JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Purification and Biochemical Characterization of Insoluble Acid Invertase (INAC-INV) from Pea Seedlings

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ABSTRACT: Invertase (EC 3.2.1.26) catalyzes the hydrolysis of sucrose into D-glucose and D-fructose. Insoluble acid invertase (INAC-INV) was purified from pea (*Pisum sativum* L.) by sequential procedures entailing ammonium sulfate precipitation, ion exchange chromatography, absorption chromatography, reactive green-19 affinity chromatography, and gel filtration. The purified INAC-INV had a pH optimum of 4.0 and a temperature optimum of 45 °C. The effects of various concentrations of Tris-HCl, $HgCl_2$, and $CuSO_4$ on the activities of the purified invertase were examined. INAC-INV was not affected by Tris-HCl and $HgCl_2$. INAC-INV activity was inhibited by 6.2 mM $CuSO_4$ up to 50%. The enzymes display typical hyperbolic saturation kinetics for sucrose hydrolysis. The K_m and V_{max} values of INAC-INV were determined to be 4.41 mM and 8.41 U (mg protein)$^{-1}$ min^{-1}, respectively. INAC-INV is a true member of the β-fructofuranosidases, which can react with sucrose and raffinose as substrates. SDS-PAGE and immunoblotting were used to determine the molecular mass of INAC-INV to be 69 kDa. The isoelectric point of INAC-INV was estimated to be about pH 8.0. Taken together, INAC-INV is a pea seedling invertase with a stable and optimum activity at lower acid pH and at higher temperature than other invertases.

KEYWORDS: insoluble acid invertase, purification, characterization, *Pisum sativum* L.

INTRODUCTION

Invertase (β-D-fructosfuranosidase, EC 3.2.1.26) catalyzes the hydrolysis of sucrose into D-glucose and D-fructose, the main forms of carbon and energy supply in plant metabolism. Invertase is one of the important commercial enzymes used in the food industry and is known as the first biocatalyst in food biotechnology.[1] Invertase from different sources is used for the synthesis of fructose syrup, which has several industrial applications such as bee feeding, cookies, production of organic acids, chocolate, and production of alcohol. In addition to the application of invertase as a typical biocatalyst, in some conditions invertase can catalyze transfructosylation to produce fructooligosaccharides such as kestose, nystose, and fructofuranosylnystose. Especially fructooligosaccharides are well-known as neo-sugars and have numerous beneficial favorable functional properties; functional foods have become significant for the promotion of human health in modern society. Recently the understanding of the thermal denaturation of invertase and its related mechanisms has offered new possibilities in improving the potential and effective use of this enzyme in such diverse and broad areas.[1−4]

Sucrose is derived from photosynthetic products generated in tissues involved in carbon and energy fixation, called "sources". Sucrose flows through the plant vascular system to nonphotosynthetic or growing tissues, called "sinks". Among these translocatable compounds, sucrose, the disaccharide fructosyl glucose, is preeminent.[5] Sucrose may be actively transferred to the cell wall, where it is actively transported into the phloem by a sucrose−proton symporter,[6] or it may move into the phloem via the plasmodesmata. Movement of sucrose within the phloem requires mass flow. The catabolism of sucrose in sink tissues, primarily by invertase, generates the necessary gradient to drive this mass flow. Invertases bound to cell wall structures have repeatedly been suggested as a means to generate concentration gradients to drive apoplastic sucrose movements.[7,8]

Invertases are widely distributed in the plant world, and numerous studies describing them have been published.[9−15] Plant invertases can be classified into three subgroups on the basis of solubility, optimum pH, isoelectric point, and subcellular localization.[16] Therefore, there are three biochemical subgroups of invertases in plants: vacuolar (soluble acid), cytoplasmic (soluble alkaline) invertase, and cell wall bound (insoluble acid) invertases. The presence of multiple isoforms of invertase in nature is probably functionally beneficial to the plants.[17]

Insoluble acid invertase (INAC-INV) is a cell wall bound invertase and a glycosylated protein with variable molecular weights ranging between 28 and 64 kDa by SDS-PAGE. INAC-INV showed a pH optimum between 4.0 and 5.0 and an isoelectric point (pI) value around 9. INAC-INV revealed a K_m for sucrose between 1.2 and 5.5 mM.[15,18−20] Histochemically, using an invertase activity stain, INAC-INV was shown to be localized in the basal endosperm and pedicel tissues in maize kernels[21] as well as in cell walls by ionic linkages.[20,22] Using immunocytochemical techniques, INAC-INV was shown to be involved in the normal development of the

Received: March 16, 2011
Revised: July 19, 2011
Accepted: September 16, 2011
Published: September 16, 2011

dx.doi.org/10.1021/jf201057c | *J. Agric. Food Chem.* 2011, 59, 11228–11233

endosperm and maternal cells in the pedicel tissues in maize.[23] In studies of broad bean (*Vicia faba* L.) seed development, an INAC-INV was found in the thin walls of the seed coat parenchyma cells, known to be the site of sucrose unloading. Higher invertase activities were associated with higher hexose concentration.[24] In this paper, we report the first purification and biochemical characterization of INAC-INV from pea shoot seedlings to make anti-INAC-INV specific antibody as well as to study its role in plant physiology and development.

MATERIALS AND METHODS

Plant Material. Seeds of the garden pea, *Pisum sativum* L. cv. Little Marvel (dwarf) or Alaska (tall), were planted and grown in the greenhouse at Sungkyunkwan University, Korea. To obtain etiolated tissue, pea seeds were surface-sterilized by washing in a 10% Clorox (commercial solution of calcium hypochlorite) solution for 10 min before rinsing in sterile distilled water and planted in autoclaved vermiculite. The seeds were grown at room temperature in the dark for 7 days before treatment with 15 μM gibberellic acid (GA_3) solution. The sprayed plants were harvested after 2 days. The required tissues were harvested separately, weighed, and stored at −80 °C.

Reagents. All common reagents were of analytical grade. Acrylamide (electrophoresis grade), *N*,*N*′-methylene-bisacrylamide, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (Temed), and ammonium persulfate were purchased from Sigma-Aldrich (Seoul, Korea). Sodium dodecyl sulfate (SDS) was purchased from Sigma-Aldrich. Molecular weight markers, low range (from 6.5 to 66 kDa) and high range (36 to 205 kDa), were obtained from Sigma. Ampholytes were the Pharmalyte brand obtained from Sigma. Low EEO agarose was purchased from Fisher Biotech (Chicago, IL).

Crude Extract Preparation. All procedures were carried out at 4 °C. Fresh or cold-stored tissue was frozen with liquid nitrogen and powdered in a Sorval blender (Omni-Mixer 117350). The powder (500 g) was stirred with 2500 mL of extraction buffer. The tissue extraction buffer was 30 mM citrate/phosphate, pH 6.5, containing 0.2% β-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1 mM benzamidine as protease inhibitors. PMSF was added from a stock solution (100 mM in 100% isopropanol). The debris, washed exhaustively with prechilled distilled water followed by one wash with cold 100 mM NaCl, was extracted by stirring with 2 M NaCl in the cold room (4 °C) overnight to obtain the insoluble acid invertase. The crude extract was collected by squeezing through cheesecloth. Polyvinyl polypyrrolidone (1% w/v) was added to the filtrate, stirred for 30 min, and centrifuged at 10000*g* for 30 min. The supernatant was used for the purification of insoluble invertases.

Ammonium Sulfate Precipitation. After the initial precipitation from 10% saturated ammonium sulfate had been rejected, the supernatant was brought to 70% saturation and the precipitate was collected. After this precipitate had been dissolved in extraction buffer, the 45−70% fraction was collected for the acid enzyme.

Ion Exchange Chromatography. The sample, dissolved in HEPES buffer (pH 6.0, 10 mM), was applied to the CM-Sepharose cation-exchange column (10 × 2.5 cm) pre-equilibrated with the same buffer. After the unbound proteins had been washed from the column with equilibration buffer, the acid invertase activity was eluted with a gradient of 0−0.5 M NaCl.

Hydroxyapatite Chromatography. The sample, dissolved in 20 mM potassium phosphate buffer (pH 6.8), was applied to a hydroxyapatite chromatography column (10 × 2.5 cm) pre-equilibrated with the same buffer. After the unbound proteins had been washed from the column with equilibration buffer, invertase activity was eluted with a gradient of 20−500 mM potassium phosphate buffer, pH 6.8.

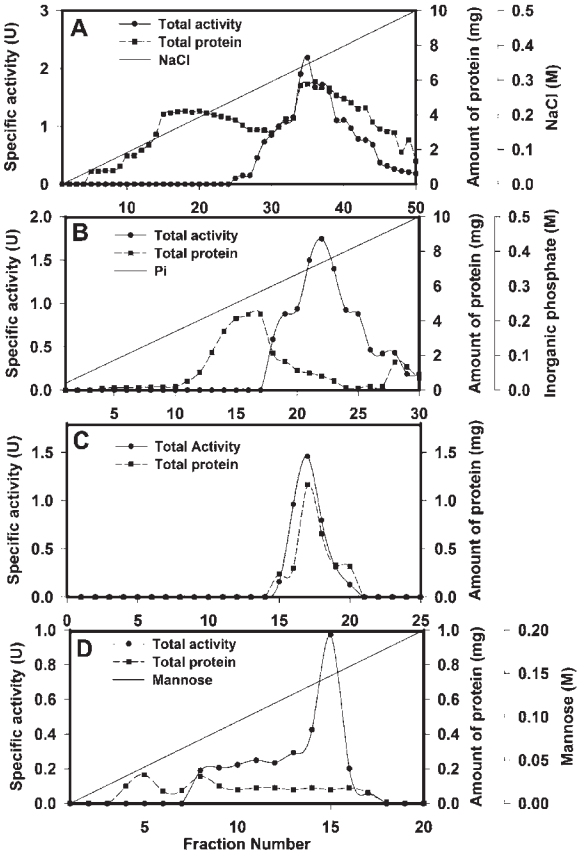

Figure 1. Purification of insoluble acid invertase from *Pisum sativum* L.: (A) elution profile from CM-Sepharose chromatography; (B) elution profile from hydroxyapatite chromatography; (C) elution profile from gel filtration chromatography; (D) elution profile from Con-A Sepharose chromatography. All experiments were performed three to five times, and results represent averages of individual data.

Gel Filtration Chromatography. The column (90 cm × 1.2 cm, Sephacryl 300) was equilibrated with HEPES buffer (10 mM HEPES, pH 6.8, containing 0.2% β-mercaptoethanol, 1 mM PMSF, 1 mM benzamidine, 500 mM NaCl).

Concanavalin-A (Con-A) Sepharose Chromatography. The sample dissolved in Con-A buffer (10 mM HEPES, pH 6.8, containing 0.2% β-mercaptoethanol, 1 mM PMSF, 1 mM benzamidine, 500 mM NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$) was applied to the column (10 × 1.5 cm) pre-equilibrated with the same buffer. After the unbound proteins had been washed from the column with equilibration buffer, invertase activity was eluted with a gradient of 0−200 mM methyl-D-mannose in the same buffer.

Invertase Assay. Invertase activity in tissue extracts or column separation fractions was determined by measuring the amount of reducing sugars by sucrose hydrolysis. For the standard assay of invertase, the final volume of digest solution was 1 mL of buffer, 50 mM citrate/phosphate, pH 6.5, containing 100 mM sucrose and 0.1 U of enzyme. Invertase assay was initiated by the addition of enzyme. The mixture was incubated at 37 °C for 60 min, followed by the addition of 1 mL of the dinitrosalicylate reagent (1% w/v 3,5-dinitrosalicylic acid, 1.6% w/v sodium hydroxide, and 30% w/v sodium potassium tartrate), which also served to stop the reaction.[25] This mixture was heated in a boiling water bath for 10 min and cooled to room temperature, and the absorbance was measured at 560 nm using a Beckman DU-40 spectrophotometer. The reducing sugar produced by invertase activity reacts with the dinitrosalicylic acid reagent, generating a red-orange color.

Table 1. Purification of Insoluble Acid Invertase Activity from *Pisum sativum* L.

purification step	volume (mL)	total protein (mg)	total activity (U)	specific activity (U/mg)	fold purification
crude extract	1300.0	1835.00	64.20	0.03	1.0
CM	60.0	50.80	14.50	0.29	8.2
hydroxyapatite	32.5	5.33	6.46	1.20	34.6
gel filtration	9.0	2.10	3.20	1.52	43.5
Con-A	2.0	0.08	0.97	12.13	346.5

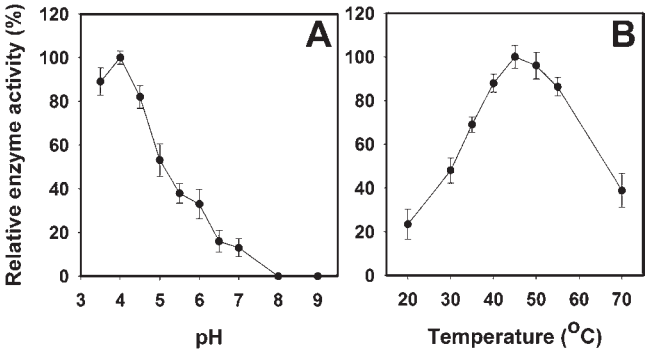

Figure 2. Effect of pH (A) and temperature (B) on activity of the insoluble acid invertase from *Pisum sativum* L.: (A) effect of pH on activity of pea invertases; (B) effect of reaction temperature on activity of pea invertases. All data were adjusted relative to the maximum activity (100%) for each enzyme. The values are the mean ± SE of three to five determinations.

A standard curve was prepared for an equi-molar mixture of glucose and fructose. A linear relationship between absorbance and glucose/fructose content covers the range from 0 to 1000 μM glucose or fructose per assay. One unit (U) of invertase activity was defined as the formation of 1 μM of reducing sugar from sucrose per minute at 37 °C. Specific activity was expressed as units of invertase activity per milligram of protein per minute.

Protein Assay. The protein content of extract solutions and column fractions was determined using the procedure of Bradford.[26] Reagents were obtained from Bio-Rad (Hercules, CA). Bovine serum albumin (BSA) was used as the standard protein in the range of 0−100 μg/assay. As a rough indicator of protein content in fractions collected from column chromatography, the absorbance of the solutions at 280 nm was measured.

Enzyme Characterization. The optimum pH for purified enzyme was determined over a pH range from 3.5 to 9.0. Incubation of sucrose at pH values of 3.5 or lower under the conditions used resulted in some nonenzymic hydrolysis. The assay mixture contained 100 μL of enzyme solution and 900 μL of reaction buffer supplemented by 50 mM citrate/phosphate buffer, 45 °C, and 50 mM sucrose to make 1 mL of solution. The reaction was conducted for 30 min at different pH values.

The optimum temperature for activity of purified enzyme was determined over the range of 20−70 °C at the optimum pH. Reaction mixtures (minus enzyme) were equilibrated at each temperature prior to initiation of the reaction by the addition of enzyme. Reactions were conducted for 30 min at different temperatures.

The substrate specificity of purified enzyme was tested by assaying for reducing sugars after incubation with sucrose (β-D-fructofuranulosyl-(2→1)-α-D-glucopyranoside), raffinose (α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-β-D-fructofuranoside), melezitose (α-D-glucopyranosyl-(1→3)-β-D-fructofuranosyl-α-D-glucopyranoside), and trehalose (α-D-glucopyranosyl-α-D-glucopyranoside).[27] Enzyme activity was assayed by measuring the production of reducing sugars. Substrate concentration was 50 mM. The amount of substrate hydrolysis was compared with that of sucrose at 100%.

The effect of a number of reagents, Tris/HCl, $CuSO_4$, and $HgCl_2$, variously reported to influence invertase activity,[28,29] was tested by assaying invertase activity in the presence of a range of concentrations of the reagents.

The enzyme kinetics of a reaction may indicate the nature of the enzyme-catalyzed reaction. The Michaelis−Menten constant (K_m) defines the substrate concentration at half-maximum velocity (V_{max}) of enzyme reaction and provides a means of comparing enzymes from different sources. For kinetic measurements the reaction mixtures consisted of a constant amount of enzyme, a range of sucrose concentration, in a constant volume of 50 mM citrate/phosphate buffer, pH 4.0, buffer. The purified protein (0.1 U) was incubated with increasing sucrose concentration. The reactions were carried out for 30 min at 45 °C. The K_m and V_{max} values were calculated by using a Lineweaver−Burk plot.

Gel Electrophoresis and Isoelectric Focusing. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis was carried out with a Hoefer Mini Slab Gel Unit (Hoefer Scientific Instrument Inc.) with a final gel concentration of 12% acrylamide from monomer stock solution (30% acrylamide, 2.7% *N,N′*-methylene-bisacrylamide). Isoelectric focusing polyacrylamide gel electrophoresis was performed using vertical tube gels or mini-slab gels. The slab gel contained 1.7 mL of PAGE monomer stock solution (which contains no SDS), 0.9 mL of glycerol, 0.5 mL of ampholytes (wide range, pH 3−10), 35 μL of initiator (10% ammonium persulfate), 25 μL of TEMED, and 0.6 mL of double-distilled water. The gel was pre-electrophoresed at 80 V for 10 min before the samples were added to each well.

Western Blotting/Immunoblotting. Protein sample was electrophoresed on denaturing SDS− or nondenaturing IEF−polyacrylamide gels. The electrophoresed protein was transferred to a nitrocellulose membrane using the protein blot apparatus (Hoefer Transfer TE 22 unit). After electrophoresis, the gel and nitrocellulose membrane were soaked in electrotransfer buffer (25 mM Tris, 190 mM glycine, 0.15% w/v SDS, and 20% v/v methanol). The nitrocellulose membranes were incubated with diluted serum (1:1000) or anti-invertase antibodies prepared from broad bean alkaline invertase. The washed membranes were treated with alkaline phosphatase assay solution. After color development, the membranes were rinsed with distilled water, air-dried, and stored in a desiccator until photographed.

Data Analyses. All data are presented as the mean ± SE. Group means were compared using one-way ANOVA followed by Duncan's multiple-range tests to identify differences among groups when appropriate. Two-group comparisons of control and Ni-exposed groups were carried out using Student's *t* test. All analyses were carried out using the statistic software GraphPAD Prism program (GraphPAD Software).

RESULTS AND DISCUSSION

Purification of Insoluble Acid Invertase. Insoluble acid invertase (INAC-INV) was extracted from the washed tissue residues with 10 mM HEPES buffer, pH 7.2, containing 1.0 M NaCl. The solution was dialyzed at 4 °C against 10 mM HEPES

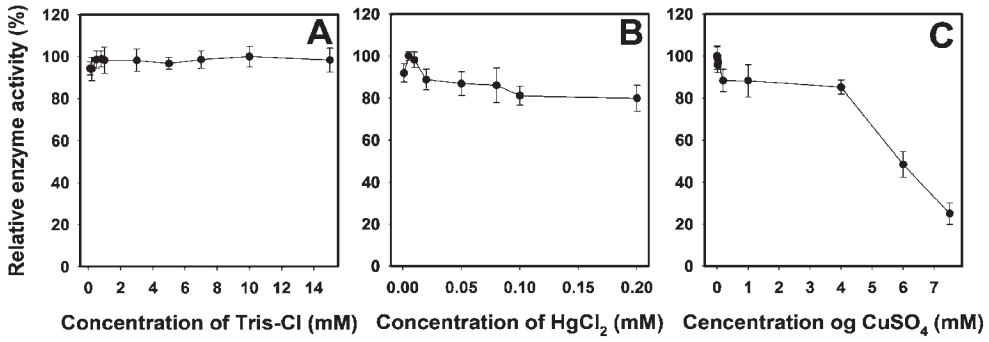

Figure 3. Effects of Tris-HCl (A), $HgCl_2$ (B), and $CuSO_4$ (C) on the activity of insoluble acid invertase from *Pisum sativum* L. In each assay, the inhibitor was preincubated with enzyme for 5 min before substrate (50 mM sucrose) was added to the reaction mixture. Results are expressed as percent initial activity. The values are the mean ± SE of three to five determinations.

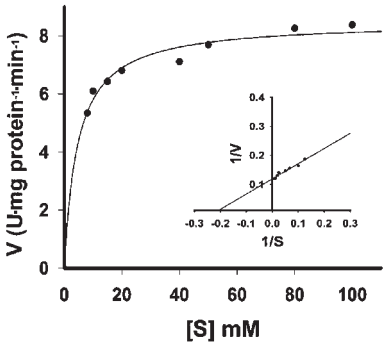

Figure 4. Saturation curves of insoluble acid invertase from *Pisum sativum* L. for sucrose. (Inset) Lineweaver–Burk plot. All experiments were performed three to five times, and results represent the average of individual data.

buffer, pH 6.0, to remove NaCl. The dialysate was passed through a CM-Sepharose column equilibrated with 10 mM HEPES buffer, pH 6.0. After the column had been washed with the same buffer used to remove unbound proteins, the bound activity was eluted with a linear increasing gradient of NaCl. A single peak of activity was observed (Figure 1A). Fractions containing high enzyme activity were concentrated by hydroxyapatite chromatography (Figure 1B). Fractions from the hydroxyapatite column containing high activity were concentrated by ultrafiltration and further purified by gel filtration chromatography (Figure 1C). Fractions containing high enzyme activity were combined and subjected to Con-A chromatography (Figure 1D). The results of a typical INAC-INV purification are presented in Table 1. The overall purification was 346-fold.

Characterization of Insoluble Acid Invertase. The optimum pH and temperature of INAC-INV were determined. INAC-INV showed activity in a range from pH 3.5 to 7.0. From Figure 2A, INAC-INV had optimum activity at pH 4.0. The activity of INAC-INV was examined from 20 to 70 °C (Figure 2B). INAC-INV had optimal activity at 45 °C.

We examined the effect of increasing concentrations of Tris-HCl, $HgCl_2$, or $CuSO_4$ on the activity of INAC-INV. Tris-HCl did not affect INAC-INV activity up to 14 mM (Figure 3A). INAC-INV activity was not affected by $HgCl_2$ (Figure 3B). The effect of $CuSO_4$ was less clear, but 6.2 mM $CuSO_4$ inhibited INAC-INV by 50% (Figure 3C). Studies on inhibition of pea enzyme activity by Hg, Cu, and Tris-HCl have shown that INAC-INV might not have sulfhydryl groups at catalytic sites.[30]

Table 2. Substrate Specificity of the Purified Invertases from *Pisum sativum* L.

substrate	acid invertase (%)	substrate	acid invertase (%)
sucrose	100	raffinose	13.45
melezitose	4.47	trehalose	N/D[a]

[a] ND, not detected.

Typical Michaelis–Menten kinetics was observed when the activity of INAC-INV was measured in sucrose concentrations up to 100 mM (Figure 4). The K_m values of the two enzymes for sucrose were determined via Lineweaver–Burk plot; the K_m and V_{max} values of INAC-INV were determined to be 4.41 mM and 8.41 U (mg protein)$^{-1}$ min^{-1}, respectively. Catalytic coefficiency (V_{max}/K_m) values for INAC-INV were 1.9. The kinetic properties of INAC-INV were similar to values for INAC-INV from several plants.[28]

To be hydrolyzed by invertase a substrate should contain an unsubstituted β-D-fructofuranosyl residue. We examined the abilities of the INAC-INV to hydrolyze a range of oligosaccharides. The results are expressed as a percentage of the substrate hydrolyzed relative to sucrose (Table 2). The tested oligosaccharides were raffinose, melezitose, and trehalose. Raffinose, a β-fructofuranoside, was hydrolyzed to about 40% of the rate at which sucrose was hydrolyzed. Melezitose, an α-fructofuranoside, was approximately 5% hydrolyzed, and trehalose, an α-glucopyranoside, was not detected. Hence, the sucrose-hydrolyzing enzymes isolated in this study appeared to be typical β-fructofuranosidase. This β-fructofuranosidase activity distinguishes between α- and β-linked fructose residues and is unable to hydrolyze glucose linkages (Table 2). This establishes the isolated enzyme as true invertase and not the α-glucosidase, sucrase.[31]

The molecular weight of INAC-INV was determined by SDS-PAGE and immunoblotting (Figure 5). Invertase preparation was separated by SDS-PAGE, followed by electrophoretic transfer to nitrocellulose membranes to be probed with antibodies against the known invertases. During the purification of INAC-INV, two bands at about 66 and 69 kDa showed an increase in relative intensity. Antibodies against carrot insoluble acid invertase[32] reacted with these two bands (Figure 5). The 66 kDa band might be the insoluble acid enzyme or perhaps an isoform of INAC-INV. Different isoforms of INAC-INV have been identified from radish hypocotyl[18,33] and carrot seedling.[34]

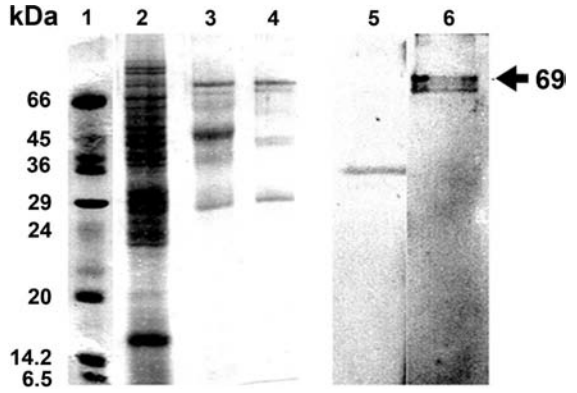

Figure 5. Molecular weight determination for insoluble acid invertases based on SDS-PAGE and immunoblotting. Approximately 20 μg of protein was added in lanes 2, 3, 4, and 6. The 10% gel was stained with Coomassie Blue (lanes 1−4). Lanes: 1 and 5, low molecular weight markers (the single band in lane 5 cross-reacts with the antibody against carrot insoluble invertase); 2, peak of invertase activity eluted from CM Sepharose; 3, peak of activity eluted from hydroxyapatite; 4, peak of activity eluted from Concanavalin-A Sepharose; 6, peak of activity eluted from Con-A. Immunoblot analysis was performed of proteins probed with anti-carrot insoluble invertase antibodies. The arrow indicates the position on the molecular weight scale of the band identified by this antibody.

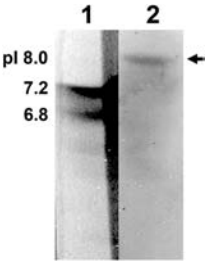

Figure 6. IEF-PAGE analysis of purified insoluble acid invertase from *Pisum sativum* L. Lanes: 1, standard p*I* markers stained with Coomassie blue; 2, immunoblot of INAC-INV probed with anti-carrot insoluble acid invertase antibody. The arrows indicate the position and estimated p*I* value for the INAC-INV in comparison with the standard markers.

However, in the present study, the presence of a single sharp band of activity on an IEF gel (Figure 6) suggests a unique enzyme, or two very similar molecules, with a common isoelectric point of pH 8.0 (Figure 6). Therefore, the apparent molecular size of the major polypeptide in the INAC-INV preparation was estimated to be approximately 69 kDa.

The purification of INAC-INV from many plant species has been reported. The binding of partially purified pea INAC-INV to CM-Sepharose, pH 6.0, and to Con-A indicates that this enzyme is glycosylated and has a relatively high p*I*. The pea INAC-INV has an apparent p*I* of about 8.0 (Figure 6). In general, INAC-INVs have been reported to have high p*I* values of pH 9.3 from *Urtica*,[35] 9.9 from carrot,[34] and 9.11 from *Arabidopsis*.[19] INAC-INV enzymes are generally glycosylated proteins.[7,36,37]

In conclusion, INAC-INV was purified from pea (*P. sativum* L.) by sequential biochemical processes. The purified INAC-INV had a pH optimum of <4.0 and a temperature optimum of 45 °C. INAC-INV displays typical hyperbolic saturation kinetics for sucrose hydrolysis. INAC-INV is a true member of the β-fructofuranosidases, which can react with sucrose and raffinose as substrates. Taken together, pea INAC-INV has a stable and optimum activity at relatively lower acid pH and at higher temperature than other invertases purified from other plant species.

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Funding Sources

This work was supported by grants from the Korea Ocean Research and Development Institute (No. PE9853B) and by grants from BioGreen 21 Project funded by Rural Development Administration of Korea (No. 20070401-034-028-009).

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