

## Characterization of Neutral Invertase from Fast Growing Pea (*Pisum sativum* L.) Seedlings after Gibberellic Acid (GA) Treatment

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Invertase ( $\beta$ -D-fructofuranosidase, EC 3.2.1.26) catalyzes the hydrolysis of sucrose into D-glucose and D-fructose. Three biochemical subgroups of invertases have been investigated in plants: vacuolar (soluble acid), cytoplasmic (soluble alkaline), and cell wall-bound (insoluble acid) invertases. An isoform of neutral invertase was purified from pea seedlings (*Pisum sativum* L.) and treated with gibberellic acid (GA) by sequential procedures consisting of ammonium sulfate precipitation, ion-exchange chromatography, absorption chromatography, and reactive green-19 affinity chromatography. The results of the overall insoluble invertase purification were a 430-fold increase. The purified neutral invertase was not glycosylated and had an optimum pH between neutral and alkaline (pH 6.8-7.5). It was inhibited by Tris, as well as by heavy metals, such as  $\text{Hg}^{2+}$  and  $\text{Cu}^{2+}$ . Typical Michaelis - Menten kinetics were observed when the activity of the purified invertase was measured, with sucrose concentrations up to 100 mM. The  $K_m$  and  $V_{max}$  values were 12.95 mM and 2.98 U/min, respectively. The molecular mass was around 20 kDa. The sucrose-cleaving enzyme activity of this enzyme is similar to that of sucrose synthase and fructosyltransferase, but its biochemical characteristics are different from those of sucrose synthase and fructosyltransferase. Based on this biochemical characterization and existing knowledge, neutral INV is an invertase isoform in plants.

**Key words** :  $\beta$ -D-fructofuranosidase, characterization, *Pisum sativum* L., neutral invertase, purification

### Introduction

Invertase ( $\beta$ -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. Invertases (INV) are widely distributed in the plant world and numerous studies describing them have been published [11, 14, 18, 19]. Plant invertases can be classified into different subgroups based on solubility, optimum pH, isoelectric point, and subcellular localization [23, 29]. In plants, three biochemical subgroups of invertases namely vacuolar (V-INVs), cell wall-bound (CW-INV), and cytoplasmic invertases (Cyto-INV) have been reported and characterized. The enzyme activities of these proteins were not inhibited by heavy metal ions such as  $\text{Hg}^{2+}$  and  $\text{Ag}^{2+}$  so that they might not have sulfhydryl groups at catalytic sites [27].

The Cyto-INV as a third type invertase is also called by

neutral/alkaline invertase (N/A-INV) due to their pH-optimum (pH 6.8-8.0) and subcellular localization [27]. Insoluble alkaline-INV is a true member of  $\beta$ -fructofuranosidase which can react with sucrose as well as raffinose as substrates. Recently, small amounts of N/A-INV genes were cloned and characterized. N/A-INVs, unlike acidic invertase, are not a member of fructofuranosidases because N/A-INVs are all non-glycosylated proteins and can hydrolyze preferentially or solely sucrose [27, 30, 34]. Their molecular weights range between 60 kDa and 70 kDa by SDS-PAGE. The isolated active enzyme is commonly described as a tetramer or octamer [3, 8, 21]. N/A-INVs seem to have other catalytic sites which are not shown in acidic invertase because their activities are strongly inhibited by glucose, fructose, or Tris but not inhibited by heavy metal ions [23].

Most known functions about invertases are specific to particular developmental stages [10]. Vacuolar (V)-INVs play a critical role in maintaining and regulating sugar balance in fruit tissues and mature tubers [25]. V-INV involves in the mobilization of vacuolar sucrose in sucrose-storing organ [12] and is necessary in normal root elongation of Arabidopsis [26]. The enzyme activity of CW-INV was increased when plants were wounded and attacked by pathogens as well as during early embryo development [28]. In

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maize, CW-INV has an important role in normal kernel development [4] and pollen tube extension [9].

Gibberellic acid (GA) has been reported to increase invertase activity and enhanced plant growth with soluble saccharides [29].

Although N/A-INV is considered to be involved in plant growth and development as a maintenance enzyme and thought to be indispensable in normal plant growth [10]. Generally neutral/alkaline invertases are believed to have low and unstable activity [23, 35] and it is very difficult to investigate the roles of neutral/alkaline invertases [2].

Although there has been little report about N/A-INV compared to other type of invertases. Recently, it was reported that N/A-INV could be localized in subcellular organelles such as chloroplast and mitochondria as well as cytoplasm [22, 31]. In addition, neutral/alkaline cytoplasmic invertase can be also localized in nucleus [13, 32]. Hence, the presence and biochemical nature of an invertase localized in subcellular organelles is under the special attention. Moreover, the important facts about the invertase localization in subcellular organelles suggest that invertases can be present not only as a soluble form but as an insoluble form. The insoluble neutral/alkaline N/A-INV invertase in plant system with the characteristics of neutral-to-alkaline pH optima (pH 6.8-8.0) and membrane-bound activity is expected. In this paper, I purified and characterized an insoluble neural INV from tissues of fast growing pea seedlings after gibberellic acid treatment.

## Materials and Methods

### Plant materials and reagents

Seeds of the garden pea, *Pisum sativum* L. cv. Little Marvel (dwarf) or Alaska (tall), were planted and grown in the greenhouse at Silla University, Korea. The surface sterilized seeds were grown at room temperature in the dark for 7 days before treatment with 15  $\mu$ M gibberellic acid (GA<sub>3</sub>) solution [14, 15]. The sprayed plants were harvested after 2

days. The required tissues were harvested separately, weighed and stored at -80°C. All common reagents were of analytical grade and were performed according to the methods of Kim et. al. [14, 15].

### Enzyme purification and characterization

For the enzyme purification, crude extract preparation, ammonium sulfate precipitation, DEAE-Sepharose anion-exchange chromatography, absorption chromatography, reactive Green-19 affinity chromatography, Q-Sepharose anion-exchange chromatography, and concentration methods were performed according to the methods of Kim et. al. [14, 15].

Determination of optimum pH and temperature, effect of a number of reagents (Tris-HCl, CuSO<sub>4</sub> and HgCl<sub>2</sub>), substrate specificity and enzyme kinetics were performed according to the methods of Kim et. al. [14, 15]. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed according to the methods of Kim et. al. [14, 15].

## Results

### Purification of insoluble invertase

The neutral invertase was obtained from the washed tissue residues after the soluble invertase activity had been removed. Insoluble invertase was extracted with enzyme extraction buffer containing 1 M NaCl. The protein sample in high-salt buffer solution was dialyzed in NaCl-free, 10 mM HEPES buffer (pH 7.2) at 4°C. The dialysate was passed through a DEAE-Sepharose column equilibrated with 10 mM HEPES buffer (pH 7.2).

The sequential procedures (data not shown) have been done with absorption chromatography, and reactive Green-19 affinity chromatography. The results of the typical IN-INV purification are presented in Table 1. The overall purification was 430-fold increase (Table 1).

### Characterization of neutral invertase

The optimum pH and temperature of the purified in-

Table 1. Purification of neutral invertase from *Pisum sativum* L.

Purification step	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold
Crude extract	1300.0	1835.00	24.00	0.0132	1.0
DEAE	45.5	18.70	2.20	0.1200	9.0
Green-19	13.8	1.30	1.70	1.3100	99.0
Hydroxyapatite	6.5	0.33	1.25	3.7900	287.0
Q anion exchanger	5.0	0.15	0.85	5.6700	430

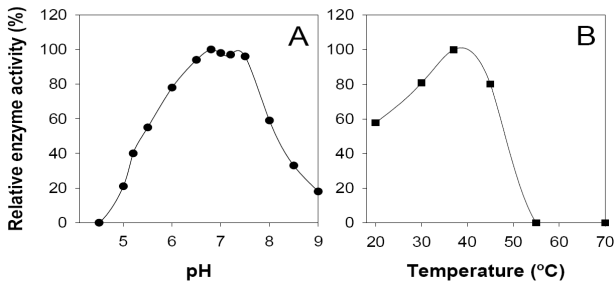


Fig. 1. Effect of pH A and temperature B on activity of the insoluble neutral invertase from *Pisum sativum* L. All data were adjusted relative to the maximum activity (100%) for each enzyme. All experiments were performed 3-5 times and results were represented by averages of individual data.

vertase were determined (Fig. 1). Enzyme showed activity in a range from pH 5 to 9. IN-INV had optimum activity at pH 6.8-7.5 (Fig. 1A). The activity of enzyme preparation was examined from 20°C to 70°C (Fig. 1B). IN-INV was most active at 37°C. We examined the effect of increasing concentrations of Tris-HCl, HgCl<sub>2</sub>, or CuSO<sub>4</sub> on the activity of the purified enzyme (Fig. 2). 10 mM Tris-HCl, 0.03 mM HgCl<sub>2</sub>, or 1.18 mM CuSO<sub>4</sub> inhibited IN-INV activity by about 50% (Fig. 2).

Typical Michaelis-Menten kinetics was observed when the activity of the purified invertase was measured sucrose concentrations up to 100 mM (Fig. 3). The K<sub>m</sub> and V<sub>max</sub> value of IN-INV were determined to be 12.95 mM and 2.98 U/min, respectively. Catalytic coefficient (V<sub>max</sub>/K<sub>m</sub>) value for IN-INV was 0.23.

A substrate should contain an unsubstituted β-D-fructofuranosyl residue. I examined the abilities of the purified IN-INV to hydrolyze a range of oligosaccharides. The results are expressed as a percentage of the substrate hydrolyzed

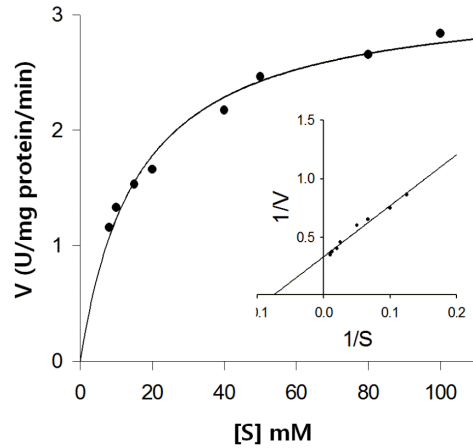


Fig. 3. Saturation curves of insoluble neutral invertase from *Pisum sativum* L. for sucrose. The inset shows the Lineweaver - Burk plot. All experiments were performed 3-5 times and results were represented by averages of individual data.

Table 2. Substrate specificity of the purified neutral invertase from *Pisum sativum* L.

Substrate	Enzyme activity (%)
Sucrose	100
Melezitose	3.08
Raffinose	66.10
Trehalose	*N/D

\*N/D, Not detected

relative to sucrose (Table 2). The tested oligosaccharides were raffinose (Gal-α-1,6-Glu-β-1,2-Fru), melezitose (Glu-α-1,3-Fru-α-1,2-Glu) and trehalose (Glu-α-1,1-Glu). Raffinose, a β-fructofuranoside, was hydrolyzed to about 66.1% of the rate at which sucrose (Glu-β-1,2-Fru) was hydrolyzed. Melezitose, an α-fructofuranoside, was approximately 3.08% hydrolyzed and trehalose, an α-glucopyranoside, was not

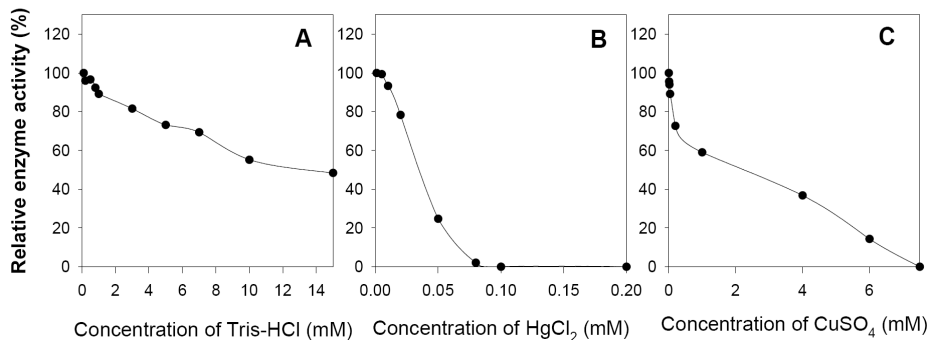


Fig. 2. Effects of Tris-HCl (A), HgCl<sub>2</sub> (B) and CuSO<sub>4</sub> (C) on activity of insoluble neutral invertase from *Pisum sativum* L. In each assay, the inhibitor was pre-incubated with enzyme for 5 min before substrate (50 mM sucrose) was added to the reaction mixture. Results are expressed as % initial activity. All experiments were performed 3-5 times and results were represented by averages of individual data.

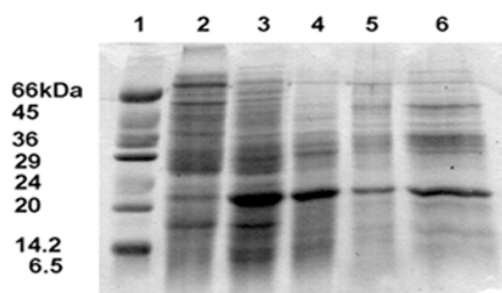


Fig. 4. SDS-PAGE analysis of protein samples from various stages of insoluble neutral invertase purification. Approximately 20  $\mu$ g of protein were added to lanes 2 - 6. One band around 20kDa was gradually increased. The 10% gels were stained with Coomassie Blue. Lane 1, low molecular weight marker; lane 2, buffer/NaCl extract after dialysis; lane 3, peak of invertase activity eluted from DEAE-Sephadex; lane 4, peak of activity eluted from Green 19; lane 5, peak of activity eluted from hydroxyapatite; lane 6, invertase activity eluted from Q-Sephadex.

detected for hydrolyzation (Table 2). Hence, the sucrose-hydrolyzing enzymes isolated in this study appeared to be typical  $\beta$ -fructofuranosidases.

The molecular weights of the purified invertases were determined around 20 kDa by SDS-PAGE (Fig. 4).

## Discussion

To isolate this enzyme from cell residue, the purification steps were performed after the readily insoluble protein was washed with HEPES buffer (pH 7.2) containing 1M NaCl. And then IN-INV proteins were sequentially eluted by DEAE- and Q-Sephadex anion exchanger, hydroxyapatite chromatography, and Green-19 adsorption chromatography. This cytoplasmic isozyme has apparently non-glycosylated (data not shown) because IN-INV did not react with concanavalin-A sephadex chromatography according to Lee and Sturm [21]. This enzyme has been purified and the product contains a complex mixture of polypeptides and possibly several related proteins such multi-mer structures as a tetramer [3, 8, 21]. SDS-PAGE results indicated two major polypeptides with molecular weights of 20 and 80 kDa after octyl-agarose chromatography (data not shown). IEF-PAGE yielded a relatively diffuse band of activity and a broad band reacting with the antibody against broad bean IN-INV (data not shown). We will study more for understanding these problems in a future.

The studies on inhibition of pea invertase activity by  $Hg^{2+}$ ,

$Cu^{2+}$ , or Tris have shown that IN-INV has properties consistent with those of other previously reported invertases [21, 24]. In the present study, pea IN-INV activity was strongly inhibited by  $Cu^{2+}$ , similar to what was observed for the activities of alkaline invertase from soybean nodules, carrot suspension cultured cells, and pea seedling [8, 14, 21]. Pea IN-INV was completely inhibited by  $Hg^{2+}$ , suggesting that one or more reduced sulfhydryl groups might be essential for the activity.

The kinetic properties of alkaline invertases were similar to those of other plants. Apparently, the responses of the enzymes to increasing sucrose concentrations followed Michaelis-Menten kinetics. The  $K_m$  value of IN-INV for sucrose was determined to be 12.95 mM in this study (Fig. 3). For other plants, the  $K_m$  values for alkaline invertase have been reported to range from 8.9 to 65 mM [3, 8, 21, 24]. IN-INV showed a selective  $\beta$ -fructofuranosidase activity, displaying clear substrate preference for sucrose and  $\beta$ -fructofuranosides. This  $\beta$ -fructofuranosidase activity distinguishes  $\alpha$ -linked fructose residues from  $\beta$ -linked fructose residues and  $\beta$ -fructofuranosidase is unable to hydrolyze glucose linkages (Table 2). This establishes the IN-INV as a true invertase and not  $\alpha$ -glucosidase, sucrase.

If IN-INV is localized in cell walls, the optimum pH should be acidic. However, in this work, IN-INV was extracted with high salt buffer and the optimum pH of IN-INV was pH 6.8-7.5. IN-INV seems to be strongly bound to cytoplasmic membranous components.

In addition to invertases, two types of sucrose cleaving enzymes, sucrose synthase and fructosyltransferase, have been reported [1, 7, 16, 23, 33]. Sucrose synthase was subcellularly localized in cytosol and was also found as a transiently membrane-bound form at plasma membrane and tonoplast membrane [16]. Fructosyltransferase showed fructan biosynthetic and degrading enzyme activity and sucrolytic activity in cytosol. Fructosyltransferase revealed the similar sequence homology with V-INV [7, 23, 33]. These two enzymes are soluble proteins and their optimal pH is acidic (pH 6.0-6.5 for sucrose synthase and pH 5.0-6.0 for fructosyltransferase). In this study, we purified and characterized a sucrose cleaving enzyme from pea seedlings. This enzyme is similar to sucrose synthase and fructosyltransferase in sucrose cleaving enzyme activity, but its biochemical characteristics are different from those of sucrose synthase and fructosyltransferase. Taken together based on the biochemical characterization and our knowledge, IN-INV is the

characterized membrane-bound isoform among neutral invertases in pea.

## Acknowledgement

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**초록 : GA 처리 후 급 성장하는 완두콩(*Pisum sativum* L.) 발아체로부터 분리된 중성 invertase의 특성**

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Invertase ( $\beta$ -D-fructofuranosidase, EC 3.2.1.26)는 설탕을 포도당과 과당으로 가수분해하는 반응을 촉매한다. 3종류의 invertases [액포(수용성 산), 세포질(수용성 알칼리) 및 세포벽 결합]가 식물에서 연구되어 왔다. 우리는 순차적인 ammonium sulfate 침전, 이온교환크로마토그래피, 흡수크로마토그래피, Green-19 친화크로마토그래피 과정을 통해 완두콩(*Pisum sativum* L.) 발아체로부터 중성 invertase의 세포막 연결 isoform을 430배 순수 분리하였다. 분리된 세포막과 결합된 insoluble invertase (IN-INV)는 최적 pH는 중성에서 알칼리 사이(pH 6.8-7.5)로 나타났다. 이 효소는 Tris 뿐만 아니라  $Hg^{2+}$  and  $Cu^{2+}$ 와 같은 중금속에 의해 저해되었다. IN-INV의  $K_m$ 과  $V_{max}$  값은 각각 12.95 mM과 2.98 U/min으로 측정되었다. IN-INV는 기질로써 과당뿐만 아니라 라피노오스와 반응하기 때문에 진정한  $\beta$ -fructofuranosidase로 판명되었다. IN-INV의 분자량 20 kDa이었다. 위 결과로 볼 때 GA 영향으로 급속히 자라는 발아체에서 단백질이 분리되었는데 특징적으로 invertase였다.