3. DEAE-Sephacel chromatography of alkaline invertase from rice. The invertase activity was assayed by the Somogyi–Nelson method (A_{520}). Protein content was monitored by A_{280}.

Sepharose affinity chromatography and DEAE-Sephacel chromatography are presented in Figs 1, 2 and 3, respectively. The final step of preparative electrophoresis yielded an electrophoretically homogeneous preparation (Fig. 4). Its SDS-PAGE (Fig. 4a, lane 2) and native PAGE (Fig. 4b, lane 4) patterns are shown. Similarly to results obtained with other plant species, the rice alkaline invertase had no affinity towards Con A and thus could be separated from the Con A-binding acid forms (Fig. 2). DEAE-Sephacel chromatography was an effective step; a large number of proteins were not retained by the DEAE matrix, whereas alkaline invertase was eluted at 0.08–0.14 M NaCl.

Molecular mass

The molecular mass of the native enzyme was 240 kDa. In denaturing SDS-PAGE, it gave a single band of 60 kDa. Therefore it was thought to be a tetrameric enzyme. Its N-terminal amino acid sequence was Ser-Asp-Ser-Asp-Arg.

Kinetic properties and substrate specificity

The optimum pH was 7.0 (Fig. 1); the enzyme also had the greatest stability at pH 7.0. The thermal stability of the enzyme decreased as the incubation time and temperature increased. When the incubation temperature reached 40°C, it lost over 60 % of its activity. The enzyme responded to sucrose concentration in accordance with Michaelis-Menten kinetics; no substrate inhibition was shown even at 600 mM sucrose. The apparent K_m values for sucrose and raffinose were 2.53 and 4.59 mM, respectively. No hydrolytic activity towards maltose or lactose was detected. Hence, the alkaline invertase is a β-D-fructofuranosidase. The pI of the enzyme was 4.4 (results not shown).

Inhibition by products

Fructose and glucose were competitive and non-competitive inhibitors, with K_i values of 38 mM (Fig. 5a) and 72 mM (Fig. 5b), respectively. These results indicated that the enzyme activity could be modulated by its end-products.

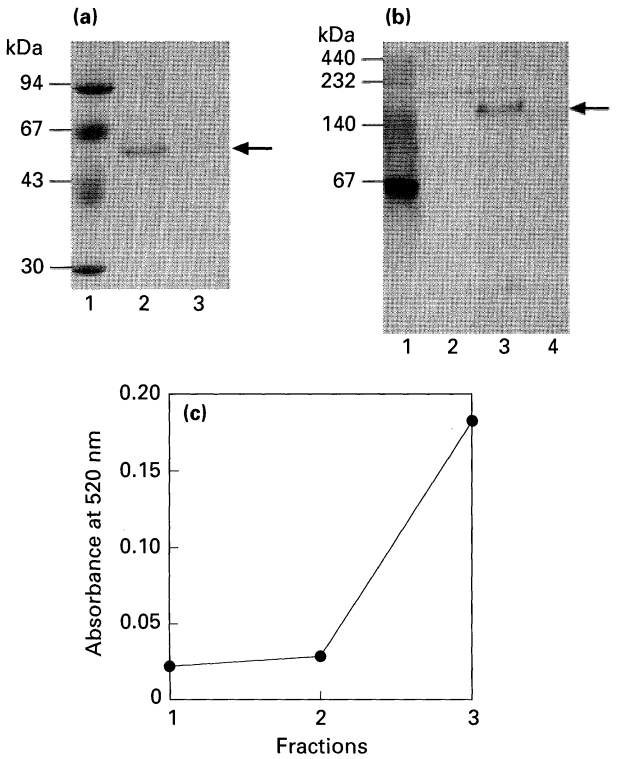

Fig. 4. (a) SDS-PAGE (7.5 %, m/v) pattern of alkaline invertase from rice purified by preparative electrophoresis. Coomassie Blue R-250 was used to reveal the protein. The arrow indicates the location of alkaline invertase. Lane 1, low-molecular-mass protein markers; lanes 2 and 3, fraction II of preparative electrophoresis. (b) Silver staining profiles of native PAGE (7.5 %, m/v). Lane 1, high-molecular-mass protein markers; lane 2, upper fraction of preparative electrophoresis; lane 3, fraction I of preparative electrophoresis; lane 4, fraction II of preparative electrophoresis. (c) Activity profile of preparative electrophoresis. Lane 1, upper fraction of preparative electrophoresis; lane 2, fraction I of preparative electrophoresis; lane 3, fraction II of preparative electrophoresis.

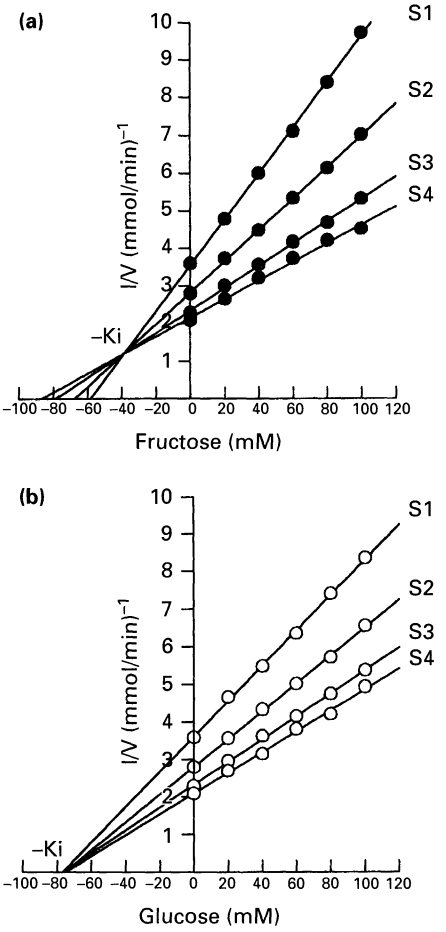

Fig. 5. Graphical determination (Dixon's method) of the K_i for (a) fructose and (b) glucose of rice alkaline invertase. Velocities were determined at sucrose concentrations of 25, 50, 100, and 200 mM (lines labelled S1, S2, S3 and S4, respectively).

Absence of glycoconjugates

Most purified plant acid invertases are glycoproteins (Copeland, 1990; Fahrendorf & Beck, 1990; Krishnan *et al.*, 1985), but the glycosylation status of alkaline invertase was uncertain. Similarly to the result from soybean, rice acid and alkaline invertases could be inferred as containing and lacking glycoconjugates, respectively, by their behaviour under Con A-Sepharose chromatography (Fig. 2). To obtain additional evidence, we performed glycoconjugate staining on PAGE plates of partly purified alkaline invertase along with two known glycoproteins (acid invertases from baker's yeast and *Candida utilis*) and one non-glycoprotein (BSA). Acid invertases were stained by the periodic acid-Schiff reagent (Fig. 6b, lanes 2 and 4), whereas BSA and the alkaline invertase were not (Fig. 6b, lanes 1 and 3). We therefore concluded that the alkaline invertase from rice is not a glycoprotein.

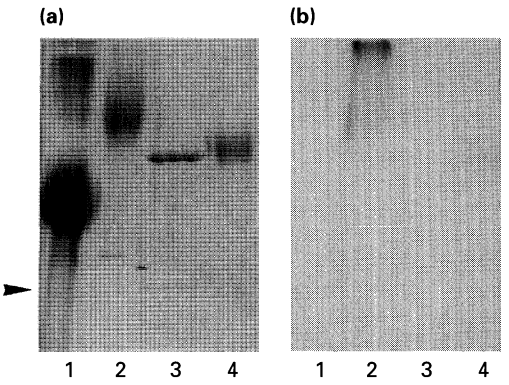

Fig. 6. Glycoprotein staining with rice alkaline invertase in comparison with Coomassie Blue R-250 staining. (a) SDS-PAGE (10%, m/v) analysis of partly purified alkaline invertase stained with Coomassie Blue R-250. Lane 1, BSA (non-glycoprotein); lane 2, acid invertase from baker's yeast (glycoprotein); lane 3, partly purified (DEAE-Sephacel step) alkaline invertase; lane 4, acid invertase from *Candida utilis* (glycoprotein). (b) The same gel as in (a) after staining for glycoprotein (periodic acid-Schiff method).

Effectors

The effects of metal ions and various chemicals on the activity of the alkaline invertase are shown in Table 2. Ca^{2+} and Mg^{2+} had no effect on activity, whereas heavy metal ions, especially Hg^{2+}, Cu^{2+} and Ag^{+}, decreased activity. Several reagents known to inhibit plant invertases (Karuppiah *et al.*, 1989; Lin & Sung, 1993; Charng *et al.*, 1994; Sung & Huang, 1994; Lee & Sturm, 1996) were tested (Table 2). At 5 mM, pyridoxine, pyridoxal, aniline and iodoacetamide decreased the activity to 40%, 58%, 30% and 65%, respectively. The serine proteinase inhibitor phenylmethanesulphonyl fluoride had little effect. These results indicated that acidic amino acids are important for the enzyme's catalytic activity and that the serine residue does not seem to participate directly in the catalysis. The enzyme activity was completely inhibited by $HgCl_2$ at 1 mM and was strongly inhibited by Tris at 20 mM with a K_i of 8 mM. Treatment with an alkaline phosphatase

Table 2. *Effects of metal ions and various chemicals on the activity of rice alkaline invertase*

Metal ions	Concentration (mM)	Relative activity (%)	Chemicals	Concentration (mM)	Relative activity (%)
None	–	100	None	–	100
Ca^{2+} ($CaCl_2$)	1	101	PMSF	0.01	95
	5	95		0.05	89
Mg^{2+} ($MgCl_2$)	1	100	Iodoacetamide	1	81
	5	108		5	65
Pb^{2+} ($Pb(OAc)_2$)	1	72	DTT	0.1	102
	5	30		1	125
Zn^{2+} ($Zn(OAc)_2$)	1	67	Aniline	1	52
	5	49		5	30
Cu^{2+} ($CuSO_4$)	1	40	Pyridoxine	1	76
	5	25		5	40
Hg^{2+} ($HgCl_2$)	1	2	Pyridoxal	1	90
	5	0		5	58
Ag^{+} ($AgNO_3$)	1	39			
	5	10			

Table 3. *Effects of some proteins on the activity of acid and alkaline invertases of rice*

Protein	POA (mM)		Maximal activation (%)	
	Acid invertase[a]	Alkaline invertase	Acid invertase[a]	Alkaline invertase
Control	–	–	–	–
Alkaline phosphatase	4.0	6.0	35	50
BSA	3.0	–	25	–
Urease	1.2	–	41	–
Con A	1.0	–	100	–

The point of optimal activation (POA) is the minimal concentration of effector that produces the maximal enzyme activation. –, no effect.
[a]Data from Isla *et al.*, 1995.

enhanced the activity, but other proteins (BSA, Con A and urease) did not. This indicates that no interaction between protein and alkaline invertase takes place *in vitro*. These results are different from those obtained with a rice acid invertase (Isla *et al.*, 1995), towards which proteins are activators (Table 3). The mechanism of activity enhancement by alkaline phosphatase is unknown and needs further study.

DISCUSSION

We have shown that the rice alkaline invertase is a tetrameric enzyme with a molecular mass of 240 kDa. This is similar to the enzyme from soybean hypocotyls (Morell & Copeland, 1984). The molecular masses of alkaline invertases from other plants are in the range 60–280 kDa (Copeland, 1990; Stommel & Simon, 1990; Chen & Black, 1992; Ross *et al.*, 1992; Van den Ende & Van Laere, 1995). The rice enzyme is smaller than carrot alkaline invertases. We therefore suspect that the monomeric form of the enzyme might be catalytically active. Its N-terminal amino acid sequence shows no similarity to the published sequences for acid invertases and alkaline/neutral invertase (Gallagher & Pollock, 1998). Recently we constructed a complementary DNA (cDNA) library from etiolated rice seedlings and used a specific probe containing the two conserved amino acid sequences of invertase found in other plant species to screen the cDNA library. We isolated three types of invertase cDNA; one type might be the alkaline invertase cDNA because its sequence shows no conserved active site (Trp-Glu-Cys-Val/Pro-Asp). It is similar to the first alkaline/neutral invertase from *Lolium temulentum* (Gallagher & Pollock, 1998).

The optimum pH (7.0) at which the rice enzyme is also most stable, agrees with those reported for alkaline invertases from various plants (Morell & Copeland, 1984; Schaffer, 1986; Chen & Black, 1992; Ross *et al.*, 1996). Most of the highly purified invertases, especially the acidic forms, are reported to be glycoproteins (Anderson & Ewing, 1978; Krishnan *et al.*, 1985; Copeland, 1990; Fahrendorf & Beck, 1990). Both the vacuolar and cell-wall-bound forms have an acid pH optimum in the range 4.0–5.5; they are acid invertases. Therefore the glycosylation of acid invertase synthesized in the cytoplasm would be necessary for its transport across either the tonoplast or the plasma membrane. The lack of glycoconjugate in the rice alkaline invertase is consistent with the tentative identification of its location in the cytoplasm (Ricardo & ap Rees, 1970).

The phenomenon of product inhibition for invertases has been known for a long time. It is well known that alkaline invertase participates in the regulation of hexose and sucrose levels in the cytoplasm. For example, an increase in the concentration of glucose and fructose due to a decreased demand for hexoses would decrease sucrose metabolism directly through the inhibition of invertases (Lee & Sturm, 1996). We found that fructose and glucose inhibited rice alkaline invertase. This finding is in support of the view that the enzyme is a regulatory agent of hexose and sucrose levels in plant cells. The inhibitory effects exerted by heavy metal ions, thiol reagents and Tris are in agreement with enzymes from other plants (Pollock & Lloyd, 1977; Chen & Black, 1992).

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REFERENCES

Anderson RS, Ewing EE. 1978. Partial purification of potato tuber invertase and its proteinaceous inhibitor. *Phytochemistry* **17**: 1070–1081.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248–254.

Charng YY, Juang RH, Su JC, Sung HY. 1994. Partial purification and characterization of invertase isozymes from rice grains (*Oryza sativa*). *Biochemistry and Molecular Biology International* **33**: 607–615.

Chen JQ, Black CC. 1992. Biochemical and immunological properties of alkaline invertase isolated from sprouting soybean hypocotyls. *Archives of Biochemistry and Biophysics* **295**: 61–69.

Cooper RA, Greenshields RN. 1964. The partial purification and some properties of two sucrases of *Phaseolus vulgaris*. *Biochemical Journal* **92**: 357–364.

Copeland L. 1990. Enzymes of sucrose metabolism. In: Lea PJ, ed. *Methods in plant biochemistry, vol. 3. Enzymes of primary metabolism*. London, UK: Academic Press, 73–85.

Doehlert DC, Felker FC. 1987. Characterization and distribution of invertase activity in developing maize (*Zea may*) kernels. *Physiologia Plantarum* **70**: 51–57.

Ebell LF. 1969. Specific total starch determination in conifer tissue with glucose oxidase. *Photochemistry* **8**: 25–36.

Fahrendorf T, Beck E. 1990. Cytosolic and cell wall-bound acid invertases from leaves of *Urtica dioica* L.: a comparison. *Planta* **180**: 237–244.

Fay L, Ghorbel A. 1983. Studies on β-fructosidase from radish seedlings. III. Comparative structure of soluble and wall bound forms. *Plant Science Letters* **29**: 33–48.

Gallagher JA, Pollock CJ. 1998. Isolation and characterization of a cDNA clone from *Lolium temulentum* L. encoding for a sucrose hydrolytic enzyme which shows alkaline/neutral invertase activity. *Journal of Experimental Botany* **49**: 789–795.

Hatch MD, Glasziou KT. 1963. Sugar accumulation cycle in sugar cane. II. Relationship of invertase activity to sugar content and growth rate in storage tissue of plants grown in controlled environments. *Plant Physiology* **38**: 344–348.

Isla MI, Salerno G, Pontis H, Vattuone MA, Sampietro AR. 1995. Purification and properties of the soluble acid invertase from *Oryza sativa*. *Phytochemistry* **38**: 321–325.

Karuppiah N, Vadlamudi B, Kaufman PB. 1989. Purification and characterization of soluble (cytosolic) and bound (cell wall) isoforms of invertase in barley (*Hordeum vulgare*) elongating stem tissue. *Plant Physiology* **91**: 993–998.

Krishnan HB, Blanchette JT, Okita TW. 1985. Wheat invertase.

Characterization of cell wall-bound and soluble forms. *Plant Physiology* **78**: 241–245.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Lee H, Sturm A. 1996. Purification and characterization of neutral and alkaline invertase from carrot. *Plant Physiology* **112**: 1513–1522.

Lin SS, Sung HY. 1993. Partial purification and characterization of soluble acid invertase from rice (*Oryza sativa*) leaves. *Biochemistry and Molecular Biology International* **31**: 945–953.

Lopez ME, Vattuone MA, Sampietro AR. 1988. Partial purification and properties of invertase from *Carica papaya* fruits. *Phytochemistry* **27**: 3077–3081.

Masuda H, Sugawara S. 1980. Purification and some properties of cell wall-bound invertases from sugar beet seedlings and aged slices of mature roots. *Plant Physiology* **66**: 93–96.

Masuda H, Takahashi T, Sugawara S. 1987. The occurrence and properties of alkaline invertase in mature roots of sugar beets. *Agricultural and Biological Chemistry* **51**: 2309–2314.

Masuda H, Takahashi T, Sugawara S. 1988. Acid and alkaline invertase in suspension cultures of sugar beet cells. *Plant Physiology* **86**: 312–317.

Morell M, Copeland L. 1984. Enzymes of sucrose breakdown in soybean nodules. *Plant Physiology* **74**: 1030–1034.

Nelson N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biological Chemistry* **153**: 375–380.

Pollock CJ, Lloyd EJ. 1977. The distribution of acid invertase in developing leaves of *Lolium temulentum* L. *Planta* **133**: 197–200.

Ricardo CPP. 1974. Alkaline β-fructofuranosidases of tuberous roots: possible physiological function. *Planta* **118**: 333–343.

Ricardo CPP, ap Rees T. 1970. Invertase activity during the development of carrot roots. *Phytochemistry* **9**: 239–247.

Ross HA, McRae D, Davis HV. 1996. Sucrolytic enzyme activities in cotyledons of the faba bean. Developmental changes and purification of alkaline invertase. *Plant Physiology* **11**: 329–338.

Schaffer AA. 1986. Invertase in young and mature leaves of *Citrus sinensis*. *Phytochemistry* **25**: 2275–2277.

Segrest JP, Jackson RL. 1973. Molecular weight determination of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. In: Ginsburg V, ed. *Methods in enzymology*, vol. 28. San Diego, USA: Academic Press, 54–63.

Stommel JR, Simon PW. 1990. Multiple forms of invertase from *Daucus carota* cell cultures. *Phytochemistry* **29**: 2087–2089.

Sturm A, Chrispeels MJ. 1990. cDNA cloning of carrot extracellular β-fructosidases and its expression in response to wounding and bacterial infection. *Plant Cell* **2**: 1107–1119.

Sung HY, Huang WC. 1994. Purification and characterization of cell-wall-bound invertase from rice (*Oryza sativa*) *Biotechnology and Applied Biochemistry* **19**: 75–83.

Van den Ende W, Van Laere A. 1995. Purification and properties of a neutral invertase from the roots of *Cichorium intybus*. *Physiologia Plantatum* **93**: 241–248.