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行政院國家科學委員會補助專題研究計畫■期中進度報告

（計畫名稱）

甘藷胰蛋白酶抑制因子生理活性探討(1/3)

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ABSTRACT
Proteases were purified successively by trypsin-Sepharose 4B, sweet potato (*Ipomoea batatas* [L.] Lam) trypsin inhibitor (SPTI)-Sepharose 4B, benzamidine-Sepharose 6B, and arginine-Sepharose 4B affinity columns from crude extracts of SP dormant roots. One of them, Arg-1, was specific to the substrate benzoyl-arginine-*p*-nitroanilide, with an optimal pH 8.0. Arg-1 migrated as a single band of 20 kDa in SDS-PAGE, detected by activity staining. The activity was completely inhibited by SPTI in a dose-dependent manner. The activity was inhibited by aprotinin and soybean TI, but not by E-64, pepstatin A or EDTA. This suggested that Arg-1 was a serine type protease, inhibited endogenously by SPTI. Denatured SPTI could be degraded by Arg-1 in vitro. The physiological role of SPTI in the regulation of Arg-1 activity was discussed.

Keywords: physiological function; regulation; serine protease; sweet potato; trypsin inhibitor

1. Introduction

Proteases play important roles in post-translational modification, protein turnover, activation and inactivation of specific proteins, and nutrient supplementation [1]. In plant storage organs or tissues, specific proteases involved in the mobilization of reserve proteins [2-7], developmental processes [8-11], and senescence [12-15] have been studied intensively.

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 [16,17]. Sohonie and Bhandarker [18] reported for the first time the presence of trypsin inhibitors (TIs) in sweet potato (SP). Later, we indicated that TI activities in SP are positively correlated with concentrations of water-soluble protein [19], and that a large negative correlation exists between the natural logarithm of TI activities and cumulative rainfall, which suggests that SPTI activities may vary in response to drought [20]. Polyamines, including cadaverine, spermidine and spermine, bound covalently to SPTI which might participate in regulating the growth and developmental processes of SP [21]. SPTIs were also proved to have both dehydroascorbate reductase and monodehydroascorbate reductase activities and might respond to environmental stresses [22]. We found that TIs in SP roots accounted for about 60% of total water-soluble proteins and could be recognized as storage proteins [19]. Maeshima et al. [23] identified the sporamin as the major storage protein in SP root, which accounted for 80% of total proteins in root; a dramatic decrease to 2% of original value was found during sprouting. Lin [24] proposed that sporamin should be one form of TIs in SP, which was confirmed later by Yeh et al. [25].

Until now most reports of plant proteinaceous protease inhibitors focus on the aspect of potential insecticidal activities [17, 26, 27] and no report concerning the physiological function of them in the endogenous regulation of various protease activities has been presented. In this work we report for the first time that SP proteinaceous TIs inhibit their cognate serine protease activity.

2. Materials and methods

2.1. Isolation and purification of serine proteases from SP

The dormant storage roots of SP were used to isolate and purify serine proteases. The storage roots were cut into strips which were extracted immediately with four volumes (W/V) of 100 mM Tris-HCl buffer (pH 7.9) containing 100 mM NaCl, 10 mM EDTA and 1% ascorbate. After centrifugation at 14000 × g twice, the crude extracts were loaded directly onto a trypsin-Sepharose 4B affinity column (1.0×10 cm) to remove SPTIs according to the method of Hou and Lin [22]. The unbound fraction which was SPTI-free was collected and concentrated with centriprep 10 (the molecular weight cutoff is 10 kDa). The concentrates were loaded onto a SPTI-Sepharose 4B affinity column, which was prepared according to the method of Hou and Lin [22] to adsorb serine proteases. After washing with 100 mM Tris-HCl buffer (pH 7.9) containing 100 mM NaCl the bound serine proteases were eluted batchwise with 200 mM KCl (pH 2.0). The flow rate was 32 ml/h and each fraction contained 4.8 ml. The eluents from SPTI-Sepharose 4B column were saved with pH value being adjusted to 7.4, then the solution was desalted and concentrated with centriprep 10 to small volumes for next purification step. For the 3rd purification step, the desalted solution was loaded onto a benzamidine-Sepharose 6B affinity column (1.0×10 cm) and washed with buffer A [20 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl]. The unbound fraction, which could hydrolyze benzoyl-arginine-*p*-nitroanilide (BR*p*NA), was named as Ben-1 and collected for further purification. The bound serine protease was eluted batchwise with buffer B [50 mM phosphate buffer (pH 11.5) containing 500 mM NaCl] and was named as Ben-2. The flow rate was 32 ml/h and each fraction contained 4.8 ml. For the 4th purification step, the Ben-1 fraction was loaded onto an arginine-Sepharose 4B affinity column (1.0×10 cm) and washed with buffer A. The unbound

fraction, which could hydrolyze BR*p*NA, was named as Arg-1 and collected for further characterization. The bound serine protease was eluted batchwise with buffer B which was named as Arg-2. The flow rate was 32 ml/h and each fraction contained 4.8 ml.

2.2. Determination of Arg-1 activity using synthetic substrates

The BR*p*NA was used as a specific synthetic substrate for Arg-1 activity determination. The reaction mixtures contained 300 μl of 100 mM Na-phosphate buffer (pH 8.0), 300 μl of 4 mM BR*p*NA (dissolved in 10 % dimethylformamide), 100 μl enzymes and water was added to make a total volume of 800 μl. The mixtures were incubated at 37 °C for 30 min and 300 μl of 50 % acetic acid was added to stop the reaction. The absorbance at 410 nm was measured to determine the amounts of *p*-nitroaniline released. The *p*-nitroaniline was used to plot a standard curve. One unit of Arg-1 activity was defined as the amount of enzyme that catalyzed the formation of 1 nmole *p*-nitroaniline per hour at 37 °C, pH 8.0. Other synthetic compounds including Lys-*p*NA, *r*-Glu-*p*NA, Ala-Ala-Ala-*p*NA, Met-*p*NA, Pro-*p*NA, benzyl-Cys-S-*p*NA and acetyl-Tyr-*p*NA were also examined for their substrate specificity.

2.3. Optimal pH for Arg-1 activity using BRpNA as a substrate

Optimal pH for Arg-1 activity using BR*p*NA as a substrate was determined in both 100 mM Na-phosphate buffer (pH 6.5, 7.0, 7.5, 8.0 and 8.5) and 100 mM Tris-HCl buffer (pH 7.0, 7.5, 8.0 and 8.5). The reaction conditions for Arg-1 activity were described above.

2.4. Arg-1 and TI activity stainings on 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 with or without 2-mercaptoethanol for Arg-1 and TI activity stainings, respectively, at 4 °C overnight. Coomassie brilliant blue G-250 was used for protein staining [28]. Arg-1 activity staining was carried out on a 15% SDS-PAGE gel co-polymerized with 0.1 % (W/V) gelatin [11]. After electrophoresis, gels were washed with 25% isopropanol in 10 mM Tris-HCl buffer (pH 7.9) for 10 min twice to remove SDS [29]. For Arg-1 activity staining, the gel was shaked in 100 mM Na-phosphate buffer (pH 8.0) overnight and then stained with coomassie brilliant blue R-250. For inhibition of Arg-1 activity by SPTIs on a gelatin-SDS-PAGE gel, the gel after removal of SDS was overlaid with 1 mg/ml SPTIs solution and then incubated in a moist petri dish overnight. After washing with 500 mM Tris-HCl buffer (pH 7.9) for 20 min twice to remove SPTIs on the gel surface, the gel was stained with coomassie brilliant blue R-250. For SPTI activity staining, the gel was stained according to the method of Hou and Lin [29].

2.5. Dose effects of SPTIs or inhibitors on the inhibition of Arg-1 activity in vitro

One hundred μl with different amounts (50 ng, 100 ng, 150 ng, 200 ng, 400 ng, 500 ng, 1 μg, 5 μg, 10 μg and 15 μg) of SPTIs in distilled water were premixed with 100 μl Arg-1 (57.6 units) and 300 μl of 100 mM Na-phosphate buffer (pH 8.0) at room temperature for 20 min, and then 300 μl of 4 mM BR*p*NA was added. These mixtures were incubated at 37 °C for 30 min, then 300 μl of 50 % acetic acid was added to stop the reaction. The absorbance at 410 nm was measured to determine the amounts of *p*-nitroaniline released. Distilled water was used instead of SPTIs as a control for Arg-1 standard activity. Other protease inhibitors including 10 μM of E-64 for cysteine protease, 10 μM of pepstatin A for aspartic type protease, or 10 μg of aprotinin or commercial soybean TI (T-9128, Sigma Chemical Co.) for serine protease were also examined for inhibition of Arg-1 activity .

2.6. Arg-1 degraded the denatured 33 kDa SPTI in vitro

The 10 % preparative SDS-PAGE gel was used to isolate the 33 kDa SPTI from SPTI mixtures obtained by a trypsin affinity column [21]. After electrophoresis and SDS removal [29], the 33 kDa SPTI 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 centricon10 and then lyophilized for further use. For SPTI denaturation, 100 μg of 33 kDa SPTI was dissolved in 100 μl of 20 mM DTT in distilled water, and then heated at 100 °C for 10 min. Forty μl Arg-1, 40 μl of the denatured 33 kDa SPTI and 20 μl of 100 mM Na-phosphate buffer (pH 8.0) were mixed and hydrolysis reaction was carried out at 37 °C for 48 and 72 hr, then the hydrolysate was heated at 100 °C for 5 min to stop the reaction followed by 15% SDS-PAGE analysis.

2.7. Materials

All chemicals and reagents were of the highest purity available. Trypsin (TPCK-treated, 40 U/mg) and N-benzoyl-L-arginine-4-nitroanilide were purchased from E. Merck Inc. (Darmstadt, Germany); Seeblue prestained markers for SDS-PAGE were from Novex (San Diego, CA); CNBr-activated Sepharose 4B, benzamidine-Sepharose 6B, arginine-Sepharose 4B were from Pharmacia

Biotech AB (Uppsala, Sweden). Other chemicals and reagents including protease inhibitors and synthetic substrates were from Sigma Chemical Co. (St. Louis, MO, USA).

3. Results and discussion

3.1. Isolation and purification of serine proteases

In SP, about 60 % of total water-soluble proteins were TIs [19]. Maeshima et al. [23] reported that the storage proteins of SP were reduced from 4.41 to 0.067 mg/g tissue after sprouting. Li and Oba [30] also reported that the storage proteins of SP were reduced from 3.22 to 0.18 mg/g tissue after one year storage at 10 to 12 °C. It is clear that SPTIs serve as storage proteins to provide nitrogen sources during sprouting or storage. Yeh et al. [26] reported that SPTI expressed in transgenic tobacco plants confers resistance against *Spodoptera litura*. Until now, no endogenous protease has been reported that is inhibited by SPTIs. Hence, we sought to isolate and partially purify on endogenous serine protease from the dormant storage roots of SP that was regulated by TIs.

To initiate the research it was necessary to remove the large amounts of TI from SP. For this purpose, we used a trypsin-Sepharose 4B affinity column [21] to adsorb SPTIs, and the SPTI-free extracts were saved for serine protease purifications. As a second step, a SPTI-Sepharose 4B affinity column was prepared to select SPTI inhibited proteases, which were eluted with 0.2 M KCl buffer (pH 2.0) (Fig. 1). These eluted proteins may be regulating by or interacting with SPTIs. The benzamidine-Sepharose 6B [31, 32] affinity column was used for the 3rd purification step (Fig. 2A). The unbound fraction, which could hydrolyze BR*p*NA, was named as Ben-1 and collected for further purification. The bound protease which was eluted batchwise with buffer B (see Materials and methods) was named as Ben-2. A arginine-Sepharose 4B affinity column [31] was used for the 4th purification step. The Ben-1 fraction was loaded onto an arginine-Sepharose 4B affinity column (Fig. 2B). The unbound fraction, which could hydrolyze BR*p*NA, was named as Arg-1. The bound protease, which was eluted batchwise with buffer B, was named as Arg-2. Although three fractions (Ben-2, Arg-1 and Arg-2) were detected to have protease activities using gelatin-SDS-PAGE method (data not shown), only Arg-1 was active toward synthetic substrate BR*p*NA. Hence, only Arg-1 was used for further characteriaztion. Arg-1 could hydrolyze BR*p*NA but it can not adsorb onto arginine-Sepharose 4B affinity column. It is possible that Arg-1 belongs to an endoprotease. Arg-1 was not trypsin itself because it was not adsorbed onto benzamidine- and arginine-Sepharose affinity column.

3.2. Optimal pH for Arg-1 activity using BRpNA as a substrate

Both the Na-phosphate buffer and Tris-HCl buffer (pH 7.0 to pH 8.5) were used to determine the optimal pH of the Arg-1 protease toward BApNA (Fig. 3). Arg-1 exhibited a higher activity in Na-phosphate buffer than in Tris-HCl buffer at the same pH values. The optimal pH for Arg-1 was pH 8.0 no matter which buffer was used.

3.3. Substrate specificities of Arg-1

*S*ynthetic compounds including BR*p*NA, Lys-*p*NA, *r*-Glu-*p*NA, Ala-Ala-Ala-*p*NA, Met-*p*NA, Pro-*p*NA, benzyl-Cys-S-*p*NA and acetyl-Tyr-*p*NA were used for substrate specificity tests of Arg-1 (Table 1). No other compounds served as a better substrate than BR*p*NA in Na-phosphate buffer (pH 8.0). So BR*p*NA was used as a specific substrate of Arg-1 in the following investigations.

3.4. Effects of various Inhibitors or SPTIs on Arg-1 activity

Table 2 showed effects of different protease inhibitors on Arg-1 activity. Only serine type protease inhibitors, including aprotinin, soybean TI and SPTI, could inhibit Arg-1 activity. Hence Arg-1 was identified to be a serine type protease. It is interesting that Arg-1 purified from dormant roots of SP was completely inhibited by SPTIs. Therefore, different amounts of SPTI were used to determine the inhibition on Arg-1 activity. Fig. 4 showed the dose-dependent profile of inhibition of Arg-1 activity by SPTIs. It was clear that the activity of Arg-1 was completely inhibited by 5 μg or more of purified SPTIs (Fig. 4). The inlet of Fig. 4 showed the protease activity staining (lane C) and inhibition of Arg-1 by SPTIs (lane 1) on gelatin gels after SDS-PAGE. Arg-1 moved as a single activity band with a molecular mass of 20 kDa determined by gelatin-SDS-PAGE activity staining (lane C). However, the activity was diminished while SPTIs were overlaid on gel's surface (lane 1). This is the first report demonstrating that a plant protease activity was inhibited by its endogenous proteinaceous inhibitors, namely inhibition of SP Arg-1 by SPTIs, the storage proteins of sweet potato roots.

3.5. The inhibition of Arg-1 by SPTIs under different treatments

Arg-1 activity in Na-phosphate buffer (pH 8.0) served as a control (Fig. 5A). Treatment 1 was Arg-1 activity in Na-phosphate buffer containing 5 mM DTT (pH 8.0). In treatment 2, 10 μg SPTIs was premixed with 10 mM DTT

at 37 °C for 20 h and then an equal volume of Arg-1 was added for Arg-1 activity determination. In treatment 3, 10 μg SPTIs was premixed with 10 mM DTT and heated at 100 °C for 10 min, cooled down, and then an equal volume of Arg-1 was added for Arg-1 activity determination. It was showed that 5 mM DTT could activate Arg-1 activity (Fig. 5A, treatment 1) to about 130% of the control. Treatment 2 showed no Arg-1 activity (Fig. 5A). Fig. 5B showed profile of SPTI activity staining under different treatments. Comparing with original SPTIs (Fig. 5B, lane C), it was showed that SPTIs still had trypsin inhibitory activity under reduction conditions (Fig. 5B, lane 2). Therefore, no Arg-1 activity was detectable in treatment 2 of Fig. 5A. The characteristics of SPTIs were different from those reported for other TIs. Soybean TI lost its inhibitory activity after DTT treatments [33] and ovomucoid also lost its inhibitory activity when disulfide bonds were reduced [34]. Treatment 3 showed the recovery of full Arg-1 activity when SPTIs were premixed with 10 mM DTT and heated at 100 °C for 10 min (Fig. 5A). Comparing with original SPTIs (Fig. 5B, lane C), it was showed that SPTIs lost trypsin inhibitory activity completely after heating under reducing conditions (Fig. 5B, lane 3). It means that Arg-1 activity was inhibited only by close contact with SPTIs having appropriate molecular conformations.

3.6. Arg-1 degraded the denatured 33 kDa SPTI in vitro

Using preparative SDS-PAGE, the 33 kDa SPTI was isolated from partially purified SPTIs. Comparing protein staining (Fig. 6A) with TI activity staining (Fig. 6B) under non-reducing conditions, the purified 33 kDa SPTI was identified. This protein was denatured by premixing with 10 mM DTT, heated at 100 °C for 10 min, and was tested for degradations by Arg-1. The purified 33 kDa SPTI contained three bands (bands 1 to 3) after heating under reducing conditions that were degraded gradually by Arg-1 after 48 and 72 h, while a hydrolysis product (band 4) increased gradually. This means that the denatured SPTI could serve as a substrate for Arg-1. Maeshima et al. [23] indicated that the storage proteins of SP decreased from 4.41 to 0.067 mg/g tissue after sprouting. The results of Figs 5 and 6 suggest that root storage proteins of sweet potato, TIs or sporamins, could regulate Arg-1 activity under different physiological conditions. SPTIs in their native or active state could completely inhibit Arg-1 activity. However, when SPTIs lost their inhibitory activities under special physiological conditions or growth stages, Arg-1 could degrade the storage proteins to provide nitrogen sources. It is possible that other proteases may also degrade the denatured SPTIs. During sprouting, the storage proteins of SP roots, trypsin inhibitor or sporamin, decreased dramatically [23]. Kobrehel et al. [35, 36] showed that, TIs of both Kunitz type and Bowman-Birk type, could be reduced by NADP/thioredoxin system and could facilitate their hydrolysis by proteases. Apart from the reported physiological functions of TIs, storage proteins of SP roots [21-23, 25, 26], this work provides the first line of evidence that SPTIs or sporamins could regulate endogenous protease activity. The detailed mechanisms deserve further investigations.

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B.C. Yee, B.B. Buchanan, Specific reduction of wheat storage proteins by thioredoxin *h*, Plant Physiol. 99 (1992) 919-924.

Table 1
Substrate specificities of Arg-1 from sweet potato roots

Substrate (4 mM)	Relative activity (%)
BR*p*NA	100
Ala-Ala-Ala-*p*NA	0
acetyl-Tyr-*p*NA	3
benzyl-Cys-S-*p*NA	0
r-Glu-*p*NA	3
Lys-*p*NA	6
Met-*p*NA	0
Pro-*p*NA	0

* The reaction mixtures contained 300 μl of 100 mM Na-phosphate buffer (pH 8.0), 300 μl of 4 mM of each substrate (dissolved in 10 % dimethylformamide), 100 μl Arg-1 (52 units) and water was added to make a total volume of 800 μl. The mixtures were incubated at 37 °C for 30 min and then 300 μl of 50 % acetic acid was added to stop the reaction. The activity of Arg-1 toward BR*p*NA was expressed as 100 %.

Table 2
Effects of protease inhibitors on the activity of Arg-1 from dormant sweet potato roots

	Target protease	Relative activity* (%)
Control		100
E-64 (10 μM)	cysteine protease	89
Pepstatin A (10 μM)	aspartic protease	143
EDTA (5 mM)	metalloprotease	102
Aprotinin (10 μg)	serine protease	0
Soybean trypsin inhibitor (10 μg)	serine protease	0
Sweet potato trypsin inhibitor (10 μg)	serine protease	0

* Each protease inhibitor was premixed with 100 μl Arg-1 (30 units) and 300 μl of 100 mM Na-phosphate buffer (pH 8.0) at room temperature for 20 min, and then 300 μl of 4 mM BR*p*NA was added. The mixtures were incubated at 37 °C for 30 min, then 300 μl of 50 % acetic acid was added to stop the reaction. The control contained no protease inhibitor and expressed as 100 % for comparisons.

Legends

Fig. 1. The chromatograms of endogenous proteases from SPTI-free crude extracts on a sweet potato trypsin inhibitor-Sepharose 4B column. After washing with 100 mM Tris-HCl buffer (pH 7.9) containing 100 mM NaCl the bound proteases were eluted batchwise with 200 mM KCl (pH 2.0). The flow rate was 32 ml/h and each fraction contained 4.8 ml.

Fig. 2. The chromatograms of SP endogenous proteases from Figure 1 on a benzamidine-Sepharose 6B affinity column (1.0×10 cm) (A); after washing with 20 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl, the fraction which could hydrolyze BR*p*NA was named as Ben-1 (circle, blank). Ben-1 fraction was further purified by an arginine-Sepharose 4B affinity column (1.0×10 cm) (B), the fraction which could hydrolyze BR*p*NA was named as Arg-1 (circle, blank). The bound serine protease fraction (Ben-2 or Arg-2) was eluted batchwise with 50 mM phosphate buffer (pH 11.5) containing 500 mM NaCl. The flow rate was 32 ml/h and each fraction contained 4.8 ml.

Fig. 3. Optimal pH of Arg-1 activity using BRpNA as a substrate. Both Na-phosphate buffer (pH 6.5 to pH 8.5) and Tris-HCl buffer (pH 7.0 to pH 8.5) were used.

Fig. 4. Dose effects of SPTIs (50 ng, 100 ng, 150 ng, 200 ng, 400 ng, 500 ng, 1 μg, 5 μg, 10 μg and 15 μg) on the inhibition of Arg-1 activity. The inlet showed the protease activity staining (lane C) and inhibition of Arg-1 by SPTIs (lane 1) on gelatin gels after SDS-PAGE. After electrophoresis, gels were washed with 25% isopropanol in 10 mM Tris-HCl buffer (pH 7.9) for 10 min twice to remove SDS. For inhibition of Arg-1 activity by SPTIs on a gelatin-SDS-PAGE gel, the gel after removal of SDS was overlaid with 1 mg/ml SPTIs and then incubated in a moist petri dish overnight.

Fig. 5. The inhibition of Arg-1 by SPTIs under different treatments. (A) Arg-1 activity determinations. Arg-1 activity in Na-phosphate buffer (pH 8.0) served as a control. Treatment 1 was Arg-1 activity in Na-phosphate buffer containing 5 mM DTT (pH 8.0). In treatment 2, 10 μg SPTIs was premixed with 10 mM DTT at 37 °C for 20 h and then an equal volume of Arg-1 was added for activity determination. In treatment 3, 10 μg SPTIs was premixed with 10 mM DTT and heated at 100 °C for 10 min, cooled down, and then an equal volume of Arg-1 was added for activity determination. (B) SPTIs activity staining. lane C served as a control; lane 2 was SPTIs under reduction conditions; lane 3 was SPTIs after heating under reducing conditions.