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Glutathione peroxidase-like activity of 33 kDa trypsin inhibitor from roots of sweet potato (*Ipomoea batatas* [L.] Lam 'Tainong 57')

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Abstract

The hydrogen peroxide, glutathione (GSH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and phenazine methosulfate (PMS) were used for spectrophotometric assay of commercial glutathione peroxidase (GPx) activity at 570 nm. A positive correlation ($r^2 = 0.998$) was found between level of GPx activity and the absorbance changes (%). The 33 kDa trypsin inhibitor (TI) was purified from the storage roots of sweet potato (SP) (*Ipomoea batatas* [L.] Lam 'Tainong 57') by trypsin-Sepharose 4B affinity chromatography and preparative acrylamide gel electrophoresis. The first 15 amino acids in the N-ternimal region of 33 kDa TI were SSETPVLDINGDEVR, which were identical to that of deduced sequence of sopramin A or B. A positive correlation ($r^2 = 0.979$) was found between the amounts of 33 kDa TI added and absorbance changes. Absorbance changes of 5.18, 16.35 and 25.83%, respectively, were obtained when 100, 200 and 250 μg 33 kDa TI were added, which were equal to 0.56, 1.21 and 1.76 GPx units. Using TI and GPx activity stainings, it was confirmed that 33 kDa TI exhibited GPx-like activity. The physiological significance of TIs with GPx activities is discussed.

Keywords: Glutathione (GSH); Glutathione peroxidase (GPx); *Ipomoea batatas* (L.) Lam; Trypsin inhibitor (TI)

1. Introduction

Glutathione peroxidase (GPx, EC 1.11.1.9) is a family of multiple isozymes involved in scavenging oxygen radicals in animals including four distinct types, namely, classical (or cytosolic) GPx, phospholipid hydroperoxide GPx, gastrointestinal GPx, and plasma GPx [2]. GPx consists of either 20 kDa monomer or homotetramers of a 23 kDa subunit, and each isozyme has distinct properties with respect to substrate specificity and tissue and organelle expression [2]. GPx reduces reactive oxygen species such as hydrogen peroxide and alkyl hydroperoxide at the expense of GSH to remove cytotoxicity. In doxorubicin-treated human breast carcinoma cells, the overexpressed GPx could prevent ceramide production and partially inhibite apoptosis [3]. Plant extracts with increased GPx-like activity were also observed during cold treatment of apple [4]. In contrast to the mammalian GPx, only few plant GPxs have been isolated [5,6], including a 16 kDa one from *Aloe vera* [7] and a 22 kDa one from *Citrus sinensis* [8]. Lin et al. [9] reported a GPx activity staining method on acrylamide gels using hydrogen peroxide, glutathione (GSH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and phenazine methosulfate (PMS). Hou et al. [10] used this method to stain GPx activities in leaves of three cultivars of *Liriope spicata* L.

Proteinaceous protease inhibitors in plants may be important in regulating and controlling endogenous proteases and in acting as protective agents against insect and/or microbial proteases [11,12]. Sohonie and Bhandarker [13] reported for the first time the presence of trypsin inhibitors (TIs)

Abbreviations: APS, ammonium persulfate; Bis, *N*,*N*′-methylenediacrylamide; CBG, commassie brilliant blue G-250; GSH, glutathione; GPx, glutathione peroxidase; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SP, sweet potato; TEMED, *N*,*N*,*N*′,*N*′-tetramethylenediamine; TI, trypsin inhibitor

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in sweet potato (SP). Later, we indicated that TI activity levels in SP are positively correlated with concentrations of water-soluble proteins [14], and that a large negative correlation exists between the natural logarithm of TI activity levels and cumulative rainfall, which suggests that SPTI activity may vary in response to drought [15]. Polyamines, including cadaverine, spermidine and spermine, bound covalently to SPTI that might participate in regulating the growth and developmental processes of SP [1]. SPTIs were also shown to have both dehydroascorbate reductase and monodehydroascorbate reductase activities, and that they might respond to environmental stresses [16]. Recently, we found that SPTI could regulate one endogenous serine-type proteinase activity from sweet potato [17]. TIs in SP roots accounted for about 60% of total water-soluble proteins and could be recognized as storage proteins [14]. Maeshima et al. [18] identified sporamin as the major storage protein in SP root, which accounted for 80% of total proteins in root; a dramatic decrease to 2% of the original value was found during sprouting. Lin [19] proposed that sporamin was one form of TIs in SP, which was confirmed later by Yeh et al. [20]. In this work, we used spectrophotometric and activity-staining methods based on hydrogen peroxide, GSH, MTT and PMS to determine GPx activity, and found that SPTI (33 kDa TI) exhibited GPx-like activity. The physiological significance of this finding is discussed.

2. Materials and methods

2.1. Plant materials and TI purification

Fresh roots of SP (*Ipomoea batatas* (L.) Lam 'Tainong 57') were purchased from a local market. Extraction and purification processes were according to Hou and Lin [1] as follows. The storage roots were cut into strips that were extracted immediately with four volumes (w/v) of 100 mM Tris–HCl buffer (pH 7.9) containing 100 mM NaCl, 1% (w/v) ascorbate and 1% (w/v) polyvinylpolypyrrolidone. After centrifugation twice at 14,000 × *g*, the crude extracts were loaded directly on to a trypsin-Sepharose 4B affinity column, and the adsorbed TIs were eluted by changing pH value with 0.2 M KCl buffer (pH 2.0) [1]. Preparative 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were used to isolate the 33 kDa TI after trypsin-affinity column purification. After electrophoresis, sodium dodecylsulfate was removed [17] and the 33 kDa TI band on the gel was cut and extracted with 100 mM Tris–HCl buffer (pH 7.9) overnight. The extracts were desalted and concentrated with Centricon 10 and then lyophilized for further use.

2.2. Electroblotting and protein sequencing

The purified 33 kDa TI was subjected to 15% SDS-PAGE according to Laemmli [21]. After electrophoresis, gels were equilibrated in 250 mM sodium borate, pH 9.6, 0.5% (w/v) SDS and transferred to an Immobilon PVDF membrane (Millipore, Bedford, MA). A 33 kDa TI band was cut off with a sharp razor blade for protein sequencing. Automated cycles of Edman degradation were performed with an Applied Biosystems gas/liquid phase Model 470A/900A sequencer with an on-line Model 120A phenylthiohydantoin-amino acid analyzer [22].

2.3. Determination of GPx activity by spectrophotometry

The GPx activity was determined as follows. Commercial GPx (1.25–5 U) in 100 mM Tris–HCl buffer (pH 8.0) was added to a solution containing 390 μM PMS, 32 μM MTT, 10 μl of 0.2% hydrogen peroxide and 500 μg GSH with a final total volume of 1.5 ml. Means of duplicate absorbance changes at 570 nm during 5 min (ΔA570/min) were recorded. Reaction solutions without GPx were used as controls. Regression curves between GPx activity levels and absorbance changes (%) were plotted. The absorbance changes (%) were calculated with the equation: $[(\Delta A570_{control}/min) - (\Delta A570_{enzyme}/min)] \div (\Delta A570_{control}/min) \times 100\%$. The 33 kDa SPTI was added (100, 200 and 250 μg) instead of GPx for GPx-like activity assays.

2.4. Protein, TI activity and GPx activity staining of SPTIs on 12.5% SDS-PAGE gels

Four parts of samples were mixed with one part of sample buffer, namely 60 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 25% glycerol and 0.1% bromophenol blue without 2-mercaptoethanol for TI and GPx activity stainings at 4 °C overnight. Coomassie brilliant blue G-250 was used for protein staining [23].

After electrophoresis, gels were washed with 25% isopropanol in 10 mM Tris–HCl buffer (pH 7.9) for 10 min twice to remove SDS [24]. For SPTI activity staining, the gel was stained according to the method of Hou and Lin [24]. For GPx activity staining, the gel was stained according to the method of Lin et al. [9].

2.5. Chemicals

Commercial GPx from bovine erythrocytes (500 U, 616 U/mg protein, G-6137), GSH, GSSG, MTT, PMS, hydrogen peroxide solution (30%) were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acrylamide, *N*,*N*′-methylenediacrylamide (Bis), *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED), ammonium persulfate (APS), Tris and ammonium sulfate were obtained from E. Merck Inc. (Germany). See Blue™ pre-stained standard kit for SDS-PAGE was from Invitrogen Co. (Carlsbad, CA, USA). The kit contains myosin (250 kDa), BSA (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin (30 kDa),

lysozyme (16 kDa), aprotinin (6 kDa) and insulin B chain (4 kDa).

3. Results

3.1. Determination of GPx activity by spectrophotometry

The GPx activity determination was modified from Ukai and Sekiya [25] and was based on sulfur-containing compounds, such as GSH, being able to reduce MTT in the presence of PMS to form formazan. Lin et al. [9] used this method to develop GPx activity staining in which the clear zone of GPx activity against the purple background was found in both native and SDS-PAGE gels. We further modified the activity staining method [9] to suit GPx activity determination by spectrophotometry (Fig. 1). From the results of Fig. 1A, it was found that the more GPx added the less absorbance at 570 nm was obtained. From calculations, the absorbance changes (%) were positively correlated ($r^2 = 0.988$) with levels of GPx activity (Fig. 1A, inset). The effects of GSH and hydrogen peroxide on GPx assay system were shown at Fig. 1B. Without GSH, the MTT in the reaction mixture was not reduced even in the presence of hydrogen peroxide and PMS. Omitting hydrogen peroxide in this assay system did not affect the MTT reduction; however, GPx activity (1.25 U) could not be detected compared to the control (Fig. 1B). It is clear that this new assay system is suitable for GPx activity determinations.

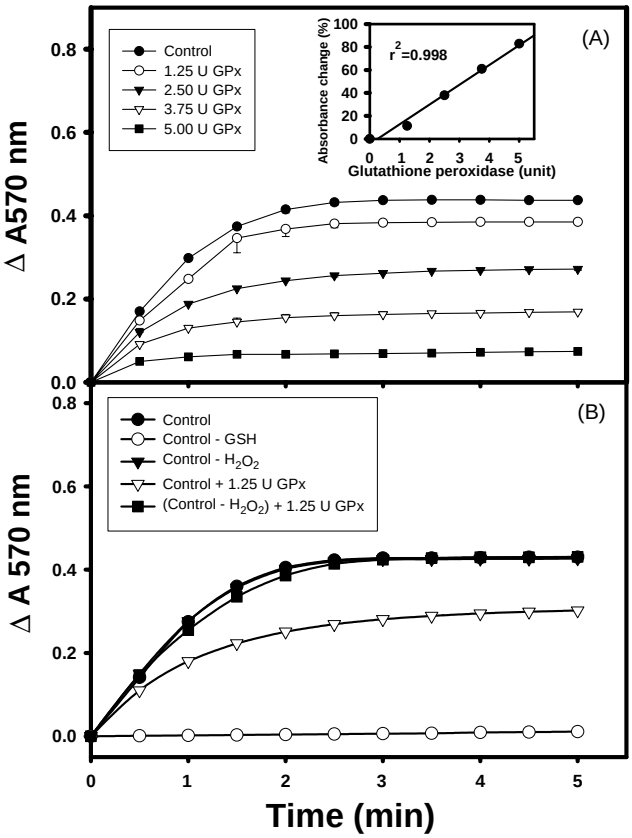

Fig. 1. (A) The commercial glutathione peroxidase (1.25, 2.50, 3.75 and 5.0 units) activity was determined by spectrophotometry. (B) The effects of GSH and hydrogen peroxide on glutathione peroxidase assay system. The absorbance readings at 570 nm during 5 min were recorded. Reaction solutions without glutathione peroxidase were used as controls. The regression curves between levels of glutathione peroxidase activity and absorbance changes (%) at 570 nm were plotted (inset), and the absorbance changes (%) were calculated based on the equation: $[(\Delta A570_{control}/min) - (\Delta A570_{enzyme}/min)] \div (\Delta A570_{control}/min) \times 100\%$.

3.2. GPx-like activity of 33 kDa SPTI by spectrophotometry

Under the same assay system, the purified 33 kDa SPTI after trypsin-affinity column and preparative SDS-PAGE was used for GPx-like activity determinations (Fig. 2). In the first, the purified 33 kDa TI was used to analyze the N-terminal amino acid sequence. The first 15 amino acids in the N-ternimal region of 33 kDa TI were SSETPVLDINGDEVR, which were identical to that of deduced sequence of sopramin A or B, which was reported to exhibit trypsin inhibitory activity [20]. When 100, 200 and 250 μg 33 kDa SPTI were added instead of GPx, there were 5.18, 16.35 and 25.83% absorbance changes, respectively. A positive correlation ($r^2 = 0.979$) between amounts of 33 kDa TI (μg) and absorbance changes (%) was found (Fig. 2 inset). By calculating from the linear regression of Fig. 1 (inset), the 100, 200 and 250 μg TI, respectively, was equal to 0.56, 1.21 and 1.76 GPx units. In average, one mg 33 kDa SPTI was equal to about 6.23 GPx Units.

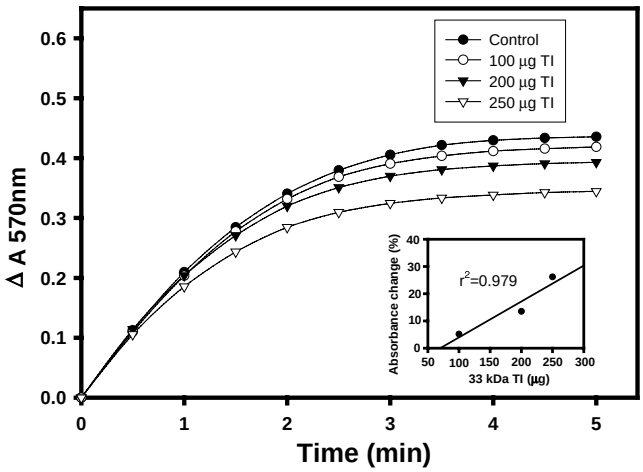

Fig. 2. Glutathione peroxidase-like activity of sweet potato 33 kDa trypsin inhibitor determined by spectrophotometry. Sweet potato trypsin inhibitor of 100, 200 and 250 μg were added instead of glutathione peroxidase. The absorbance readings at 570 nm during 5 min were recorded. Reaction solutions without glutathione peroxidase were used as controls. The regression curves between amounts of 33 kDa trypsin inhibitor and absorbance changes (%) at 570 nm were plotted (inset), and the absorbance changes (%) were calculated based on the equation: $[(\Delta A570_{control}/min) - (\Delta A570_{enzyme}/min)] \div (\Delta A570_{control}/min) \times 100\%$.

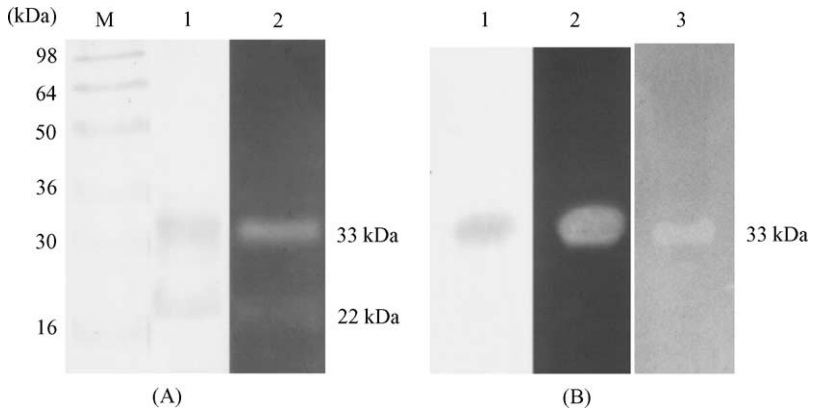

Fig. 3. Protein (lane 1), trypsin inhibitory activity (lane 2), and glutathione peroxidase activity (lane 3) stainings of sweet potato trypsin inhibitors on 12.5% SDS-PAGE gels (A) after the trypsin-affinity column and (B) then after the preparative acrylamide gel. Each lane contained 20 μg purified SPTIs. "M" indicated the see Blue™ pre-stained markers for SDS-PAGE.

3.3. Protein, TI activity and GPx activity staining of SPTIs on 12.5% SDS-PAGE gels

Fig. 3 showed protein (lane 1), TI activity (lane 2) and GPx activity (lane 3) stainings of SPTIs on 12.5% SDS-PAGE gels after the trypsin-affinity column (Fig. 3A) and then after the preparative acrylamide gel (Fig. 3B). Each lane contained 20 μg purified SPTI. After affinity column purification, two SPTI activity bands (22 and 33 kDa) were found (Fig. 3A). Therefore, the preparative SDS-PAGE gels were used to purify the major band (33 kDa SPTI). From the GPx activity staining (lane 3, Fig. 3B), it is clear that 33 kDa SPTI exhibited both trypsin inhibitory (lane 2, Fig. 3B) and GPx-like activities.

3.4. Purity of protein preparation used in Fig. 3B

The first 15 amino acids in the N-ternimal region of 33 kDa TI were SSETPVLDINGDEVR, which were identical to that of the deduced sequence of sopramin A or B being reported to exhibit trypsin inhibitory activity [20]. It means that the purified 33 kDa protein is indeed one form of sweet potato TIs. If there is any contaminant protein in our sample, then its amount is beyond the detectable limit of N-terminal amino acid determination.

4. Discussion

Although GPx is important for its protective role in cells [3–7,26–30], few plant GPxs have been isolated [5,6], e.g. one with 16 kDa from *Aloe vera* [7] and another one with 22 kDa from *Citrus sinensis* [8]. Lin et al. [9] proposed a GPx activity staining method on acrylamide gels, by which different isozymes of GPx in ammonium sulfate fractions of ginger were found. We reported here for the first time that the 33 kDa SPTI displayed unique GPx activity detected by both spectrophotometric and activity staining methods. From N-terminal amino acid sequence, the first 15 amino acids in the N-ternimal region of 33 kDa TI were identical to that of deduced sequence of sopramin A or B, which was reported to exhibit trypsin inhibitory activity [20].

The mammalian GPx was a homotetramer selenoprotein with four cysteine residues on each subunit [31]. The monomer of GPx was shown to consist of 198 amino acids with molecular mass about 21,900 Da [31]. It was reported that the substituted selenocysteine for catalytic cysteine 41 enhanced enzymatic activity of plant phospholipid hydroperoxide GPx [6]. Yeh et al. [20] reported that the deduced amino acid sequence from sporamin cDNA contained four cysteine residues with molecular mass of 22 kDa. From the TI activity staining (lane 2, Fig. 3A), the SPTIs purified by trypsin-Sepharose 4B exhibited two trypsin inhibitory activity bands. After the peparative polyacrylamide gel step, the 33 kDa SPTI exhibited GPx activity (lane 3, Fig. 3B). From calculations, 100, 200 and 250 μg 33 kDa TI, respectively, were equivalent to 0.56, 1.21 and 1.76 GPx units (Fig. 2). On average, one mg 33 kDa SPTI was equivalent to 6.23 GPx units.

Maeshima et al. [18] identified sporamin as the major storage protein in SP root, which accounted for 80% of total proteins, and about 4.41 mg/g storage roots. Lin [19] proposed that sporamin should be one form of TIs in SP, which was confirmed later by Yeh et al. [20]. We reported recently that SPTIs mimicked dehydroascorbate reductase [16] to reduce dehydroascorbate to regenerate ascorbate and intermolecular thiol-disulfide interchanges occurred during the catalytic reaction. Kobrehel et al. [32] suggested that in the absence of compartmental barriers, some protein inhibitors, including Kunitz type TIs and Bowman-Birk type TIs, can be reduced within the cell. The multifunctions played by TIs, which occur in extremely high amounts, might be beneficial for SP root tissues or cells under stress conditions. Lin et al. [33] purified cytosolic Cu/Zn-superoxide dismutase from SP variety Tainong 57. The Cu/Zn-superoxide dismutase (EC 1.15.1.1) catalyzes the dismutation of superoxide

to dioxygen and hydrogen peroxide to protect the organisms from oxidative damage [34]. The peroxide-scavenging was accomplished through the ascorbate-glutathione pathway [35–37] or peroxidase (such as GPx). Our results of SPTI's GPx-like activity in this paper, together with SPTIs' activities of dehydroascorbate reductase and monodehydroascorbate reductase [16], are indicative of their roles in SP roots against environmental oxidative stresses.

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