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Purification and Characterization of Neutral and Alkaline Invertase from Carrot

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Neutral and alkaline invertase were identified in cells of a suspension culture of carrot (*Daucus carota* L.) and purified to electrophoretic homogeneity. Neutral invertase is an octamer with a molecular mass of 456 kD and subunits of 57 kD, whereas alkaline invertase is a tetramer with a molecular mass of 504 kD and subunits of 126 kD. Both enzymes had sharp pH profiles, with maximal activities at pH 6.8 for neutral invertase and pH 8.0 for alkaline invertase, and both hydrolyzed sucrose with typical hyperbolic kinetics and similar K_m values of about 20 mM at pH 7.5. Neutral invertase also hydrolyzed raffinose and stachyose and, therefore, is a β-fructofuranosidase. In contrast, alkaline invertase was highly specific for sucrose. Fructose acted as a competitive inhibitor of both enzymes, with K_i values of about 15 mM. Glucose was a noncompetitive inhibitor of both neutral and alkaline invertase, with a K_i of about 30 mM. Neither enzyme was inhibited by $HgCl_2$. Alkaline invertase was markedly inhibited by $CaCl_2$, $MgCl_2$, and $MnCl_2$, and neutral invertase was not. In contrast to alkaline invertase, neutral invertase was inhibited by the nucleotides ATP, CTP, GTP, and UTP.

Invertases (β-fructofuranosidases, EC 3.2.1.26), which hydrolyze Suc into Glc and Fru, are a group of ubiquitous plant enzymes with different pH optima and subcellular localizations. The pH optima of the acid invertases are 4.5 to 5.5 and those of the neutral and alkaline invertases are 6.8 to 8.0. The acid invertases are *N*-linked glycoproteins (Sturm, 1991; Unger et al., 1992) and are located either as soluble proteins in the vacuole or ionically bound to the cell wall (Leigh et al., 1979; Laurière et al., 1988; Obenland et al., 1993). The neutral and alkaline invertases are thought to be nonglycosylated cytoplasmic polypeptides (Copeland, 1990; Chen and Black, 1992).

Acid invertase activity is mainly found in immature plant organs and declines rapidly and concomitantly with the accumulation of Suc as the organ matures (Ricardo and ap Rees, 1970; Masuda et al., 1987; Iwatsubo et al., 1992). In several instances a direct correlation between the hexose level and acid invertase activity was found (Krishnan and Pueppke, 1990; Ranwala et al., 1992; Klann et al., 1993). It has been suggested that acid invertases are involved in Suc metabolism (ap Rees, 1984), phloem unloading (Eschrich, 1980), control of sugar composition in storage organs (Klann et al., 1993; Sturm et al., 1995), osmoregulation (Wyse et al., 1986; Perry et al., 1987), gravitropism (Wu et al., 1993), and response to pathogens (Sturm and Chrispeels, 1990; Storr and Hall, 1992) and to wounding (Matsushita and Uritani, 1974; Sturm and Chrispeels, 1990).

The neutral and alkaline invertases seem to be confined to mature tissues (Ricardo and ap Rees, 1970; Masuda et al., 1987). The results of earlier studies suggested that the function of these enzymes is the hydrolysis of Suc in the cytoplasm of cells that lack significant amounts of acid invertase activity (Ricardo and ap Rees, 1970). More recent studies have shown the presence of neutral/alkaline and different isoforms of acid invertase in the same tissue (Morell and Copeland, 1984; Masuda et al., 1988; Ranwala et al., 1991). These invertase isoforms, with different subcellular locations and unique biochemical properties, may allow independent control of Suc metabolism, translocation, and storage (Chen and Black, 1992).

Several isoforms of carrot acid invertase have been purified and characterized at the physiological, biochemical, and molecular levels (Stommel and Simon, 1990; Sturm, 1996). Fractions enriched for carrot alkaline invertase have been studied (Ricardo and Sovia, 1974; Stommel and Simon, 1990), but the enzyme was never purified. The purification to electrophoretic homogeneity of an alkaline invertase from soybean hypocotyl (Chen and Black, 1992) and faba bean (Ross et al., 1996) and a neutral invertase from roots of chicory (Van den Ende and Van Laere, 1995) has been reported. Despite these studies, the relationship of the neutral and alkaline invertases to the acid invertases is not clear; nor are the mechanisms of their biochemical and molecular regulation known. The main goal of our current work is the purification, characterization, and cDNA cloning of neutral and alkaline invertases from carrot. In this paper we describe their purification and our first results concerning their biochemical properties.

MATERIALS AND METHODS

Cells of *Daucus carota*, Queen Anne's lace (wild carrot cell culture line W001C; Sung, 1976), were grown in Murashige-Skoog medium (Murashige and Skoog, 1962) supplemented with 0.1 mg/L 2,4-D at 26°C in the dark. The suspension cells were transferred at weekly intervals into fresh Murashige-Skoog medium.

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Purification of Neutral and Alkaline Invertase

Preparation of Extracts

Carrot cells (400 g) that were collected from suspension cultures in the exponential growth phase were homogenized four times for 20 s at full speed with a Polytron homogenizer (Kinematika, Kriens/Luzern, Switzerland) in 2.5 volumes of an ice-cold extraction buffer A (50 mM Hepes-KOH, pH 7.5, containing 0.5 mM EDTA, 10 mM Lys, 0.5 mM $MgCl_2$, 0.5% 2-mercaptoethanol, and 100 μM PMSF). The homogenate was centrifuged for 20 min at 6,000*g* in a Sorvall GSA-rotor. The supernatant was collected and kept cold. The 6,000*g* pellet was resuspended in 2.5 volumes of ice-cold buffer A, homogenized with a Polytron homogenizer three times for 20 s at full speed, and centrifuged for a further 20 min. The combined supernatants were centrifuged at 16,300*g* for 30 min and then poured through four layers of Miracloth (Calbiochem-Behring). The filtrate was used for further protein purification. If not stated otherwise, all steps were carried out at 4°C.

Ammonium Sulfate Precipitation

Solid ammonium sulfate was slowly added to the crude extract with gentle stirring, and the protein that precipitated between 20 and 45% saturation was collected by centrifugation for 30 min at 16,300*g*. The precipitate was dissolved in buffer B (100 mL of 25 mM Hepes-KOH, pH 7.5, containing 190 mM NaCl, 0.5% 2-mercaptoethanol, and 100 μM PMSF) and dialyzed against buffer B overnight.

Anion-Exchange Chromatography on Q-Sepharose

The dialysate was loaded onto a Q-Sepharose column (2.5 × 25 cm, Pharmacia), which had been equilibrated previously with buffer B. The column was washed with buffer B until the A_{280} was less than 0.01. Bound protein was eluted with a linear gradient of 240 mL of 190 to 550 mM NaCl in 25 mM Hepes-KOH, pH 7.5, containing 0.5% 2-mercaptoethanol and 100 μM PMSF. Active fractions (fraction size 5 mL) were pooled, precipitated with ammonium sulfate at 60% saturation, and centrifuged for 30 min at 16,300*g*. The precipitate was dissolved in 5 mL of 5 mM potassium phosphate buffer, pH 7.5, containing 0.1% 2-mercaptoethanol (buffer C), and dialyzed against buffer C overnight.

Chromatography on HA-Ultrogel

The dialysate was applied to an HA-Ultrogel column (2.5 × 25 cm, Sigma), which had been equilibrated previously with buffer C. The column was washed with buffer C and eluted with 200 mL of a linear gradient of 5 to 500 mM potassium phosphate buffer, pH 7.5, containing 0.1% 2-mercaptoethanol. The column was eluted at a flow rate of 40 mL/h and fractions of 5 mL were collected. Fractions in the flow-through containing neutral invertase activity and fractions in the eluate containing alkaline invertase activity were combined separately, precipitated with ammonium sulfate at 60% saturation, and centrifuged for 30 min at 16,300*g*. The two protein pellets were individually dissolved in 5 mL of 25 mM potassium phosphate buffer, pH 7.5, containing 0.1% 2-mercaptoethanol (buffer D), and dialyzed against buffer D overnight.

Affinity Chromatography on Green 19 Dye

The dialyzed protein solutions (5 mL each) were divided into 0.5-mL aliquots and then applied to 10 prepacked green 19 dye columns (4.5 × 0.7 cm, Sigma), which had been equilibrated previously with buffer D. The columns were washed with 15 mL of buffer D and then step-eluted with NaCl at 0.35 and 1.5 M (15 and 25 mL, respectively), and the eluate was collected in 2-mL fractions. Neutral invertase activity was detected in the flow-through, whereas alkaline invertase activity was eluted by 1.5 M NaCl. Fractions containing enzyme activity were pooled and precipitated with ammonium sulfate at 60% saturation. The precipitated proteins were collected by centrifugation for 30 min at 16,300*g*.

Gel-Filtration Chromatography I on Sephacryl S-300

Each protein pellet was dissolved in 7 mL of 100 mM Hepes-KOH, pH 7.5, containing 0.1% 2-mercaptoethanol (buffer E). The protein solutions were individually applied to a Sephacryl S-300 column (2.6 × 100 cm, Pharmacia), which had been equilibrated previously with buffer E and calibrated with blue dextran (V_o), thyroglobulin (669 kD), apoferritin (443 kD), β-amylase (200 kD), alcohol dehydrogenase (150 kD), BSA (66 kD), and carbonic anhydrase (29 kD). The column was eluted at a flow rate of 110 mL/h, and fractions of 5 mL were collected. Fractions containing enzyme activity were pooled, dialyzed overnight against 25 mM Hepes-KOH, pH 8.0, containing 200 mM NaCl and 0.1% 2-mercaptoethanol (buffer F) for alkaline invertase, and 25 mM Hepes-KOH, pH 7.2, containing 275 mM NaCl and 0.1% 2-mercaptoethanol (buffer G) for neutral invertase.

Anion-Exchange Chromatography II on Macro-Prep

For further purification of alkaline invertase, the dialysate was applied to a Macro-Prep column (1.5 × 20 cm, Bio-Rad), which had been equilibrated previously with buffer F. The column was washed with buffer F and eluted with 200 mL of a linear gradient of 200 to 450 mM NaCl in 25 mM Hepes-KOH, pH 8, containing 0.1% 2-mercaptoethanol.

For further purification of neutral invertase, the dialysate was applied to a Macro-Prep column (1.2 × 25 cm), which had been equilibrated previously with buffer G. The column was washed with buffer G and eluted with 200 mL of a linear gradient of 275 to 360 mM NaCl in 25 mM Hepes-KOH, pH 7.2, containing 0.1% 2-mercaptoethanol.

Fractions containing activity of the two enzymes were combined separately and used for kinetic studies and the determination of the properties of neutral and alkaline invertases. The remaining protein solutions were precipitated with ammonium sulfate at 60% saturation and centrifuged for 30 min at 16,300*g*.

Hydrophobic Interaction Chromatography on Propyl Agarose

The protein pellet with alkaline invertase activity was dissolved in 5 mL of 25 mM Hepes-KOH, pH 8.0, containing 1.5 M ammonium sulfate and 0.1% 2-mercaptoethanol (buffer H). The solution was applied to a propyl agarose column (10 × 1.5 cm, Sigma), which had been equilibrated previously with buffer H. The column was washed with buffer H, then eluted with 25 mM Hepes-KOH, pH 8.0, containing 0.1% 2-mercaptoethanol, and collected in 3-mL fractions. Fractions containing enzyme activity were pooled, dialyzed against 10 mM Hepes-KOH, pH 8.0, containing 0.1% 2-mercaptoethanol, and stored in 50% glycerol at −20°C.

Gel-Filtration Chromatography II on Sephacryl S-300

The protein pellet with neutral invertase activity was dissolved in 5 mL of 100 mM potassium phosphate buffer, pH 7.0, containing 0.1% 2-mercaptoethanol (buffer I) and applied to a Sephacryl S-300 column (2.6 × 100 cm), which had been equilibrated previously with buffer I. Fractions of 5 mL were collected. Fractions containing enzyme activity were pooled, dialyzed against 10 mM potassium phosphate buffer, pH 7.0, containing 0.1% 2-mercaptoethanol, and stored in 50% glycerol at −20°C.

SDS-PAGE and Western Blot Analysis

SDS-PAGE with gels of 10% polyacrylamide for neutral invertase and 12.5% for alkaline invertase was carried out by the procedure of King and Laemmli (1971). For western blot analysis, highly enriched fractions of neutral and alkaline invertase were used. As a control, a 20 to 45% ammonium sulfate precipitate of a soluble protein extract of carrot suspension cells was desalted with Sephadex G-25 and also tested with polyclonal antibodies (1:1000 dilution) against alkaline invertase from *Vicia faba* (Ross et al., 1994). The nitrocellulose membrane was blocked with 3% gelatin (Bio-Rad) and then incubated for 2 h with the primary antiserum in TBS (10 mM Tris-HCl, pH 7.5, and 150 mM NaCl) containing 1% gelatin. The blot was subsequently washed twice for 15 min in TBS containing 0.1% Tween 20 with 1% gelatin. For the detection of the bound antibodies, the blot was incubated for 2 h with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Bio-Rad) in TBS with 1% gelatin at a dilution of 1:1000. After the blot was washed once for 15 min in TBS with 1% gelatin and twice for 15 min each in TBS containing 0.1% Tween 20 and no gelatin, the color reaction was carried out according to the manufacturer's protocol (Bio-Rad). For the detection of *N*-linked glycoproteins, the nitrocellulose membrane was decorated with concanavalin A or an antiserum against Xyl-containing complex glycans, as described by Faye and Chrispeels (1985) and Laurière et al. (1989).

Enzyme and Protein Assays

Enzyme activity was determined in reaction mixtures containing 50 mM potassium phosphate buffer (pH 6.8 or 8.0), 100 mM Suc, and an appropriate volume of enzyme in a final volume of 1 mL. The mixture was incubated at 37°C for 30 min. The amount of reducing sugar liberated was determined according to the method of Laurière et al. (1988). Enzyme activity (units) was expressed as the amount (micromoles) of reducing sugar (Glc and Fru) released per minute. Invertase activity is inhibited by ammonium ions at high concentrations, which required that protein solutions were quickly dialyzed after ammonium sulfate precipitation prior to activity determination.

For the determination of the K_m of the two enzymes, the concentration of Suc was varied between 0 and 400 mM. To assess the effect of Glc (0–40 mM) on enzyme activity, the Fru released was determined by Fru dehydrogenase according to the method of Prado and Sampietro (1994). To study the effect of Fru (0–40 mM) on invertase activity, Glc was determined with Glc oxidase-peroxidase (Sigma) according to the manufacturer's protocol. The effect of metal ions (0–10 mM $CaCl_2$, $MgCl_2$, or $MnCl_2$, 0 to 200 μM $CuSO_4$, and 0 to 100 μM $HgCl_2$) on neutral and alkaline invertase was studied in reaction mixtures of 50 mM imidazole-HCl buffer (pH 6.8 for neutral invertase) and 50 mM Hepes-KOH (pH 8.0 for alkaline invertase) instead of potassium phosphate buffer. Protein concentrations were determined with Coomassie blue reagent (Bio-Rad) according to the manufacturer's instructions, using BSA as a standard. For the inhibition of neutral and alkaline invertase, 0 to 15 mM Tris-HCl, pH 7.5, was used. As possible effectors of enzyme activity, 0 to 30 mM MgATP, ATP, CTP, GTP, or UTP was tested.

RESULTS AND DISCUSSION

Purification of Carrot Neutral and Alkaline Invertase

Cells of a suspension culture of carrot contained soluble Suc-cleaving activities with distinct pH optima above and below pH 6.0 (alkaline and acid invertase, respectively). The two activities were efficiently separated by an ammonium sulfate precipitation at 20 to 45% saturation, which is in accordance with previous reports (Ricardo and ap Rees, 1970; Chen and Black, 1992). Activity of alkaline invertase was detected in the protein pellet, whereas that of acid invertase remained in the supernatant. The 20 to 45% ammonium sulfate fraction was chromatographed on Q-Sepharose, and two peaks of invertase activity with only poor separation were obtained (Fig. 1A). Fractions containing activity were combined and further purified by chromatography on HA-Ultrogel followed by affinity chromatography on green 19, leading to the efficient separation of the two activities. A Suc-cleaving activity with a neutral pH optimum (neutral invertase, H_1) was identified in the nonbound protein fraction (Fig. 1, B and C). An activity with a more basic pH optimum (alkaline invertase, H_2) bound to the HA-Ultrogel and green 19 dye columns and was eluted with salt-containing buffers (Fig. 1, B and D). At this stage of the purification, neutral invertase accounted for about one-third of the invertase activity, and alkaline invertase accounted for about two-thirds (Table I). The pooled fractions containing neutral and alkaline invertase were indi-

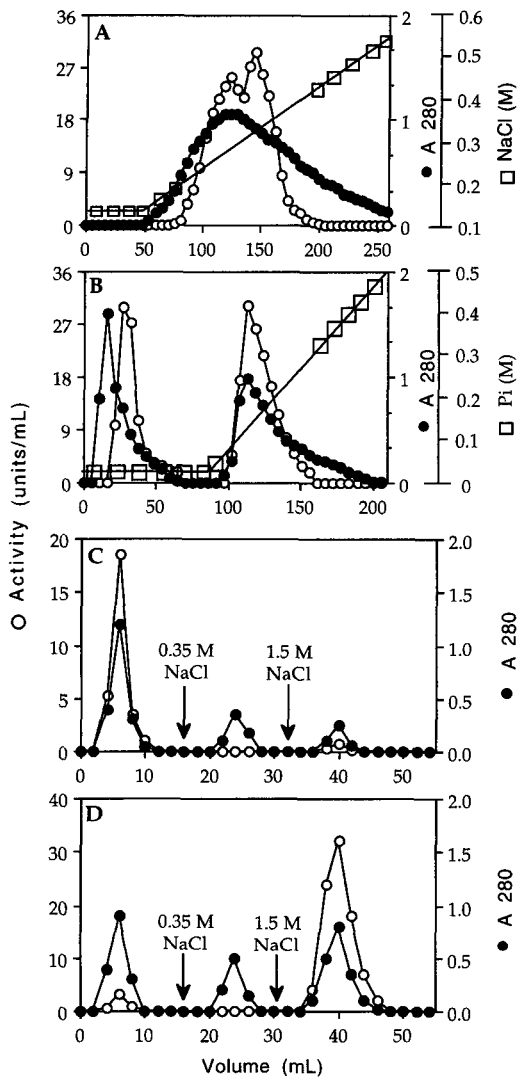

Figure 1. Separation of neutral and alkaline invertase by column chromatography. Protein (●) was monitored by A_{280} and invertase activity (○) was measured as described in "Materials and Methods." A, Anion-exchange chromatography on Q-Sepharose. Bound protein was eluted with a linear gradient of 190 to 550 mM NaCl (□). B, Chromatography on hydroxyapatite (HA-Ultrogel). The nonbound protein fraction contained neutral invertase (H_1). Bound proteins containing alkaline invertase (H_2) were eluted with a linear gradient of 2.5 to 500 mM potassium phosphate (□). Fractions containing Suc-cleaving activity were pooled, concentrated by ammonium sulfate precipitation, and used for affinity chromatography. C, Affinity chromatography on green 19. Nonbound proteins containing neutral invertase were applied to a column of green 19 and the activity was recovered in the flow-through. D, Affinity chromatography on green 19. Bound proteins containing alkaline invertase were applied to a column of green 19. The enzyme activity bound to the column and was eluted with 1.5 M NaCl.

vidually purified further by gel-filtration chromatography, a second ion-exchange chromatography, a second gel-filtration chromatography, and hydrophobic interaction chromatography (Table I).

Macro-Prep anion-exchange chromatography for neutral invertase was the most effective procedure for the removal of contaminating proteins from the preparations. Although propyl agarose chromatography did not increase the specific activity of alkaline invertase, it was a necessary step to obtain electrophoretically pure enzyme. At the end of the purifications, the recoveries of the two enzymes were less than 10% (Table I). Most likely, the marked losses were a consequence of the large number of purification steps used (Table I) and the low stabilities of the enzymes (see below).

Characterization of the Purified Neutral and Alkaline Invertase

Molecular Mass

Neutral invertase eluted from the gel-filtration column as a polypeptide with a molecular mass of approximately 456 kD. The purified enzyme migrated as a single band in a denaturing SDS-polyacrylamide gel with an estimated molecular mass of 57 kD (Fig. 2A; Table II) and, therefore, is an octamer in its native form. The molecular mass of alkaline invertase that was obtained by gel-filtration chromatography was 504 kD. The purified alkaline invertase migrated as a polypeptide of 126 kD in a denaturing SDS-polyacrylamide gel (Fig. 2B; Table II) and, therefore, appeared to be a tetramer in its native form. The molecular masses of native neutral and alkaline invertases from other plant tissues were in the range of 240 to 280 kD (Stommel and Simon, 1990; Chen and Black, 1992; Van den Ende and Van Laere, 1995; Ross et al., 1996) and were thought to be tetramers. The carrot neutral invertase fits into this size range, whereas the carrot alkaline invertase appears to be larger than the enzymes from other plant species.

Effect of pH

The dependence of the activity of a crude enzyme preparation after ammonium sulfate precipitation on the pH is shown in Figure 3A. A major activity was detected between pH 7.5 and 8.0, with a shoulder of approximately pH 6.5. The purified enzymes had sharp pH profiles with maximal activities at pH 6.8 and 8.0 (Fig. 3B), corresponding to the activity maxima observed with the enzyme mixture. The activity of neutral invertase was suppressed when the pH was increased above 9.0, whereas alkaline invertase was not active when the pH was decreased below 6.0. There was no effect on the activity of the two enzymes when phosphate buffer was replaced in reaction mixtures by buffers containing 50 mM citrate, imidazole-HCl, Mops-KOH, Tes-KOH, or Hepes-KOH (data not shown).

Specific Activity

The purified proteins had specific activities of about 23.4 units/mg protein for neutral invertase and 14.9 units/mg protein for alkaline invertase. Comparable specific activities have been reported for the purified preparations of neutral and alkaline invertase from nodules (Morell and Copeland, 1984) and hypocotyls of soybean (Chen and Black, 1992) and roots of chicory (Van den Ende and Van Laere, 1995).

Table I. *Purification scheme for neutral (H_1) and alkaline (H_2) invertase from carrot*

Procedure	Enzyme Activity	Protein	Specific Activity	Recovery	Purification
	units	*mg*	*units/mg protein*	*%*	*fold*
Crude extract	338	1680	0.2	100	1
20–45% $(NH_4)_2SO_4$	259	495	0.5	77	3
Q-Sepharose	169	151	1.1	50	6
HA-Ultrogel					
H_1	42	26	1.6	12	8
H_2	93	75	1.2	28	6
Green 19					
H_1	29	16	1.8	9	9
H_2	61	15	4.1	18	20
Sephacryl S-300					
H_1	24	3.1	7.7	7	39
H_2	46	4.2	11.0	14	55
Macro-Prep					
H_1	19	0.9	21.1	6	106
H_2	27	1.8	15.8	8	75
Sephacryl S-300					
H_1	15	0.6	23.4	4	117
Propyl agarose					
H_2	21	1.4	14.9	6	75

Substrate Specificity

Neutral invertase cleaved raffinose and stachyose (100 mM) with efficiencies of 15 and 29%, respectively, when compared with the cleavage of Suc. The enzyme did not utilize maltose, melezitose, *p*-nitrophenyl-β-glucoside, or trehalose (Table III), suggesting that neutral invertase is a β-fructofuranosidase. In contrast, alkaline invertase was highly specific for Suc, and of the other sugars tested it hydrolyzed only melezitose with very low efficiency (Table III). A high specificity for Suc has been reported for neutral and alkaline invertase from other plant tissues, with no or a minimal amount of cleavage activity toward β-Fru-containing sugars.

Kinetic Properties and Inhibition by Products

Typical hyperbolic kinetics, with linear reciprocal plots of velocity versus substrate concentration, were observed for both enzymes when Suc was the varied substrate (data not shown). The K_m of alkaline invertase for Suc was 20.7 mM at pH 7.5 and 15.1 mM at pH 8.0, and the K_m of neutral invertase was 18.7 mM at pH 7.5 and 14.3 mM at pH 6.8 (Table II). Similar K_m values were observed for neutral and alkaline invertase from other plant species. Suc concentrations greater than 350 mM caused a slight inhibition of carrot alkaline invertase activity but not of carrot neutral invertase.

Both enzymes were inhibited by their reaction products Glc and Fru (Figs. 4 and 5). Fru was a competitive inhibitor of neutral (K_i 13.4 mM) and alkaline (K_i 16.3 mM) invertase. In contrast, Glc inhibited neutral (K_i 28 mM) and alkaline (K_i 33 mM) invertase noncompetitively. The neutral and alkaline invertases from other plant tissues are also inhibited by hexoses with identical inhibition types. Carrot tap roots contain about 1 to 2% of Glc and Fru (Phan and Hsu, 1973; Hole and McKee, 1988), suggesting that the regulation of neutral and alkaline invertase activity by hexoses may be important. On the other hand, if the hexoses accumulate mainly in the vacuole, the physiological relevance of the observed in vitro inhibition is less clear.

Stability

The activities of neutral and alkaline invertase were highly labile and, on average, only 4% (H_1) and 6% (H_2) of the initial activities were recovered after the final purification steps. The purified proteins were stable for several days when stored in 50% glycerol at 4°C, whereas their activities were rapidly lost at temperatures above 20°C (Fig. 6). Without the addition of glycerol, both enzymes lost approximately one-half of their activities in 48 h at 4°C. In addition, the activity of neutral invertase was abolished by freezing in an aqueous protein solution. Both enzymes were most stable when kept at pH 7.0 to 8.0 in the presence of protease inhibitors such as PMSF and reagents such as DTT and 2-mercaptoethanol. Maximal activities of the two enzymes were measured between 37 and 40°C (Table II). The activities of the two enzymes rapidly decreased above 50°C and were abolished above 70°C (data not shown).

Effects of Inhibitors

Both enzymes were strongly inhibited by Tris and $CuSO_4$ (data not shown). Tris at 0.8 mM inhibited the activity of neutral invertase 50%, whereas 6.8 mM Tris was required to inhibit one-half of the activity of alkaline invertase. About 50% inhibition by $CuSO_4$ was observed at 17 μM for neutral invertase and 80 μM for alkaline invertase. Thus, neutral invertase was more sensitive to inhibition by Tris and $CuSO_4$ than was alkaline invertase (Table II). Comparable results have been reported for other plant neutral and

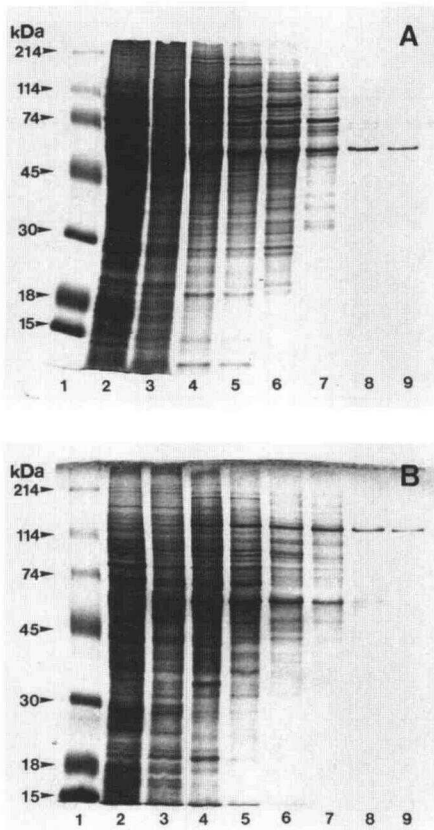

Figure 2. SDS-PAGE of protein fractions obtained during the purification of neutral (A) and alkaline (B) invertase. The gels contained 10 and 12.5% acrylamide, respectively, and were stained with Coomassie blue. A, Lane 1, Molecular mass standards; lane 2, crude protein extract; lane 3, 20 to 45% ammonium sulfate precipitate; lane 4, pooled fractions after chromatography on Q-Sepharose; lane 5, pooled fractions after chromatography on HA-Ultrogel; lane 6, pooled fractions after chromatography on green 19; lane 7, pooled fractions after gel-filtration chromatography; lane 8, pooled fractions after chromatography on Macro-Prep; lane 9, pooled fraction after the second gel-filtration chromatography. B, Lane 1, Molecular mass standards; lane 2, crude protein extract; lane 3, 20 to 45% ammonium sulfate precipitate; lane 4, pooled fractions after chromatography on Q-Sepharose; lane 5, pooled fractions after chromatography on HA-Ultrogel; lane 6, pooled fractions after chromatography on green 19; lane 7, pooled fractions after gel-filtration chromatography; lane 8, pooled fractions after chromatography on Macro-Prep; lane 9, pooled fractions after chromatography on propyl agarose. The masses of the molecular standards in kD are indicated by arrowheads.

alkaline invertases (Morell and Copeland, 1984; Van den Ende and Van Laere, 1995).

The addition of the chloride salts of Na^+ and K^+ at 100 mM had no effect on either enzyme (data not shown). In contrast, alkaline invertase was strongly inhibited by $CaCl_2$, $MgCl_2$, and $MnCl_2$ at 10 mM, but neutral invertase was not (Fig. 7; Table II). Similar results were obtained for alkaline invertase from nodules of soybean (Morell and Copeland, 1984), whereas only slight inhibition was reported for neutral invertase from roots of chicory (Van den Ende and Van Laere, 1995).

Unlike the acid plant invertases, neither enzyme was inhibited by 100 μM $HgCl_2$ (data not shown), suggesting

Table II. *Characterization of neutral and alkaline invertase from carrot*

Characteristics	Neutral Invertase (H_1)	Alkaline Invertase (H_2)
Native molecular mass (kD)	456	504
Molecular mass on SDS-polyacrylamide gels (kD)	57	126
Optimal pH for activity	6.8	8.0
K_m for Suc (mM)	(pH 6.8) 14.3	(pH 8.0) 15.1
	(pH 7.5) 18.7	(pH 7.5) 20.7
K_i for Fru (mM)	(pH 6.8) 13.4	(pH 8.0) 28
K_i for Glc (mM)	(pH 6.8) 16.3	(pH 8.0) 33
Inhibition by $HgCl_2$	N.I.[b]	N.I.
$I_{0.5}$ for Tris-HCl (mM)[a]	0.8	6.8
$I_{0.5}$ for $CaCl_2$ (mM)	(pH 6.8) N.I.	(pH 8.0) 8.7
	(pH 7.5) N.I.	(pH 7.5) 5.5
$I_{0.5}$ for $MgCl_2$ (mM)	(pH 6.8) N.I.	(pH 8.0) Inhibited[c]
	(pH 7.5) N.I.	(pH 7.5) 7.5
$I_{0.5}$ for $MnCl_2$ (mM)	(pH 6.8) N.I.	(pH 8.0) 5.4
	(pH 7.5) N.I.	(pH 7.5) 2.4
$I_{0.5}$ for $CuSO_4$ (μM)	17	80
Temperature optimum (°C)	40	40

[a] $I_{0.5}$, Concentration for 50% inhibition. [b] N.I., Not inhibited. [c] Inhibition less than 50%.

that a reduced sulfhydryl group is not essential for the activity of either enzyme from carrot. This finding is in contrast to previous reports of other neutral and alkaline invertases, which were all inhibited by $HgCl_2$ (Masuda et al., 1987; Ranwala et al., 1991; Chen and Black, 1992; Van den Ende and Van Laere, 1995).

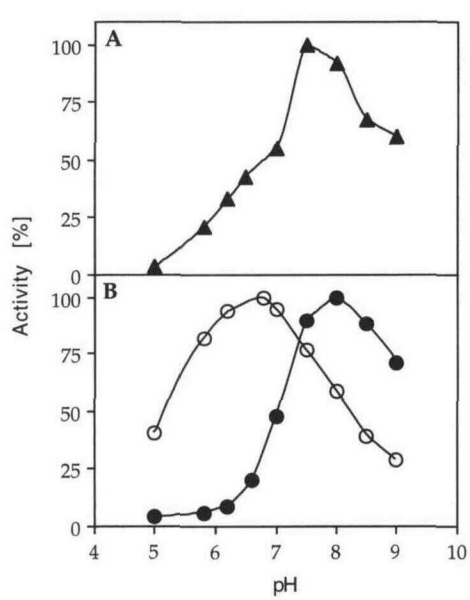

Figure 3. Effect of pH on invertase activity in the 20 to 45% ammonium sulfate precipitate (▲, A) and on the activities of the purified neutral (●) and alkaline invertase (○, B). The buffers used were citrate-phosphate (pH 5.0–7.0), imidazole-HCl (pH 6.2–7.8), Hepes-KOH (pH 7.2–8.2), and Gly-NaOH (pH 8.6–9.0). The reactions were carried out for 30 min at 37°C. All data were adjusted relative to maximal activity for each enzyme.

Table III. *Substrate specificities of neutral and alkaline invertase from carrot*

Sugar	Neutral Invertase (H_1)	Alkaline Invertase (H_2)
	%	%
Suc (Glc β-1,2-Fru)	100	100
Maltose (Glc α-1,4-Glc)	0	0
Melezitose (Glc α-1,3-Fru α-2,1-Glc)	0	2
Raffinose (Gal α-1,6-Glc β-1,2-Fru)	15	0
Stachyose (Gal α-1,6-Gal α-1,6-Glc β-1,2-Fru)	29	0
Trehalose (Glc α-1,1-Glc)	0	0
p-Nitrophenyl-α-glucoside	0	0

Effects of Nucleotides

The effects of nucleotides on the activities of carrot neutral and alkaline invertases is shown in Table IV. Neutral invertase was inhibited by ATP, CTP, GTP, UTP, and MgATP under standard assay conditions; ATP caused the strongest inhibition. Neutral invertase was not inhibited by Mg^{2+} ions and, therefore, its inhibition by MgATP was most likely due to the ATP and not to the Mg^{2+} ions. In contrast, the nucleotides tested had no effect on the activity of alkaline invertase (Table IV). The inhibition of alkaline invertase by MgATP was due most likely to Mg^{2+} ions and not to ATP.

Immunological Comparison and Glycoprotein Status

To determine the immunological relationship between the neutral/alkaline carrot invertases and related enzymes,

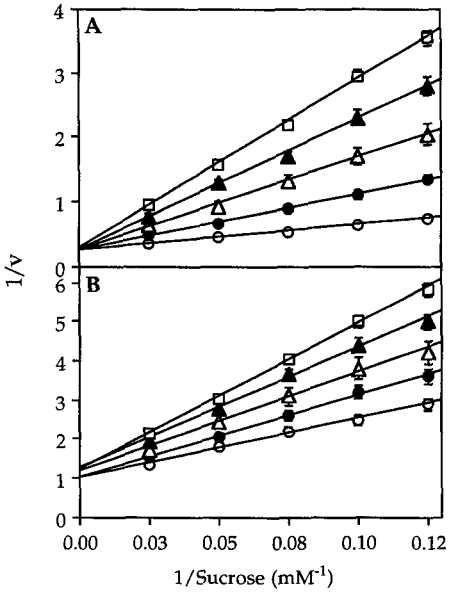

Figure 4. Inhibition of neutral (A) and alkaline (B) invertase activity by Fru. Reaction mixtures were as described for the standard assay except that the concentration of Suc was varied between 0 and 400 mM. Fru was added to a final concentration of 0 mM (○), 10 mM (●), 20 mM (△), 30 mM (▲), or 40 mM (□). The specific activity is expressed as μmol Glc produced min^{-1} mg^{-1} protein.

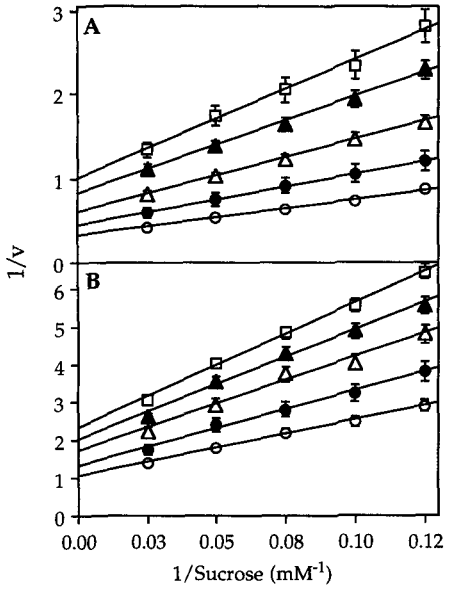

Figure 5. Inhibition of neutral (A) and alkaline (B) invertase activity by Glc. Reaction mixtures were as described for the standard assay except that the concentration of Suc was varied between 0 and 400 mM. Glc was added to a final concentration of 0 mM (○), 10 mM (●), 20 mM (△), 30 mM (▲), or 40 mM (□). The specific activity is expressed as μmol Glc produced min^{-1} mg^{-1} protein.

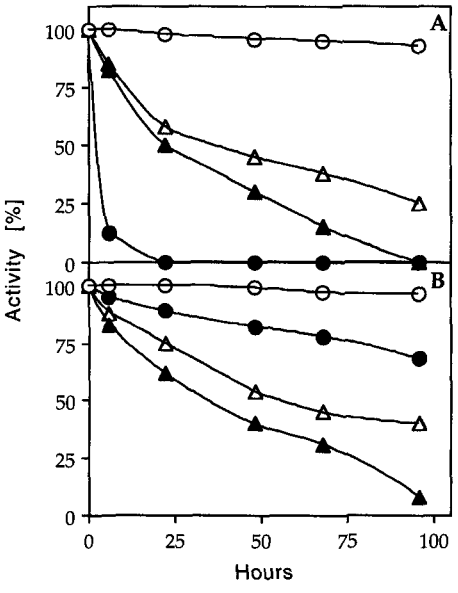

Figure 6. Temperature stability of neutral and alkaline invertase. Reaction mixtures were as described for the standard assay. The purified enzymes were stored in the presence of 0.1% 2-mercaptoethanol in 10 mM potassium phosphate (pH 7.0) for neutral invertase (A) or 10 mM Hepes-KOH (pH 8.0) for alkaline invertase (B) at 25°C (▲), 4°C (△), −20°C (●), or 4°C with 50% glycerol (○). The enzyme assays were carried out at 37°C for 30 min. All data were adjusted relative to maximal activity for each enzyme.

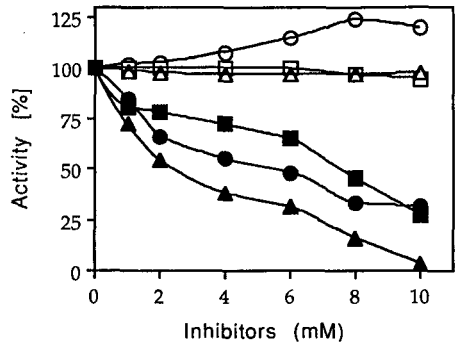

Figure 7. Inhibition of neutral and alkaline invertase activity by $CaCl_2$ (○, ●), $MgCl_2$ (□, ■), or $MnCl_2$ (△, ▲). All assays were performed at optimal pH for each enzyme (○, □, and △ for alkaline invertase; ●, ■, and ▲ for neutral invertase). All data were adjusted relative to maximal activity for each enzyme.

we analyzed highly enriched enzyme preparations on a western blot. An antibody against alkaline invertase from *V. faba* (Ross et al., 1994) bound to both enzymes, and those were also the only polypeptides recognized in an ammonium sulfate precipitate of 20 to 45% saturation (data not shown).

To test whether the purified carrot enzymes were *N*-linked glycoproteins, they were decorated on western blots with concanavalin A (Faye and Chrispeels, 1985) or an antibody against Xyl-containing complex *N*-linked glycans from plants (Laurière et al., 1989). Neither the lectin nor the anti-carbohydrate antibody bound to the proteins (data not shown). Hence, it appears that neither the neutral nor the alkaline invertase are *N*-glycosylated. Similar results were obtained with neutral and alkaline invertases from hypocotyls of soybean (Chen and Black, 1992) and roots of chicory (Van den Ende and Van Laere, 1995), which supports the proposal that these enzymes are located in the cytoplasm.

Detection of Neutral and Alkaline Invertase in Crude Protein Extracts

We attempted to determine separately the activities of neutral and alkaline invertases in crude extracts of suspension-cultured cells of carrot (Table V). For this purpose, the activities of the acid invertases were inhibited by

Table IV. *Effect of nucleotides on neutral and alkaline invertase from carrot*

All assays were performed at optimal pH for both enzymes.

Nucleotide	Neutral Invertase (H_1)	Alkaline Invertase (H_2)
	$I_{0.5}$ (mM)[a]	
MgATP	11.5	Inhibited[b]
ATP	7.5	N.I.[c]
CTP	26.9	N.I.
GTP	25.5	N.I.
UTP	22.5	N.I.

[a] $I_{0.5}$, Concentration for 50% inhibition. [b] Inhibition less than 50%. [c] N.I., Not inhibited.

Table V. *Detection of invertase activities in crude protein extracts obtained from 25 g of cells*

Addition to Extract	Invertase Activity			
	Cell-wall extract	Soluble extract		
	pH 5.0	pH 5.4	pH 6.8	pH 8.0
	μmol/min			
None	219	5.4	11.7	23.2
+100 μM $HgCl_2$	N.D.[a]	3.7	11.4	23.4
+100 μM $HgCl_2$ +20 mM ATP	N.D.	1.2	5.1	16.2
+100 μM $HgCl_2$ +15 mM $MnCl_2$	N.D.	2.8	7.9	4.9
+100 μM $HgCl_2$ +20 mM ATP +15 mM $MnCl_2$	N.D.	N.D.	N.D.	0.7

[a] N.D., Not detected.

100 μM $HgCl_2$ (Unger et al., 1992). Alkaline invertase activity was measured in the presence of 20 mM ATP, which causes inhibition of neutral invertase. The assay contained 50 mM potassium phosphate buffer, pH 8.0, 100 mM Suc, 100 μM $HgCl_2$, 20 mM ATP, and an appropriate volume of enzyme. The activity of neutral invertase was determined in the presence of 15 mM $MnCl_2$, which inhibited the activity of alkaline invertase. Here, the assay contained 50 mM potassium phosphate buffer, pH 6.8, 100 mM Suc, 100 μM $HgCl_2$, 15 mM $MnCl_2$, and an appropriate volume of enzyme.

Analysis of invertase activities in crude extracts showed that the activity of cell-wall invertase was about 5 times higher than that of the soluble invertases. Because of the overlapping pH curves, the activities determined for soluble acid invertase, neutral invertase, and alkaline invertase should be higher than their absolute activities. The addition of $HgCl_2$ led to the inhibition of cell wall invertase and soluble acid invertase. The residual activity of soluble acid invertase of 3.7 units most likely reflects the activity of the neutral invertase at pH 5.4. The addition of ATP, the inhibitor of neutral invertase, reduced the activity of alkaline invertase by about one-third, which corresponds to the activity of neutral invertase at pH 8.0. The activities measured under these conditions at pH 6.8 (5.1 units) and pH 5.4 (1.2 units) most likely stem from the activity of alkaline invertase at these pH values. $MnCl_2$, the inhibitor of alkaline invertase, reduced the activity of neutral invertase by one-third, which, again, approximately corresponds to the activity of alkaline invertase at pH 6.8. The activities determined under these conditions at pH 5.4 (2.8 units) and at pH 8.0 most likely originate from the activity of neutral invertase at these pH values. A combination of $HgCl_2$, ATP, and $MnCl_2$ inhibited the invertase activities almost completely.

CONCLUSIONS

A comparison of a number of physical and biochemical properties of carrot neutral and alkaline invertase showed

marked differences. The native polypeptides and their subunits have (a) different masses, (b) different pH optima, (c) different chromatographic behavior on HA-Ultrogel and green 19 dye columns, (d) different stability at −20°C, and (e) different behavior toward $CaCl_2$, $MgCl_2$, $MnCl_2$, and nucleotides. Although these findings suggest that neutral and alkaline invertase are the products of different genes, they appear to be immunologically related. Furthermore, the data presented do not exclude the possibility that neutral invertase is encoded by the same gene and is generated by differential splicing or proteolytic cleavage of alkaline invertase. A definite answer to this question can be given when cDNA clones for both enzymes are available.

The inhibition of both enzymes by their respective products Glc and Fru may be significant as a mechanism for the regulation of cytoplasmic Suc metabolism. Glc and Fru are substrates of hexokinase (EC 2.7.1.1) (Turner and Copeland, 1981) and fructokinase (EC 2.7.1.4) (Copeland et al., 1978). High activities of these enzymes reduce the concentrations of their substrates and thereby increase the cleavage of Suc by neutral and alkaline invertases. However, an increase in the concentration of Glc and Fru, for example, due to decreased demand for hexoses, would decrease Suc metabolism directly through inhibition of both invertases. This hypothetical regulation is consistent with a role for neutral and alkaline invertases in feeding Suc into cytoplasmic carbon metabolism. The inhibition of neutral invertase by MgATP further supports this hypothesis, although the MgATP concentration needed for 50% inhibition in vitro is above the physiological concentration of this nucleotide.

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