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Immobilized Zinc Affinity Chromatography of Pectin Hydroxamic Acids for Purification of Trypsin Inhibitors from Soybean and Sweet Potato

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Commercial pectin (with a 94% degree of esterification, DE94) suspended in methanol was reacted with methanolic alkaline hydroxylamine at room temperature for 20 h to prepare pectin hydroxamic acids (PHAs). The prepared PHA was coupled to the epoxy-activated Sepharose 6B gel to get immobilized PHA resins. The immobilized PHA resin was then balanced in column with 2 mM $ZnCl_2$ in 50 mM Tris-HCl buffer (pH 7.9) to test the immobilized Zn−PHA gel as solid phase for immobilized metal affinity chromatography for the purification of trypsin inhibitors (TIs) from soybean and sweet potato. Using TI activity staining, it was found that purified TIs from the commercial soybean and sweet potato after trypsin affinity column purification could be adsorbed onto an immobilized Zn−PHA affinity column and eluted by 100 mM EDTA in 10 mM Tris-HCl buffer (pH 7.9). The immobilized Zn−PHA affinity column was used for TI purifications from crude extracts of sweet potato. The recovery of TI activity for one step was 90%, with 19.74-fold purification increase.

KEYWORDS: Hydroxamic acid; immobilized metal affinity chromatography; pectin; trypsin inhibitors

INTRODUCTION

A variety of hydroxamic acid derivatives has been reported to have pharmacological and biological activities against cancer, cardiovascular diseases, Alzheimer's disease, and tuberculosis (*1*). Succinimide hydroxamic acids have proven to be potent inhibitors of histone deacetylase and tumor cell proliferation (*2*). Hydroxamic acid derivatives of salicylic acid include cyclooxygenase (COX)-1 and COX-2 inhibitors (*3*). Oxal hydroxamic acid derivatives are potent inhibitors of matrix metalloproteinases (*4*). The aspartic acid β-hydroxamate exhibits antitumor activity on L5178Y leukemia (*5*), therapeutic effects on friend erythroleukemia (*6*), and antiproliferative activity on friend virus-infected erythropoietic progenitor cells (*7*). We also reported that pectin hydroxamic acids (PHAs) at different degrees of esterification (DE) exhibited both semicarbazide-sensitive amine oxidase and angiotensin converting enzyme (ACE) inhibitory activities (*8*). The monohydroxamates of aspartic acid and glutamic acid also exhibited ACE inhibitory activities (*9*). The above-mentioned biological activities of hydroxamic acid derivatives were correlated with their metal-chelating properties (*1*, *10*). Ahuja and Rai (*11*) introduced glycine hydroxamate and acetic acid hydroxamate into the epoxy ether of guaran polymers. Both hydroxamate derivatives of guaran polymers exhibited pH-dependent metal adsorptions, including Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}. Lutfor et al. (*12*) prepared hydroxamate derivatives of sago starch as metal absorbents in various salt solutions.

Immobilized metal affinity chromatography (IMAC) has frequently been used for everything from purifications of recombinant proteins with histidine tags (*13*, *14*) and metalloproteins (*15*) to a tool for proteomics (*16*). Then, it was found that IMAC could purify trypsin inhibitors (TIs) (*17*, *18*). 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 (*19*, *20*). We found that polyamines, including cadaverine, spermidine and spermine, were bound covalently to TIs from sweet potato and might participate in regulating the growth and developmental processes of sweet potato (*21*). Sweet potato TIs were also proved to have both dehydroascorbate reductase and monodehydroascorbate reductase activities and might respond to environmental stresses (*22*). Maeshima et al. (*23*) identified the sporamin as the major storage protein in sweet potato root, accounting for 80% of the total proteins there; a dramatic decrease to 2% of the original value was found during sprouting. Yeh et al. (*24*) confirmed that sporamin exhibited trypsin inhibitory activities. In this research, we prepared PHAs from 94% degree of esterification (DE94) pectin and coupled them to epoxy-activated Sepharose 6B gel to get immobilized PHA gels. These gels were then balanced in a column with 2 mM

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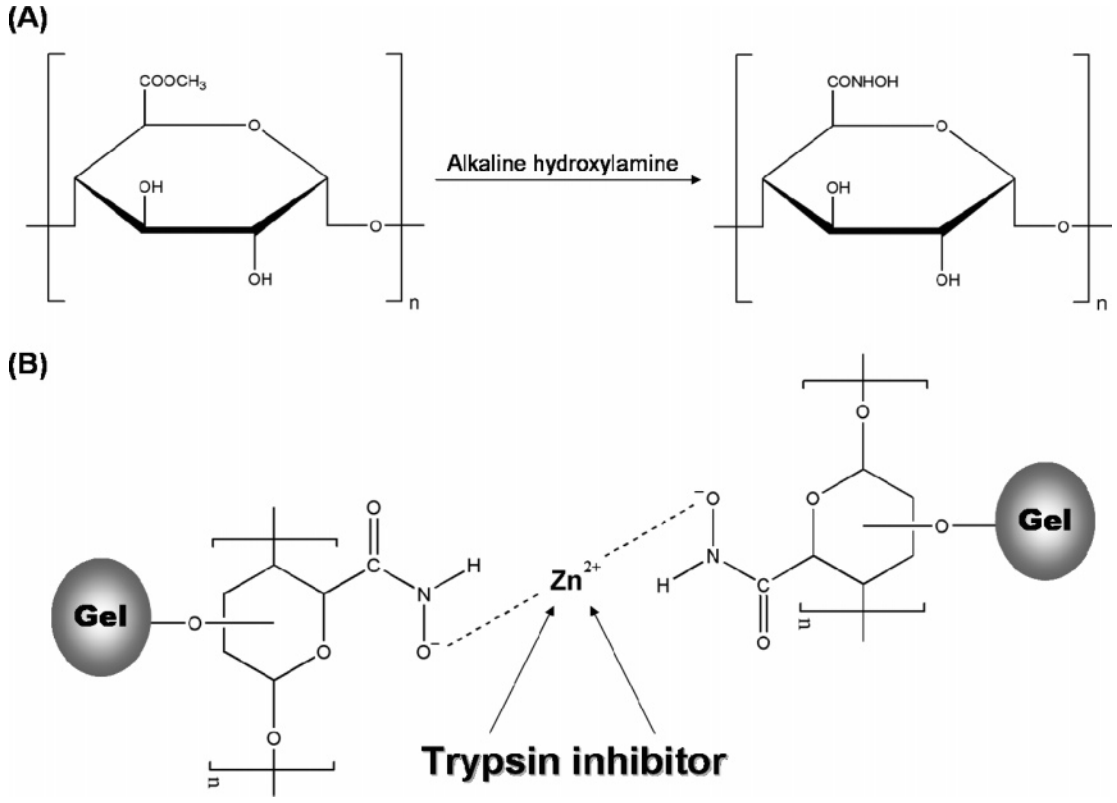

Figure 1. The diagrams of (**A**) pectin hydroxamic acid (PHA) prepared from pectin and alkaline hydroxylamine and (**B**) the immobilized PHA-Sepharose 6B gel prepared through ether linkages. The use of immobilized Zn–PHA gels as IMAC column for trypsin inhibitor purification.

$ZnCl_2$ in 50 mM Tris-HCl buffer (pH 7.9) to test the immobilized Zn−PHA affinity chromatography for the purification of TIs from soybean and sweet potato.

MATERIALS AND METHODS

Materials. Commercial pectin (from citrus fruit, DE94%), hydroxylamine, EDTA•2Na, Tris-HCl, TPCK-treated trypsin (12 400 units/mg of protein, T-1426), and zinc chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Casein (bovine milk) was from Calbichem Co. (Darmstadt, Germany). Epoxy-activated Sepharose 6B and CNBr-activated Sepharose 4B were purchased from Amersham Biosciences (Uppsala, Sweden). Soybean TI was purchased from Roche Applied Science (Mannheim, Germany). The prestained protein marker for SDS−PAGE was from Bioman Sci. Co. Ltd. Other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO).

Preparation of Pectin Hydroxamic Acid. The hydroxamate derivatives of pectin were generated according to the method of Gee et al. (*25*). An 8-g portion of commercial pectin with DE 94 suspended in 500 mL of methanol was stirred at room temperature for 20 h with a mixed solution (insoluble salt was removed by filtration) containing 13 g of potassium hydroxide in 50 mL of methanol and 12 g of hydroxylamine-HCl in 150 mL of methanol to get PHAs. After filtration by a G3 glass filter, the PHAs were washed with 70% methanol and readjusted to neutral pH. The PHAs were redissolved in water and then dialyzed against deionized water overnight. After being precipitated with 2-propanol, washed with methanol, and rinsed with acetone, the PHAs were dried at 37 °C for further use.

Preparation of Immobilized PHA−Sepharose 6B Gel. The PHA was coupled to the epoxy-activated Sepharose 6B gel to get the immobilized PHA gel according to the manufacturer's instructions. Briefly, the suitable amounts of epoxy-activated Sepharose 6B powders were swollen in distilled water. After filtration by a G3 glass filter, the swollen gel was being shaken in 50 mL of sodium carbonate buffer (pH 11.0) containing 500 mg of PHAs at 37 °C overnight. The immobilized PHA gel was washed repeatedly with bicarbonate buffer (0.1 M, pH 8.0) and acetate buffer (0.1 M, pH 4.0) and then was blocked with 1 M glycine overnight under the same conditions. After washing alternatively with solution containing 0.5 M NaCl of high and low pH, the immobilized PHA gels were collected for further usage.

Immobilized Zn−PHA Gel for IMAC Purification of Trypsin Inhibitor. The storage roots of sweet potato were cut into strips and extracted immediately with four volumes (w/v) of 100 mM Tris-HCl buffer (pH 7.9) containing 1% ascorbate and 1% (w/v) PVP in a homogenizer for 30 s (4×). The homogenates were filtered through four layers of cheesecloth and centrifuged twice at 12 000*g* for 30 min. The supernatants were saved as crude extracts. The crude extracts were loaded directly onto a self-prepared trypsin−Sepharose 4B affinity column (1.0 × 10 cm) to absorb TIs and were eluted by changing pH value with 200 mM KCl buffer (pH 2.0) as previously reported (*21*, *22*, *26*). The trypsin−Sepharose 4B affinity gels were self-prepared according to the manufacturer's instructions using CNBr-activated Sepharose 4B gels. The purified sweet potato TIs were lyophilized and stored at −18 °C for further use. The immobilized PHA Sepharose 6B gel was then balanced in column with 2 mM $ZnCl_2$ (prepared as stock solutions of 200 mM $ZnCl_2$ in 1 N HCl) in 50 mM Tris-HCl buffer (pH 7.9) for the purification of trypsin inhibitors from soybean and sweet potato. Two milligrams of purified TIs from soybean or sweet potato was dissolved in 4 mL of 2 mM $ZnCl_2$ in 50 mM Tris-HCl buffer (pH 7.9) and loaded directly onto an immobilized Zn−PHA affinity column. After being washed with the packed-column volume of the same buffer for several times, the adsorbed proteins were eluted by 100 mM EDTA in 10 mM Tris-HCl buffer (pH 7.9), and the absorbance was monitored at 280 nm. The flow rate was 45 mL/h, and 4.5 mL was saved for each fraction. The trypsin inhibitory activity was determined using casein as substrate (*27*) and expressed as micrograms of trypsin inhibited. The amount of protein was determined by the method of Bradford (*28*) using bovine serum albumin as standard.

Protein and Trypsin Inhibitory Activity Stains on the 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 activity stains at 4 °C overnight. Coomassie brilliant blue R-250 was used for protein staining (*29*). After electrophoresis, 12.5% gels were washed with 25% 2-propanol in 10 mM Tris-HCl buffer (pH 7.9) for 10 min twice to remove SDS (*30*) and then for TI activity stain.

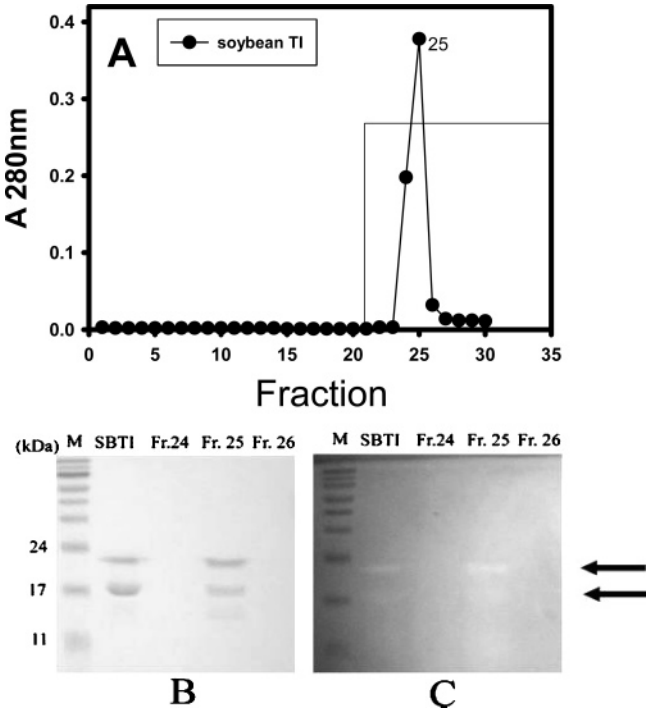

Figure 2. (**A**) The chromatogram of commercial soybean TIs on the immobilized Zn–PHA column. Two milligrams of soybean TIs was dissolved in 4 mL of 2 mM $ZnCl_2$ in 50 mM Tris-HCl buffer (pH 7.9) and loaded directly onto an immobilized Zn–PHA affinity column. After being washed with the same buffer for 20 fractions, the adsorbed proteins were eluted by 100 mM EDTA in 10 mM Tris-HCl buffer (pH 7.9) for 15 fractions, and the absorbance was monitored at 280 nm. The flow rate was 45 mL/h, and 4.5 mL was saved for each fraction. The protein (**B**) and trypsin inhibitory activity (**C**) stainings of fractions 24–26 were performed on the 12.5% SDS–PAGE gels in the comparison with soybean TI (SBTI, lane 2). Arrows indicate the locations of TI activity. M indicates the prestained markers for SDS–PAGE.

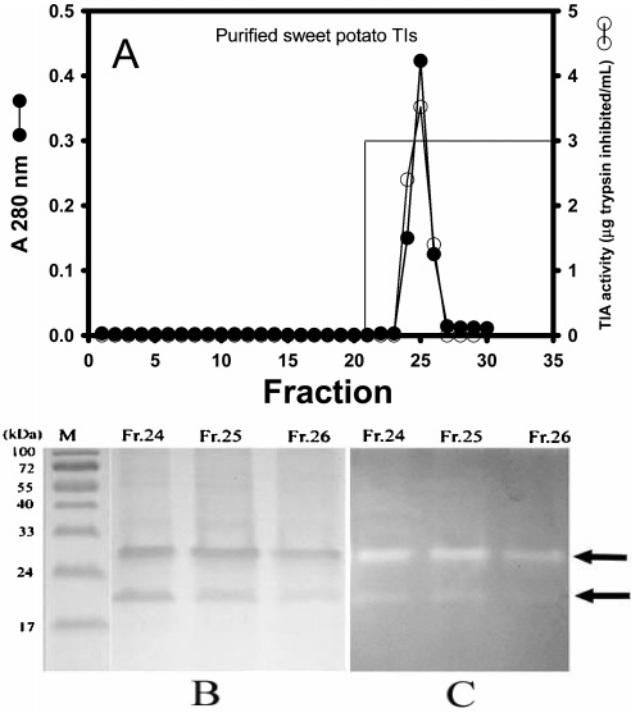

Figure 3. (**A**) The chromatogram of sweet potato TIs on the immobilized Zn–PHA column. Two milligrams of sweet potato TIs was dissolved in 4 mL of 2 mM $ZnCl_2$ in 50 mM Tris-HCl buffer (pH 7.9) and loaded directly onto an immobilized Zn–PHA affinity column. After being washed with the same buffer for 20 fractions, the adsorbed proteins were eluted by 100 mM EDTA in 10 mM Tris-HCl buffer (pH 7.9) for 15 fractions, the absorbance was monitored at 280 nm, and activity was determined. The flow rate is 45 mL/h, and 4.5 mL is saved for each fraction. The protein (**B**) and trypsin inhibitory activity (**C**) stainings of fractions 24–26 were performed on the 12.5% SDS–PAGE gels. Arrows indicate the locations of TI activity. M indicates the prestained markers for SDS–PAGE.

The gel was stained according to the method of Hou and Lin (*30*). The blank zones showed positions where the trypsin inhibitory activities were located.

RESULTS AND DISCUSSION

Preparation of Immobilized PHA and Zn−PHA Gels. The diagrams of the preparation of immobilized PHA and Zn−PHA gels are shown in **Figure 1**. Under alkaline conditions, the hydroxylamine was reacted with pectin to form PHA (**Figure 1A**), and the PHA was coupled to epoxy-activated Sepharose 6B through an ether linkage as an immobilized PHA gel (**Figure 1B**). The zinc ion was chelated by immobilized PHA gel and was used during IMAC for testing TI purifications.

Immobilized Zn−PHA Gel for IMAC Purifications of Trypsin Inhibitor. The self-prepared trypsin affinity gel was used for TI purifications (*21*, *22*, *31*−*34*). Basically, the enzyme−substrate (trypsin−trypsin inhibitor) model (*35*) was used to explain the affinity purification. However, purified trypsin was needed as a coupled ligand for affinity gel preparation. The impurities (i.e. contaminations of chymotrypsin) for affinity gel preparation might result in different types of protease inhibitors. IMAC has frequently been used for recombinant protein purification with histidine tags (*13*, *14*). It has reportedly been used for TIs purifications (*17*, *18*). It implies that available amino acids, such as histidine, cysteine, or tryptophan, on the protein surface may interact with metal ions (*36*). In this report, the immobilized Zn−PHA gels were used as IMAC gels to investigate TI purification. **Figure 2** shows the chromatographic profile (**A**) and protein (**B**) and activity (**C**) stainings of 2 mg of purified soybean TIs on immobilized Zn−PHA gels. The blank zones show positions where the trypsin inhibitory activities were located. It was clear that all of the soybean TIs could be adsorbed onto the gels and were eluted as a single peak by 100 mM EDTA in 10 mM Tris-HCl buffer (pH 7.9) (**Figure 2A**). The eluted fractions (fraction 24−26) were checked for protein (**Figure 2B**) and trypsin inhibitory activity (**Figure 2C**) on 12.5% SDS−PAGE gels in comparison with soybean TI (SBTI, lane 2, **Figure 2B,C**). Comparing the protein stains (**Figure 2B**) with TI activity stains (**Figure 2C**), the adsorbed protein (fraction 25) on immobilized Zn−PHA gel exhibited TI activity comparable with the originals.

Figure 3 shows the chromatographic profile (**A**) and protein (**B**) and activity (**C**) stains of 2 mg of purified sweet potato TIs on immobilized Zn−PHA gels. The lyophilized powders of sweet potato TIs were purified by trypsin affinity column. From the result of **Figure 3A**, the purified sweet potato TIs were also adsorbed onto the Zn−PHA gel and were eluted as a single peak by 100 mM EDTA in 10 mM Tris-HCl buffer (pH 7.9). By trypsin inhibitory activity determinations, the changes of TI activity were parallel with the contents of eluted protein (*A* 280 nm). The eluted fractions (fraction 24−26) were checked for protein (**Figure 3B**) and trypsin inhibitory activity (**Figure 3C**) on 12.5% SDS−PAGE gels. The eluted fraction contained TI activities (**Figure 3C**, arrows indicated) comparable to those of the protein stains (**Figure 3B**). From the results of **Figures**

Table 1. Purification of Trypsin Inhibitors from Sweet Potato by One Step of Immobilized Zn–PHA Affinity Chromatography

	protein (mg)	activity (μg trypsin inhibited)	specific act. (μg trypsin inhibited /mg of protein)	recovery (%)	fold
crude extracts	2.24 ± 0.03	144.68 ± 1.23	64.59 ± 0.07	100	1
Zn-PHA column	0.102 ± 0.04	130.2 ± 0.35	1276.47 ± 0.03	90	19.76

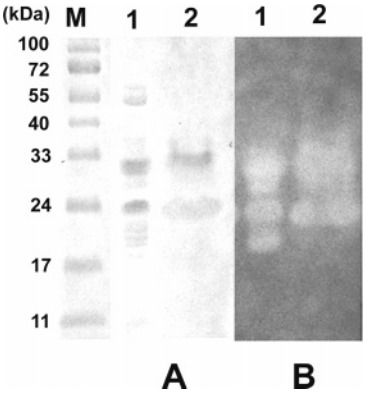

Figure 4. The protein (**A**) and trypsin inhibitory activity (**B**) stainings on the 12.5% SDS–PAGE gels of sweet potato crude extracts by one step of immobilized Zn–PHA affinity chromatography. Lane 1, crude extracts; lane 2, purified sweet potato TI after immobilized Zn–PHA affinity column. Protein (9 μg) is loaded in each well. M indicates the prestained markers for SDS–PAGE.

2 and 3, it was clear that the self-prepared immobilized Zn−PHA gels could be employed for IMAC for TI purifications.

Table 1 shows the purification of trypsin inhibitors from crude extracts of sweet potato by one step of immobilized Zn−PHA affinity chromatography. **Figure 4** shows the protein and activity stainings from crude extracts of sweet potato TI after one-step purification. Maeshima et al. (*23*) identified the sporamin as the major storage protein in sweet potato root, and it accounted for 80% of the total proteins in the root. Yeh et al. (*24*) confirmed that sporamin exhibited trypsin inhibitory activities. This meant that the crude extracts of sweet potato contained minor non-TI proteins. From the result of **Table 1**, one-step purification by the immobilized Zn−PHA can lower protein recovery up to 22-fold (from 2.24 ± 0.03 to 0.102 ± 0.04 mg); however, the recovery of the trypsin inhibitory activity remains 90% in one-step purification. It means that the recovered protein almost exhibits TI activity as confirmed in **Figure 4**. One step of the immobilized Zn−PHA affinity column from crude extracts produced an almost 20-fold purification, and the trypsin inhibitory activity matched the purified protein bands (**Figure 4**).

In conclusion, the immobilized Zn−PHA could be used for IMAC for TI purifications of soybean and sweet potato. The immobilized Zn−PHA gel may be cheaper than trypsin affinity gel for TI purifications. It might be more convenient and cheaper to use the precipitates of Zn−pectin hydroxamic acid or Cu−pectin hydroxamic acid instead of coupled gels for TI purification, and we will investigate this in the future.

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