行政院國家科學委員會專題研究計畫成果報告

計畫編號：NSC 89-2313-B-038-002
執行期限：89 年 10 月 01 日至 90 年 07 月 31 日
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一、中文摘要

文獻中指出，Trumper 等人 (1994)，從菠菜葉綠體中所純化的去氫抗壞血酸還原酶 (dehydroascorbate reductase) 的 N-端的17個胺基酸序列與大豆胰蛋白酶抑制因子 (trypsin inhibitor) 完全相同。經過氧化處理的去氫抗壞血酸還原酶喪失還原去氫抗壞血酸的能力，但是獲得部分的胰蛋白酶抑制活性；同樣地，經過還原處理的大豆胰蛋白酶抑制因子喪失其胰蛋白酶抑制的能力，但是獲得部分還原去氫抗壞血酸的能力。本計畫的目的將以甘藷(*Ipomoea batatas* [L.] Lam cv. Tainong 57) 的塊根為實驗材料，純化其儲藏性蛋白質----胰蛋白酶抑制因子，測試甘藷胰蛋白酶抑制因子是否具有去氫抗壞血酸還原酶活性，並推測其可能扮演的生理意義與角色。

關鍵詞：甘藷；儲藏性蛋白質；胰蛋白酶抑制因子；去氫抗壞血酸還原酶；生理意義

Abstract

Trypsin inhibitors (TIs) were purified from the storage roots of sweet potato (*Ipomoea batatas* [L.] Lam var. Tainong 57) with ammonium sulfate precipitation, Sephadex G-75 gel filtration chromatography, and trypsin-Sepharose 4B affinity chromatography as previously reported [1]. Dehydroascorbate (DHA) was reduced by TIs independent of glutathione to regenerate ascorbate (AsA) contrasting with the function of DHA reductase with glutathione as a cofactor. Intermolecular thiol-disulfide interchanges of TIs were found during DHA reduction. AsA was oxidized by AsA oxidase to generate monodehydroascorbate (MDA) free radicals. MDA was reduced also by TIs to AsA in the presence of NADH, which was the function of MDA reductase. The physiological significance of TIs with both DHA reductase and MDA reductase activities are discussed.

Keywords: Dehydroascorbate (DHA); DHA reductase; *Ipomoea batatas* (L.) Lam; monodehydroascorbate (MDA); MDA reductase; Trypsin inhibitor (TI)

二、緣由與目的

Protease protein inhibitors in plants may be important in regulating and controlling endogenous proteinases, in serving as storage proteins, and in acting as protective agents against insect and/or microbial proteinases [2,3]. Ever since Sohonie and Bhandarker [4] reported the presence of trypsin inhibitors (TIs) in sweet potatoes (SP), little information has been available concerning their physiological functions. We have indicated that TI activities in SP are positively correlated with water-soluble protein concentrations [5], and that a large negative correlation exists between the natural logarithm of TI activities and cumulative rainfall, which suggests that TI activities may vary in response to drought of SP [6]. Biosynthesis of TIs may be enhanced or induced by heat, exogenous plant growth regulators [7], and water-deficiency [8]. Maeshima et al. (1985) [9] identified sporamins, which accounted for 60-80% of total soluble proteins, as the major storage protein in tuberous roots of SP. However, we also found that TIs in SP variety Tainong 57 accounted for about 60% of total soluble proteins [10]. Comparing the many properties of TIs with those of sporamins in the literature, we proposed that TIs are actually the same as sporamins [11], a proposal which was later confirmed by Wang and Yeh [12].

Patatin, the potato tuber storage protein, has been demonstrated to have lipid acyl hydrolase and acyltransferase activities that are involved in tuber tissue response to wounding [13]. The soybean vegetative storage proteins VSP α and VSP β both have acid phosphatase activity [14]. Trümper et al. [15] found that the N-terminal amino acid sequence of DHA reductase (EC 1.8.5.1) purified from spinach chloroplasts is homologous to soybean TI. Both the reduced (thiol) form of soybean TI, which was reduced by dithiothreitol, and the native form (thiol) of spinach chloroplast DHA reductase can reduce DHA to regenerate AsA in the presence of glutathione; meanwhile, the oxidized (disulfide) form of spinach chloroplast DHA reductase, which was oxidized by oxidized glutathione, exhibited TI activity.

AsA plays a central role in protecting plant cells [16,17] and insects cells [18] against the action of reactive oxygen species. In plants, peroxide-scavenging was accomplished through the AsA-glutathione pathway, a coupled series of redox reactions involving four enzymes: AsA peroxidase (EC 1.11.1.11), MDA reductase (EC 1.6.5.4), DHA reductase (EC 1.8.5.1), and glutathione reductase (EC 1.6.4.2) [19-22]. This pathway was studied more often in chloroplasts, in which the possible reactive oxygen species produced by PS I during photosynthesis might cause serious damage. However, AsA-glutathione pathway was also widespread in cytosol [16,20,21,23-25]. When AsA functions as an

antioxidant in cells, it is univalently oxidized to MDA free radicals, and MDA reductase catalyzes the reduction of MDA to AsA by NAD(P)H [26]. Heber et al. [27] pointed out that MDA was a sensitive endogenous index of oxidative stress in leaf tissues. In this study, we present evidence to show that the purified TIs from storage roots of SP exhibit both DHA reductase and MDA reductase activities.

2. Materials and methods

2.1. Plant materials and TI purification

Fresh roots of SP (*Ipomoea batatas* (L.) Lam cv. Tainong 57) were purchased from a local market. After cleaning with water, the roots were cut into strips immediately for TI extraction. Extraction and purification processes were according to Hou and Lin [1]. Purified TIs were lyophilized and stored at -18°C for further use.

2.2. DHA reductase activity assay

The DHA reductase activity of TIs was assayed according to the method of Trümper et al. [15] with some modifications. Two mg lyophilized TIs were dissolved in 1 ml distilled water. Ten mg DHA were dissolved in 5.5 ml, 100 mM phosphate buffer with different pH values (pH 6.0 to pH 8.0). The reaction was carried out at 30°C by adding 0.2 ml TI solution to 1.0 ml DHA solution. The increase of absorbance at 265 nm was recorded for 20 min. Non-enzymatic reduction of DHA in phosphate buffer was measured in a separate cuvette at the same time.

2.3. Protein, activity, and thiol-labeling stainings of TIs on 15 % SDS-PAGE gels

After reaction with DHA at pH 6.0, 6.5 or 7.0 for 20 min, TIs were examined by both protein and activity stainings on 15% SDS-PAGE gels [28]. Samples were either treated with sample buffer, including 2-ME at a final concentration of 14.4 mM, and heated at 100°C for 5 min (for protein staining) or incubated in sample buffer without 2-ME at room temperature overnight (for activity staining). Thiol-labeling staining on a SDS-PAGE gel followed the method of Kobrehel et al. [29] using mBBr (Thiolyte) as a probe.

2.4. MDA reductase activity assay

The MDA reductase activity of TIs was assayed spectrophotometrically by following the decrease in absorbance at 340 nm due to NADH oxidation according to Hossain et al. [26]. MDA free radicals were generated by AsA oxidase (EC 1.10.3.3) in the assay system [30]. The reaction mixtures contained 50 mM phosphate buffer (pH 6.0, or 6.5), 0.33 mM NADH, 3 mM AsA, AsA oxidase (0.9 unit), and 200 μl TI solution (2 mg TIs dissolved in 1 ml distilled water) in a final volume of 1 ml. TI solution was replaced with distilled water for controls.

2.5. MDA reductase activity staining on a 15% SDS-PAGE gel

TIs were mixed with sample buffer (without 2-ME) and incubated at room temperature overnight before electrophoresis [28]. MDA reductase activity staining of TIs was then assayed for diaphorase activity [22] according to the methods of Kaplan and Beutler [31] on a 15% SDS-PAGE gel.

2.6. Chemicals

Ascorbic acid, dehydroascorbic acid, electrophoresis grade acrylamide and Bis, TEMED and APS were from E. Merck Inc. (Germany). Thiolyte® monobromobimane reagent was purchased from Calbiochem International (San Diego, CA, USA). Other chemicals and solvents were purchased from Sigma Chemical Company (St. Louis, MO, USA). The low molecular weight kits for electrophoresis were obtained from Pharmacia (Uppsala, Sweden).

3. Results

3.1. DHA reductase activity of TIs purified from storage roots of SP

Fig.1 shows AsA regeneration ($\Delta A_{265\ nm}$) from DHA in different pH values (pH 6.0 to pH 8.0) when TIs purified from storage roots of SP were used to examine DHA reductase activity. We found from Fig.1 that TIs had DHA reductase activity and could reduce DHA to regenerate AsA in the absence of glutathione with an optimal pH of 7.0. These results were the same as that found by Trümper et al. [15], namely the reduced (thiol) form of soybean TI could reduce DHA to regenerate AsA, except that their system was glutathione-dependent.

3.2. Protein, activity, and thiol-labeling stainings of TIs in 15 % SDS-PAGE gels

Fig. 2 shows protein, activity, and thiol-labeling stainings of TIs in SDS-PAGE gels before (lanes 1 and 5) and after reacting with DHA in phosphate buffer at pH 6.0 (lanes 2 and 6), pH 6.5 (lanes 3 and 7) and pH 7.0 (lanes 4 and 8). Stainings of trypsin inhibitors were carried out in 15% SDS-PAGE gels. Protein, TI activity, and thiol-labeling are represented by (A), (B), and (C), respectively. For electrophoresis, samples in lane 1 to lane 4 were incubated in sample buffer without 2-ME at room temperature overnight; while in lane 5 to lane 8, samples were heated in sample buffer with 2-ME at 100°C for 5 min. We observed in Fig. 2A that larger amounts of TI with M_r 38 kD and less amounts of TI with M_r 22 kD and 20 kD were found after DHA reduction to ascorbate at pH 6.0 to pH 7.0 (lane 2 to lane 4) than were found in the blank lane (lane 1). This implies that intermolecular thiol-disulfide interchanges of TIs were found during DHA reduction. Meanwhile the same protein patterns of TIs (lane 5 to lane 8) were found after treating with 2-ME and heating at 100°C for 5 min, but the 38 kD

TI was absent in lane 5 to lane 8 (Fig. 2A). Fig. 2B shows the results of TI activity staining. Although larger amounts of 38 kD TI at pH 6.0 to pH 7.0 were detected (Fig. 2A, lane 2 to lane 4), they display the same TI activity staining strength as original TI (blank, lane 1). Fig. 2C shows the results of thiol-labeling staining of TIs. Comparing Fig. 2C with Fig. 2A, we conclude that free thiol groups are detectable in all TI molecules.

3.3. MDA reductase activity assay

MDA was reduced to AsA in coupling with NADH oxidation (ΔA_{340nm}) at pH 6.0 and pH 6.5 when TIs purified from storage roots of SP were used as MDA reductase. We found that TIs exhibit MDA reductase activity at both pH 6.0 and 6.5 (Fig. 3), with pH 6.0 much more favorable than pH 6.5 in this assay system.

3.4. MDA reductase activity staining on 15% SDS-PAGE gels

MDA reductase activity staining of TIs was done for diaphorase activity [22] on SDS-PAGE gels (Fig. 4C). Comparing Fig. 4C with Fig. 4A (protein staining) and 4(B) (TI activity staining) of TIs, one can see that the major diaphorase activity staining for MDA reductase activity came from TI with M_r 22 kD.

4. Discussion

This is the first report that TIs, the major storage protein of SP roots, display both DHA reductase and MDA reductase activities. Furthermore, these activities display some unique characteristics.

Thioltransferase (glutaredoxin) and protein disulfide isomerase have already been reported to have DHA reductase activity [32]. DHA reductase and MDA reductase in chloroplasts act against oxidative stress during photosynthesis, but the physiological functions of cytosolic forms are still unclear in plants [19-22]. 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 AsA-glutathione pathway [19-22]. So far, we do not know whether the same AsA-glutathione pathway exists in the cytoplasm of SP root cells. If it does, then the DHA reductase activity of TIs represents a parallel of the known AsA-glutathione pathway since the system we found is glutathione-independent; if it does not, then the DHA reductase activity of TIs represents a vital defense in the cytoplasm of SP cells against oxidative damage. Whatever the situation is, the system we found has an unique character: TIs serve both as a hydrogen-donors and as enzymes. Since we followed the published procedure [26] in which NADH was included for determination of MDA activity of TIs, we are not sure whether TIs can also serve both as hydrogen-donors and enzymes in MDA reduction. TIs' intermolecular thiol-disulfide interchanges were found during DHA reduction to regenerate AsA (Fig. 2A). Thionin was reported to have intermolecular disulfide linkages with other proteins [35]. TI activity staining (Fig. 2B) revealed that newly formed 38 kD TI from intermolecular thiol-disulfide interchange might have mismatched disulfide linkages that resulted in lower TI activity staining intensity based on the same amount of protein. Kobrehel et al. [36] 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. This is beneficial for TIs to fulfill dual functions exhibiting trypsin inhibition activity in disulfide forms and acting against oxidative stress in thiol forms.

Figures 3 and 4 show the results of the MDA reductase activity of TIs with NADH as a hydrogen-donor. MDA reductase purified from potato was shown to contain thiol groups in their catalytic sites [22,24,37]. Fernando et al. [38] found that thioredoxin can act as a radical scavenger and facilitate the regeneration of oxidatively damaged proteins. When AsA is the sole hydrogen donor, the AsA peroxidase, guaiacol peroxidase, and AsA oxidase can produce MDA [30]. Nonenzymatic oxidations of AsA also produce MDA when cells suffer from oxidative stress [27]. Taking the above results into consideration, we construct a reduction scheme of both dehydroascorbate (DHA) and monodehydroascorbate (MDA) to ascorbate (AsA) catalyzed by trypsin inhibitors of sweet potato roots (Fig. 5). DHA and MDA can be reduced to regenerate AsA by TIs in order to prevent oxidative damage to SP roots.

References

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