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Cultivar Differences in Trypsin Inhibitory Activities of Sweet Potato Leaves and Tuberous Roots

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ABSTRACT: Trypsin inhibitor (Ti) activities in sweet potato (*Ipomoea batatas* Lam.) leaves and roots from five local cultivars were analyzed on 15% SDS-PAGE. At qualitative level, it showed that two forms of Ti were present in both organs, with Mr. 31,000 Da and 14,000 Da in leaves, and Mr. 31,000 Da as well as 21,000 Da in roots, respectively. At quantitative level, the Ti activities varied among these cultivars. The level of trypsin inhibitory activity was closely related with pest resistance. More Ti activity conferred strong insect resistance on several cultivars, i.e. Tainong 34 and 65. Finally, the data of immunostain with anti-sporamin antiserum demonstrated that Tis from leaves were different from those of roots in sweet potato.

KEY WORDS: Sweet potato, *Ipomoea batatas* , Trypsin inhibitor (Ti), Insect-resistant group, Insect-labile group.

INTRODUCTION

Trypsin inhibitors are widely present in the plant kingdom. They were first found in soybean and isolated by Kunitz (1945); subsequently, many families of trypsin inhibitors from plants were isolated and characterized. In general, they are classified as Kunitz-type trypsin inhibitor, potato inhibitor I (PI-I), potato inhibitor II (PI-II), *Bowman-Birk* and squash inhibitor, etc. Among them, the Kunitz-type trypsin inhibitors are characterized by its high molecular weight (over 20 kDa) and by its typical reactive site with Arg-X or Lys-X dipeptide (Ryan, 1989). Bowman-Birk members are unique in their low molecular weight (below 8 kDa) while the other two families, PI-I and PI-II, can be induced in response to woundings (Ryan, 1989). Although sequence divergence and variation are common features for all trypsin inhibitors, they all preserve the inhibitory function on trypsin. These observations suggest that a rapid and dramatic evolutionary change is underway in plants trypsin inhibitors (Creighton and Darby, 1989).

The presence of trypsin inhibitors in sweet potato was first reported by Sohonnie and Bhandarker (1954). They found that ingestion of trypsin inhibitors in raw sweet potatoes caused animals to initiate the disease enteritis necrotieans (Dickey and Collins, 1984). It was also found that uncooked sweet potato roots in foodstuff reduced the growth rate of swine (Boukamp *et al.*, 1985). Later, Suguira *et al.*(1973) isolated three different trypsin inhibitors

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from sweet potatoes by column chromatography, and showed a strong inhibition on trypsin but had a less effect on plasmin and kallikrein. Recent studies showed that SDS-PAGE patterns of trypsin inhibitors varied in stem and leaf, germinating roots, and dormant roots (Lin, 1993). Furthermore, various isoforms were present each organs ranging from molecular weight (Mr) 39,000 to 14,100 Da. Among them, the one with Mr. 20,500 Da was common to all organs, and those with Mr. 15,200 and 14,100 Da were specific to leaves. Variations in activity level and heat stability had been reported among different sweet potato varieties by Lin and Chen (1980); thus, prompted them to suggest that trypsin inhibitors in sweet potato could be classified as heat-resistant and heat-labile groups. In spite of a significant variability in trypsin inhibitory activity levels in sweet potato cultivars, these levels were correlated positively with total soluble protein concentrations in each season (Boukamp *et al.*, 1985).

Thus far, studies of sweet potato trypsin inhibitor have been concentrated primarily on tuberous organ related to its biochemistry, physiology and nutrition. Recently, one of the trypsin inhibitors in tuberous roots was identified as sporamin (Yeh *et al.*,1995), which was assumed to be a major storage protein (Maeshima *et al.*, 1985), and might play a protective role in plant against infectations by insects and microorganisms (Ryan, 1989; 1990). In spite of the potential of this protein to function as a natural pesticide, few study has been made in sweet potato leaves.

The purpose of this study was to examine the differences in levels of trypsin inhibitory activity in leaves and tuberous roots among sweet potato cultivars, and to correlate them with insect resistance. Anti-sporamin antiserum was employed for immunostaining to differentiate the trypsin inhibitor groups between leaves and tuberous roots.

MATERIALS AND METHODS

Plant Materials

Five cultivars (Tainong 9, 27, 34, 57and 65) of sweet potato (*Ipomoea batatas* Lam.), which represent varieties resistant (Tainong 34 and 65) and susceptible (Tainong 9 and 57) to insects, were donated by Dr. C. H. Cheng (Provincial Chia-Yi Agricultural Research Institute), and were grown at the Experimental Field of National Taiwan University for sample collections.

Crude Protein Extraction and SDS-polyacrylamide Gel Analysis

Leaves with 2-cm width in average were excised and ground immediately to fine powder in liquid nitrogen with a motar and pestle. The powder was suspended in extraction buffer consisting of 50 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 10% sucrose, 10 mM ascorbic acid, 1% dimethyl sulfoxide (DMSO), 3 mM phenylmethyl sulfonylfluoride (PMSF), 0.2% Triton X-100 and 1% β-mercaptoethanol. After centrifugation at 12,500 rpm for 10 min, the supernatant was saved, and protein concentration was quantified by the method of Bradford (1976). Equal amount of proteins for each sample was electrophoresed on 15% SDS-polyacrylamide gel and the gel was stained with coomassie blue (1% in methanol, acetic acid).

Trypsin inhibitor assay

Approximately, 80-100 μg protein was first electrophoresed on 15% SDS-polyacrylamide gel. The gel was then shaken gently for 15 min twice in 25% 2-propanol (in 10 mM Tris-HCl, pH 8.0) and once for 30 min in 10 mM Tris-HCl (pH 8.0) to remove SDS and to renature proteins. The trypsin inhibitory activity was assayed by a modified method of Chan and Delumex (1982). The gel was incubated in trypsin solution (40 μg trypsin in per ml of 50 mM Tris-HCl, pH 8.0, and 50 mM $CaCl_2$) for 30 min before transferring to a fresh dish containing substrate-dye solution prepared immediatly prior to use. The solution contained 2.5 mg N-acetyl-DL-phenylalanine β-naphthyl ester (APNE, Sigma) dissolved in per ml of dimethylformamide (DMF) and combinded with tetrazotized o-dianisidine dye solution (5 mg in per ml of 50 mM Tris-HCl, pH 8.0, and 50 mM $CaCl_2$). After incubation for 1-2 hours at room temperature, the gel was destained with 7% acetic acid for more than 30 min.

Immunostaining assay with sweet potato anti-trypsin inhibitor (anti-sporamin) anti-serum

The antiserum against sweet potato trypsin inhibitor (sporamin) was raised in a rabbit. Antigen of trypsin inhibitor was prepared from GST-SPTi fusion protein, which can be overexpressed and recovered from *Escherichia coli* (Yeh *et al*, 1995). Protein samples from roots and leaves were first electrophoresed on 15% SDS-PAGE, and blotted onto nitrocellulose (NC) paper by the capillary method (Sambrook *et al.*, 1989). The transfer buffer consisted of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM EDTA and 0.5 mM β-mercaptoethanol. The procedures of Western blot were according to the standard protocol (Sambrook *et al.*, 1989). The NC filter was treated stepwisely with blocking, and reacted to primary antibody and Horseradish peroxidase-coupled secondary antibody, respectively. Finally, diaminobenzidine tetrahydrochloride (Sigma) (6 mg in 9 ml of 0.01 M Tris-HCl, pH 7.6) and 10 μl of 30% H_2O_2 were added to the NC paper before incubation and shaking to reveal protein bands.

RESULTS AND DISCUSSION

Cultivar differences in trypsin inhibitory activities from sweet potato leaves

SDS-PAGE analysis showed that all cultivars contained two forms of trypsin inhibitors at qualitative level with Mr. 31 kDa and 14 kDa in leaves tissue (Fig. 1), a result similar to previous observation (Lin, 1993). At quatitative level, cultivar Tainong 65 contained the largest quantity of these two forms, while cultivar Tainong 9 had the least amount. In general, this result shows a good correlation of trypsin inhibitory activities to insect resistance among these cultivars. Field studies have indicated that Tainong 34 and 65 were more resistant to insect attack than Tainong 9 and 57 (Data not shown).

Cultivar differences in trypsin inhibitor activities from sweet potato tuberous root

The presence of trypsin inhibitors in sweet potato tuberous roots has been widely reported (Dickey and Collin,1984); however, there are no consistencies in molecular weight and size (Lin, 1993). Some of these discrepancies might be attributable to artifacts resulted from poor resolution of partial purified proteins on native gels. In order to improve the

resolution, we use SDS-PAGE in our experiments, and the data showed a very consistent pattern among the four cultivars studied. All of them contained two forms of trypsin inhibitory activity with Mr. 31 kDa and 21 kDa, except the cultivar 57 which showed an additional activity band with Mr. 33 kDa (Fig. 2). Although the pattern of trypsin inhibitory activity in these four cultivars was consistent at qualitative level, it varied quantitatively with cultivars 27 and 57 being more active than cultivars 34 and 65. These observations also indicate that patterns of these inhibitors in roots were different from that of leaves.

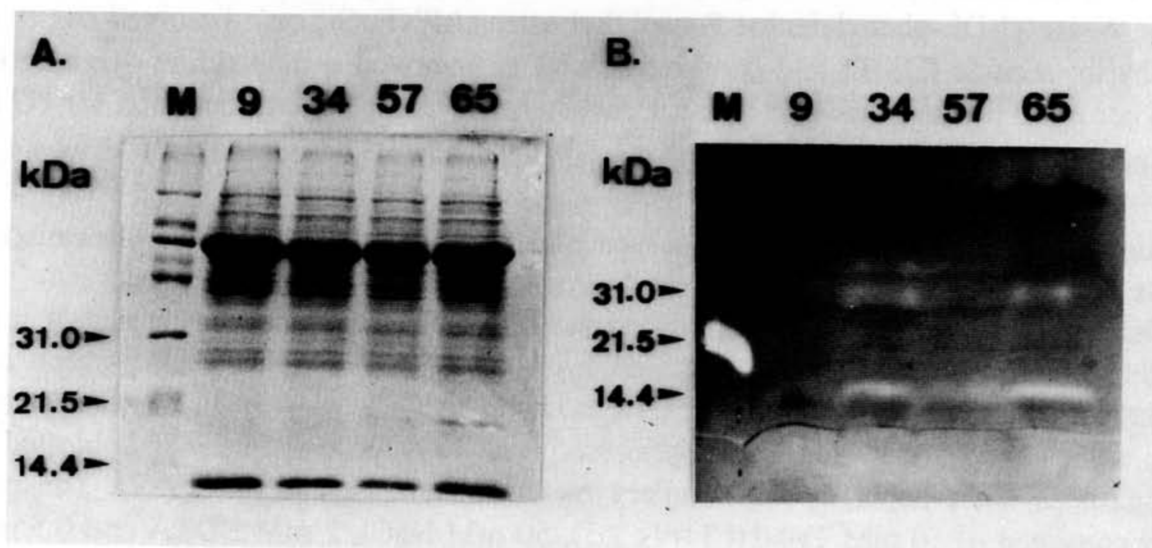

Fig. 1. Trypsin inhibitory activities of sweet potato leaves analyzed on SDS-PAGE. A. An equal amount (30 μg) of leave proteins of four cultivars (Tainong 9, 34, 57 and 65) were electrophoresed on 15% SDS-PAGE, and stained with coomassie blue. The gel was shown as standard protein quantity control for activity study. B. Activity staining gel (200 μg of leave proteins were electrophoresed on 15% SDS-PAGE) showed Ti activity varied among four cultivars (Tainong 9, 34, 57 and 65). M, protein markers containing soybean trypsin inhibitor with Mr. 21.5 kDa.

Western blot of trypsin inhibitors from sweet potato leaves and tuberous roots

When the leaf proteins were reacted with anti-Ti antiserum, which was raised against recombinant sporamin protein (Yeh *et al.*, 1995), no positive signals were observed in the reaction (Fig. 3) even though leaves and roots both contained a trypsin inhibitor with Mr. 31 kDa. Positive reactions were clearly detected with those from tuberous roots of various cultivars (Fig. 4). These results suggest that the trypsin inhibitors in leaves are distinctively different from those of tuberous roots. It is likely that at least one of the Tis in roots is a member of sporamins (Yeh *et al.*, 1995), and those found in leaves might be novel trypsin inhibitors. Protein purification and characterization are necessary to confirm this hypothesis.

This study shows that SDS-PAGE analysis is useful for resolving trypsin inhibitory activities. In general, all cultivars contain two forms of trypsin inhibitors in both roots and leaves, although leaf protiens were serologically different from those of roots. Interestingly, trypsin inhibitory activities in leaves appeared to correlate with pest resistance (Dr. Y. H. Lin, personal communication), namely, higher quantities of trypsin inhibitory activities in leaves observed in resistant cultivars, Tainong 34 and 65, and less activity in susceptible cultivars, Tainong 9 and 57. Since biosynthesis of proteinase inhibitors in plants have been shown to associate with defense mechanism against pests and pathogens (Ryan, 1989 and

1990), trypsin inhibitors in sweet potato leaves could also play a defensive role against insect infectations.

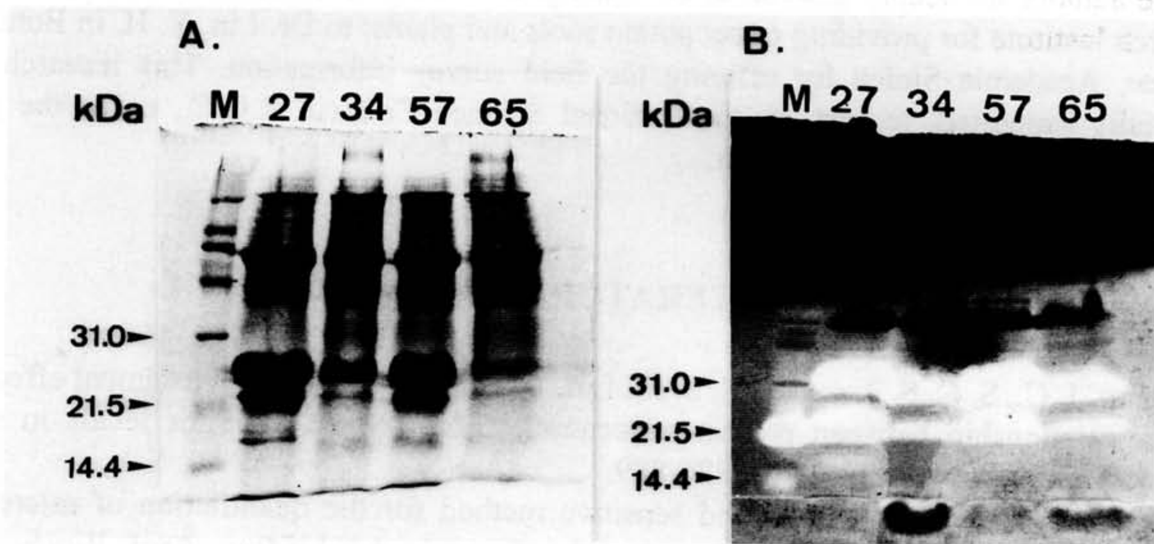

Fig.2. Trypsin inhibitory activities of sweet potato tuberous roots analyzed on SDS-PAGE. A. An equal amount (100 μg) of leave proteins of four cultivars (Tainong 27, 34, 57 and 65) were electrophoresed on 15% SDS-PAGE, and stained with coomassie blue. The gel was shown as standard protein quantity control for activity study. B. Activity staining gel (80 μg of tuberous root proteins were electrophoresed on 15% SDS-PAGE) showed Ti activity varied among four cultivars (Tainong 27, 34, 57 and 65). M, protein markers containing soybean trypsin inhibitor with Mr. 21.5 kDa.

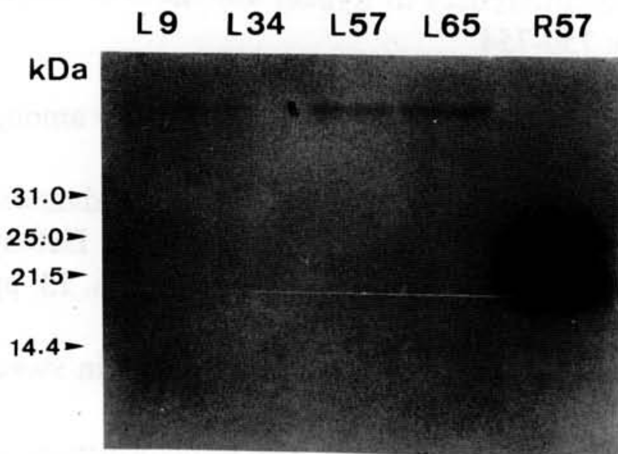

Fig.3. Western blot of sweet potato trypsin inhibitors from leaves. An equal amount (40 μg) of leaves proteins were first electrophoresed on 15% SDS-PAGE, then blotted on NC filter, and reacted with anti-Ti (sporamin) antiserum. Protein extracts from tuberous roots of sweet potato Tainong 57 were analyzed simutaneously as standard control. There are not any signals with leaves Tis samples, except the root extracts from cultivar Tainong 57.

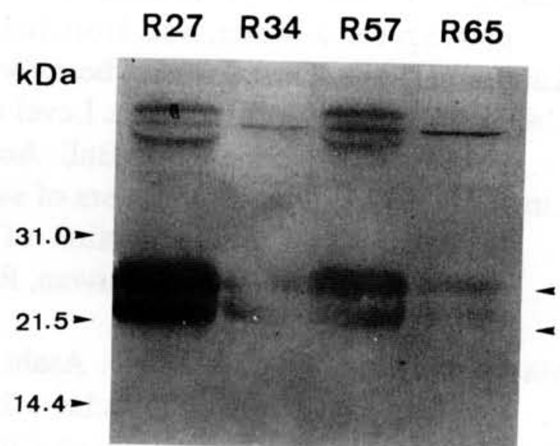

Fig.4. Western blot of sweet potato trypsin inhibitor from tuberous roots. An equal amount (80 μg) of tuberous root proteins were first electrophoresed on 15% SDS-PAGE, then blotted on NC filter, and reacted with anti-Ti (sporamin) antiserum. All cultivars showed positive signals.

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不同品系間甘薯葉部及塊根胰蛋白脢抑制因子活性之差異

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摘　　要

利用蛋白質膠體電泳技術分析不同品系間甘薯（*Ipomoea batatas* Lam.)胰蛋白脢抑制因子的活性，發現塊根及葉部的胰蛋白脢抑制因子活性在不同品系間有所差異。甘薯的葉部組織中，台農9號、34號、57號和65號均有兩種胰蛋白脢抑制因子，分子量為31 kDa及14 kDa，其中65號的胰蛋白脢抑制因子活性最強，9號的活性最弱。而在台農27號、34號、57號和65號的甘薯塊根中，均有兩種胰蛋白脢抑制因子，分子量為21 kDa及31 kDa，57號另有一個33 kDa的胰蛋白脢抑制因子。其中27號和57號的胰蛋白脢抑制因子活性較強，34號和65號的活性較弱。此結果顯示，葉部之胰蛋白脢抑制因子之活性高低，與田間栽培試驗的抗蟲能力有正相關。

經由西方轉漬法(Western blotting)分析甘薯塊根與葉部組織的胰蛋白脢抑制因子，顯示葉部之胰蛋白脢抑制因子和塊根有所不同。

關鍵詞：甘薯，胰蛋白脢抑制因子，強抗蟲力群，易感染群。

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