Bot. Bull. Acad. Sin. (2003) **44:** 267-273

# The mucilage of yam (*Dioscorea batatas* Decne) tuber exhibited angiotensin converting enzyme inhibitory activities

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(Received July 29, 2003; Accepted September 12, 2003)

**Abstract.** The tuber mucilage of yam (*Dioscorea batatas* Decne) (YTM) was extracted and purified to homogeneity, which was confirmed by the toluidine blue staining on a sodium dodecylsulfate-polyacrylamide gel electrophoresis gel treated with 2-mercaptoethanol appearing as a single band with molecular mass larger than 250 kDa. This purified YTM was shown by spectrophotometric method to inhibit angiotensin converting enzyme (ACE) in a dose-dependent manner (28.7 to 59.8% ACE inhibition, respectively, by 102.46 to 409.84 μg/mL YTM) using (*N*-(3-[2-furyl] acryloyl)-Phe-Gly-Gly) (FAPGG) as a substrate. The concentration of YTM required for 50% inhibition ($IC_{50}$) of ACE activity was 256.2 μg/mL while that of captopril was 0.00781 μM (0.0095 nmole). The commercial polysaccharide pectin (102.46 to 307.38 μg/mL) showed no inhibitory activity against ACE. Using fluorescent silica TLC or $C_{18}$ reverse phase HPLC to detect FAPGG and FAP, the results also showed that YTM inhibited ACE. The YTM showed mixed type inhibition against ACE, and the Michaelis constant in the presence of YTM was 0.33 mM. Consumption of yam tubers may benefit people's health.

**Keywords:** Angiotensin converting enzyme (ACE); HPLC; (N-(3-[2-furyl]acryloyl]-Phe-Gly-Gly) (FAPGG); Mucilage; TLC; Yam.

## Introduction

Several risk factors are associated with stroke, including age, gender, elevated cholesterol, smoking, alcohol, excessive weight, race, family history, and hypertension (Mark and Davis, 2000). Although some of these risk factors cannot be modified, one factor that can be controlled and has the greatest impact on the etiology of stroke is high blood pressure (Dunbabin, 1992). Hypertension is considered to be the central factor in stroke with approximately 33% of deaths due to stroke attributed to untreated high blood pressure (Mark and Davis, 2000). Several classes of pharmacological agents have been used in the treatment of hypertension (Mark and Davis, 2000). One class of antihypertensive drugs, known as angiotensin I converting enzyme (ACE) inhibitors (ACEI, i.e. a peptidase inhibitor), are associated with a low rate of adverse side-effects and are the preferred class of anti-hypertensive agents when treating patients with concurrent secondary diseases (Fotherby and Panayiotou, 1999). ACE (peptidyldipeptide hydrolyase EC 3.4.15.1) is a dipeptide-liberating exopeptidase classically associated with the renin-angiotensin system regulating peripheral blood pressure (Mullally et al., 1996). ACE removes a dipeptide from the C-terminus of angiotensin I to form angiotensin II, a very hypertensive compound. Several endogenous peptides, such as enkephalins, β-endorphin, and substance P, were reported to be competitive substrates and inhibitors of ACE (Mullally et al., 1996). Several food-derived peptides also inhibited ACE, including α-lactalbumin and β-lactoglobulin (Mullally et al., 1996), casein (Maruyama et al., 1987), zein (Yano et al., 1996), and gelatin (Chen et al., 1999; Kim et al., 2001). Several antioxidant peptides (reduced glutathione and carnosine-related peptides) (Hou et al., 2003) and synthetic peptides also exhibited ACEI activities (Chen et al., 2003).

Yam (*Dioscorea* species) is a member of the monocotyledonous family Dioscoreaceae and is a staple food in West Africa, Southeast Asia and the Caribbean (Akoruda, 1984). Yam is recognized as an herbal plant since dried tuber slices were frequently used as Chinese herbal medicines. The tuber storage proteins of yam, dioscorin, exhibited carbonic anhydrase and trypsin inhibitor activities (Hou et al., 1999a), both dehydroascorbate reductase and monodehydroascorbate reductase activities (Hou et al., 1999b), and antioxidant activities (Hou et al., 2001). Yam tuber contained mucilages reported to be a mannan-protein complex (Misaki et al., 1972; Tsai and Tsai, 1984). Recently, it was reported that yam tuber mucilage (YTM) exhibited antioxidant activities (Hou et al., 2002). In this work we report for the first time that purified YTM exhibits novel dose-dependent ACE inhibitory activities. Captopril was used as a positive control and commercial pectin as a negative control. The YTM showed mixed type inhibition against ACE, and the Michaelis constant in the presence of YTM was also determined.

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## Materials and Methods

### *Materials*

Tris, electrophoretic reagents, and silica gel 60 $F_{254}$ were purchased from E. Merck Inc. (Darmstadt, Germany); captopril was purchased from Calbiochem Co. (CA, USA); Seeblue prestained markers for SDS-PAGE including myosin (250 kDa), BSA (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin (30 kDa), and lysozyme (16 kDa) were from Invitrogen (Groningen, The Netherlands); FAPGG, ACE (I unit, rabbit lung), nonylamine, pectin (from citrus fruit, degree of esterification 94%), toluidine blue, coomassie brilliant blue R-250, and other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### *Extraction and Purification of Mucilage from Yam Tuber*

Fresh yam (*Dioscorea batatas* Decne) tubers were purchased from a local wholesaler. After washing and peeling, the tubers were cut into strips for YTM extraction and purification according to the methods of Hou et al. (2002). One kg yam tuber was homogenized with 4 L of 50 mM Tris-HCl buffer (pH 8.3) containing 1% vitamin C. After centrifugation at 14,000 *g* for 30 min, the supernatants were saved, and isopropanol was added to a final concentration of 70%. This solution was stirred quickly and placed at 4ºC overnight. The precipitates were filtrated and dehydrated with 100% isopropanol and then washed with acetone. After drying in an oven at 40ºC, the crude YTM was ground and collected for further purification by both SDS and heating procedures. The 1.0 g crude YTM powder was dissolved in 200 mL deionized water and kept warm in water bath at 50ºC. The 40 mL of 5% SDS solution (dissolved in 45% ethanol) was added to crude YTM solution and kept at 50ºC with gentle stirring for 20 min and continuously stirred at room temperature for another 2 h. Then, this solution was placed on ice bath to lower the temperature to precipitate the SDS-protein complex. After centrifugation at 14,000 *g* at 0ºC for 30 min, the supernatants were saved, and the SDS-protein complex was precipitated by isopropanol as mentioned above. After drying in a 40ºC oven, the semipurified YTM was ground and dissolved, then heated in boiling water for 20 min. After centrifugation at 14,000 *g* at 0ºC for 30 min, the supernatants were saved and isopropanol was added to a final concentration of 70%. This purified YTM was filtrated, dehydrated, rinsed with acetone, dried, and then collected for further uses.

### *Protein and Proteoglycan Stainings on SDS-PAGE Gels*

Eighty μL samples were mixed with 20 μL sample buffer, namely 60 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 25% glycerol and 0.1% bromophenol blue with 2-mercaptoethanol, heated in boiling water for 5 min, cooled to ambient temperature, and subjected to electrophoresis according to the method of Laemmli (1970). This discontinuous gel system contained 2.5 cm, 4% stacking gel, and 4.5 cm, 15% separating gel. Coomassie brilliant blue R-250 was used for protein staining (Neuhoff et al., 1985). The 0.2% toluidine blue solution was used for proteoglycan staining (Carney, 1986).

### *Determination of ACE Inhibitory Activity of YTM by Spectrophotometry*

The ACE inhibitory activity was measured according to the method of Holmquist et al. (1979) with some modifications. The total 1.22 mL contained 20 μL (20 mU) of commercial ACE (1 U/mL, rabbit lung, Sigma Chem. Co.), 200 μL of different amounts of YTM (125, 250, 375 and 500 μg) or commercial pectin (degree of esterification, 94%, 125, 250 and 375 μg), and 1 mL, 0.5 mM *N*-(3-[2-furyl]acryloyl]-Phe-Gly-Gly) [FAPGG, dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 0.3 M NaCl]. The decreased absorbance readings at 345 nm ($\Delta A_{inhibitor}$) were recorded over 5 min at room temperature. Deionized water was used instead of sample solution for blank reading ($\Delta A_{blank}$). The captopril (MW is 217.3) was used as a positive control for ACE inhibition (0.75, 1.89, 3.77, 5.66, 7.54, 18.8, and 75.4 nM). The ACE activity was expressed as DA 345 nm and the ACE inhibition (%) was calculated as followed: $[1-(\Delta A_{inhibitor} \div \Delta A_{control})] \times 100\%$. $IC_{50}$ was defined as the concentration of samples required to inhibit 50% of ACE activity under these conditions.

### *Determination of ACE Inhibitory Activity of YTM by TLC*

The ACE inhibitory activity of YTM was determined by TLC method (Holmquist et al., 1979). The reactions of commercial ACE with purified YTM or commercial pectin were done according to the methods of Anzenbacherova et al. (2001) with some modifications. Each 100 μL sample (250 μg) was premixed with 15 mU ACE for one min and then 200 μL of 0.5 mM FAPGG was added and reacted at room temperature for 10 min. The 800 μL methanol was added to stop the reaction. In the blank experiment only FAPGG was used; in the control experiment, ACE reacted with FAPGG under the same conditions. Each reaction mixture was dried under reduced pressure, redissolved with 400 μL methanol and 50 μL of the solution were spotted on a silica gel 60 $F_{254}$ by CAMAG Linomat IV spray-on technique (CAMAG, Switzerland). The FAPGG and FAP (product of an ACE-catalyzed hydrolysis reaction) were separated by TLC with a developing system of water saturated 1-butanol : acetic acid : water, 4:1:1 (V/V/V) and detected under UV light (Holmquist et al., 1979).

### *Determination of ACE Inhibitory Activity of YTM by HPLC*

Each 50 μL of purified YTM (250 μg) or commercial pectin (250 μg) were premixed with 15 mU ACE for one min and then 200 μL of 0.5 mM FAPGG was added and reacted at room temperature for 10 min according to the methods of Anzenbacherova et al. (2001). The 800 μL methanol was

added to stop the reaction. In the blank experiment only FAPGG was used; in the control experiment, ACE reacted with FAPGG under the same conditions. Separation of FAPGG and FAP was carried out on the Shimadzu (Japan) chromatographic system at 40ºC (CTO-6A column oven) equipped with LC-10AS liquid chromatography and SIL-9A autoinjector with a 100 µL loop. The HPLC analysis was performed on a Lichrospher 100 RP-18 endcapped column (Merck, Darmstadt, Germany; 250 × 4.6 mm i.d.), with particle size 5 µm. The compounds were separated isocratically with a mobile phase consisting of a mixture of 0.02 M nonylamine (adjusted to pH 2.4 with phosphoric acid) : acetonitrile, 67.5:32.5 (V/V) (Anzenbacherova et al., 2001). The flow rate was 1 mL/min; the injection volume was 100 µL; the eluted fractions were detected at 345 nm.

### *Determination of the Kinetic Properties of ACE Inhibition by YTM*

The kinetic properties of ACE (20 mU) without or with purified YTM (250 µg) in total volume of 1.22 mL were determined using different concentrations of FAPGG as substrates (0.1 mM to 0.5 mM). The Km (without YTM) and Km´ (with YTM) were calculated from Lineweaver-Burk plots, where Km´ was the Michaelis constant in the presence of 204.92 µg/mL YTM.

## Results and Discussion

### *Extraction and Purification of YTM*

The crude YTM was obtained by isopropanol precipitation. YTM was purified by SDS treatment in the first step, during which proteins could be bound and then removed by centrifugation (Hou et al., 2002). The average recovery was 52.0 ± 0.28% by SDS treatment in two independent trials. This semipurified YTM was further purified by heating in boiling water for 20 min in the second step. The recovery of the second treatment was 92.4 ± 0.52%. The total recovery of purified YTM after two purification steps was about 48% starting from crude samples. Figure 1 shows the proteoglycan (Figure 1A) and protein (Figure 1B) stainings of YTM on SDS-PAGE gels after 2-mercaptoethanol treatment. Lanes 1 and 2 represent crude and purified YTM, respectively. The mucilage was stainable with toluidine blue dyes, and it proved to be a proteoglycan with molecular mass larger than 250 kDa on a SDS-PAGE gel (Figure 1A, indicated by an arrow). However, the crude mucilage (lane 1, Figure 1B) was found to contain proteins with molecular mass around 32 kDa, which did not appear in the purified YTM (lane 2, Figure 1B). From our previous report (Hou et al., 1999a, 2001), the yam tuber storage protein, dioscorin, exhibited molecular mass of 32 kDa after 2-mercaptoethanol treatment. The 32 kDa protein in crude YTM (lane 1, Figure 1B) could be dioscorins, and the purified YTM did not contain them.

### *ACE Inhibitory Activity of YTM by Spectrophotometry*

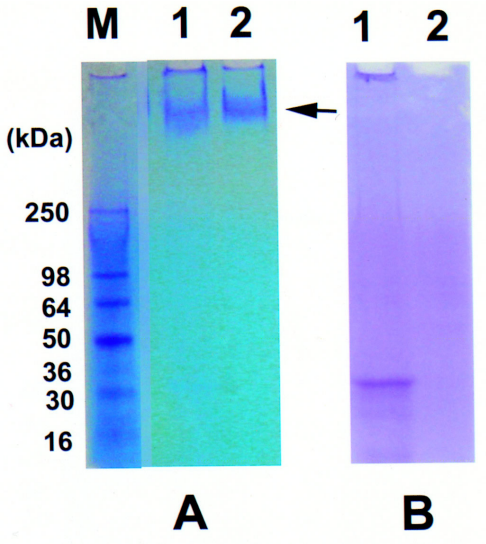


**Figure 1.** The proteoglycan (A) and protein (B) stainings of crude (lane 1) and purified (lane 2) yam tuber mucilage on SDS-PAGE gels after 2-mercaptoethanol treatment. The gel system contained 2.5 cm, 4% stacking gel and 4.5 cm, 15% separating gel. M indicates the Seeblue prestained markers of SDS-PAGE. Mucilage of 25 µg was loaded in each well. Arrow indicates the position of the mucilage.

The purified YTM was used for determinations of ACE inhibitory activity. Using FAPGG as a substrate, the concentrations of hydrolyzed product (FAP) were monitored by the decrease of the absorbance at 345 nm (Holmquist et al., 1979), found to be proportional to the hydrolysis time. Figure 2A shows the effects of purified YTM and commercial pectin on ACE activity. Figure 2B shows the effects of captopril (0.75, 1.89, 3.77, 5.66, 7.54, 18.8, and 75.4 nM) on ACE activity. In the absence of purified YTM, the ΔA/min of ACE activity was -0.0087, meaning the change in absorbance ($\Delta A_{control}$) was 0.044 after 5 min. However, in the presence of different amounts of purified YTM, the ΔA/min was changed to -0.0062, -0.0046, -0.0042, and -0.0035 for 102.46, 204.92, 307.38, and 409.84 µg/mL, respectively. The ACE inhibition (%) was calculated according to the equation $[1-(\Delta A_{inhibitor} + \Delta A_{control})] \times 100\%$, as shown in Figure 2A. Purified YTM was found to show dose-dependent ACE inhibitory activities (102.46 to 409.84 µg/mL, respectively, 28.7 to 59.8% ACE inhibition). The $IC_{50}$ of YTM in inhibiting ACE activity was 256.2 µg/mL. Captopril was 7.81 nM (Figure 2B), close to the value (7 nM) reported by Pihlanto-Leppälä et al. (1998). Captopril (3-mercapto-2-methylpropanoyl-1-proline) was the first ACE inhibitor designed and marketed for treating hypertension and was an analogue of dipeptide of Ala-Pro (Mark et al., 2000). However, the commercial polysaccharide pectin (102.46, 204.92, and 307.38 µg/mL) showed no inhibitory activity against ACE (Figure 2A). Pectins are principal constituents of the middle lamella in the cell wall. It seems that we can not explain the inhibition of ACE by YTM as an interference of enzyme-substrate interaction by

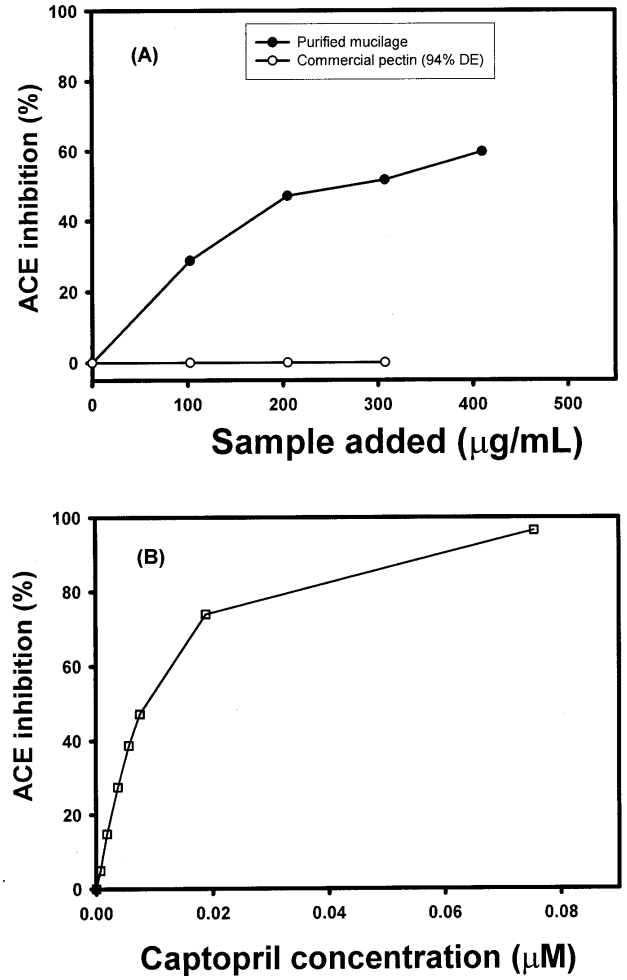


**Figure 2.** The effects of purified YTM, pectin, and captopril on ACE activity determined by spectrophotometry. (A) Purified YTM (102.46, 204.92, 307.38, and 409.84 μg/mL) or commercial pectin (102.46, 204.92, and 307.38 μg/mL); (B) Captopril (0.75, 1.89, 3.77, 5.66, 7.54, 18.8, and 75.4 nM). The inhibition of ACE (%) was calculated according to the equation $[1-(\Delta A_{inhibitor} \div \Delta A_{control})] \times 100\%$.

macromolecules. Both pectin and purified YTM were macromolecules; however, only the purified YTM showed special dose-dependent ACE inhibitory activity. In the literature, the protein hydrolysates were used as resources for purification of peptides as ACE inhibitors (Maruyama et al., 1987; Mullally et al., 1996; Yano et al., 1996; Chen et al., 1999; Kim et al., 2001; Chen et al., 2003; Hou et al., 2003), and the YTM was the first reported mucilage to exhibit ACE inhibition. The YTM was reported to be a mannan-protein complex (Misaki et al., 1972; Tsai and Tsai, 1984). Whether the ACE inhibitory activity of YTM was due to mannan, or protein portion, or the mannan-protein complex must be investigated further. From calculations, the $IC_{50}$ of YTM in inhibiting ACE activity was 256.2 μg/mL, less than that of the synthetic peptides $\alpha$-lactorphin (YGLF, 322.7 μg/mL) and $\beta$-lactotensin (HIRL, 507.4 μg/mL) (Mullally et al., 1996). Several identified peptide fragments (Pihlanto-Leppälä et al., 1998) also exhibited much higher $IC_{50}$ values than that of purified YTM; for example, LAHKAL of $\alpha$-lactalbumin hydrolysates, 406 μg/mL; GLDIQK of $\beta$-lactoglobulin hydrolysates, 391 μg/mL; and VAGTWY of $\beta$-lactoglobulin hydrolysates, 1171 μg/mL.

### *Determinations of ACE Inhibitory Activity of YTM by TLC*

The FAPGG and FAP (product of ACE catalyzed hydrolysis reaction) were separated by TLC using water saturated 1-butanol : acetic acid : water, 4:1:1 (V/V/V) as developing solvents according to the methods of Holmquist et al. (1979). Figure 3 shows the qualitative results of TLC chromatograms of a silica gel 60 $F_{254}$ for the effects of commercial pectin and purified YTM (250 μg, lanes 3 and 4, respectively) on 15 mU ACE. Lane 1 is a blank test (FAPGG only); lane 2 is a control test (ACE reacted with FAPGG to produce FAP). Compared to the control test (lane 2), purified YTM (lane 4) was found to inhibit ACE activity, resulting in smaller amounts of produced FAP observed under UV light. However, similar amounts of FAP were found between control test (lane 2) and commercial pectin (lane 3). The results demonstrated again that purified YTM exhibited ACE inhibitory activity.

### *Determinations of ACE Inhibitory Activity of YTM by HPLC*

The FAPGG and FAP were separated isocratically with a mobile phase consisting of a mixture of 0.02 M nonylamine (adjusted to pH 2.4 with phosphoric acid): acetonitrile, 67.5:32.5 (V/V) according to the methods of Anzenbacherova et al. (2001). Figure 4 shows the HPLC chromatograms of Lichrospher 100 RP-18 endcapped col-

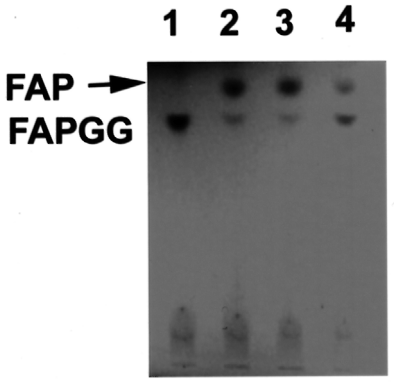


**Figure 3.** The TLC chromatograms of a silica gel 60 $F_{254}$ for the effects of yam tuber mucilage or commercial pectin on ACE activity. Lane 1, blank test (FAPGG only); lane 2, control test (ACE reacted with FAPGG to produce FAP); lane 3, 250 μg commercial pectin added; lane 4, 250 μg yam tuber mucilage added. Each solution was dried under reduced pressure and redissolved with 400 μL methanol. Each 50 μL was spotted on a silica gel 60 $F_{254}$ by CAMAG Linomat IV spray-on technique (CAMAG, Switzerland). The FAPGG and FAP were separated by water saturated 1-butanol : acetic acid : water, 4:1:1 (V/V/V). Arrow indicated the position of FAP.

umn for the inhibitory activity of yam tuber mucilage or commercial pectin on ACE. (A) is a blank test (FAPGG only with a retention time of 7.16 min); (B) is a control test (ACE reacted with FAPGG to produce FAP, with a retention time of 13.57 min); (C) is 250 µg commercial pectin; (D) is 250 µg yam tuber mucilage against 15 mU ACE. Compared with the control test (Figure 4B), it was found that purified YTM (Figure 4D, 250 µg) inhibited ACE activity since the FAP area was reduced to about 52.8% of the original value (control test, Figure 4B). However, the addition of pectin (250 µg, Figure 4C) showed no effect on FAP production compared with control (Figure 4B). This meant that purified YTM did have inhibitory activity against ACE. By using different methods, including spectrophotometry, TLC and HPLC for the determination of ACE assay, it was confirmed that purified YTM exhibited ACE inhibitory activity.

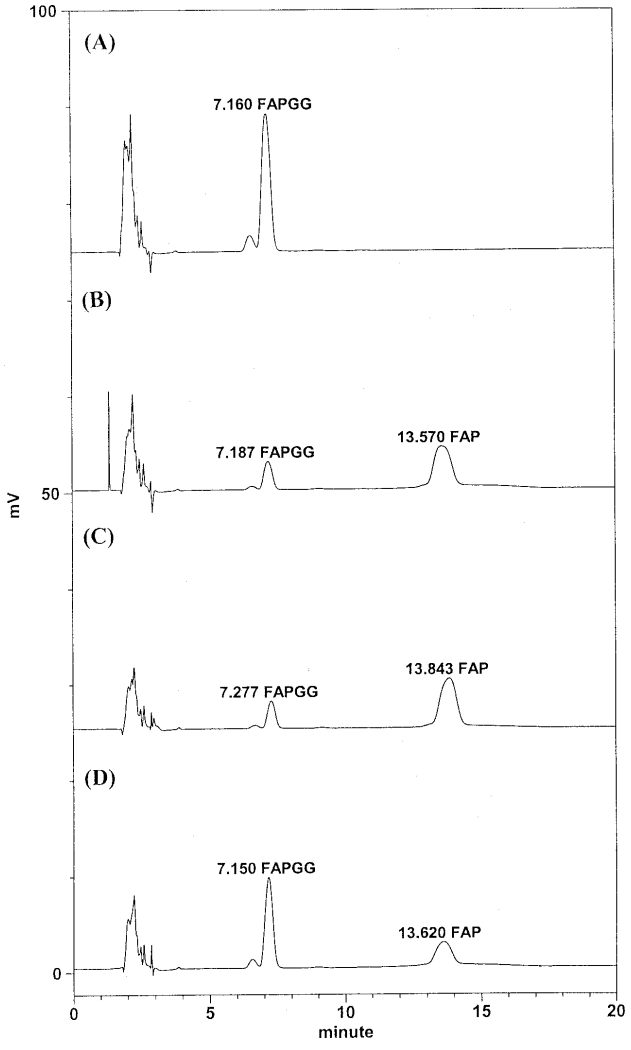


**Figure 4.** The HPLC chromatograms of Lichrospher 100 RP-18 endcapped column for the effects of yam tuber mucilage or commercial pectin on ACE inhibitory activity. (A) blank test (FAPGG only with a retention time of 7.16 min); (B) control test (ACE reacted with FAPGG to produce FAP which had a retention time of 13.57 min); (C) 250 µg commercial pectin; (D) 250 µg yam tuber mucilage against 15 mU ACE. The mobile phase consisted of a mixture of 0.02 M nonylamine (adjusted to pH 2.4 with phosphoric acid) : acetonitrile, 67.5:32.5 (V/V). The flow rate was 1 mL/min; the injection volume was 100 µL; and the eluted fractions were detected at 345 nm.

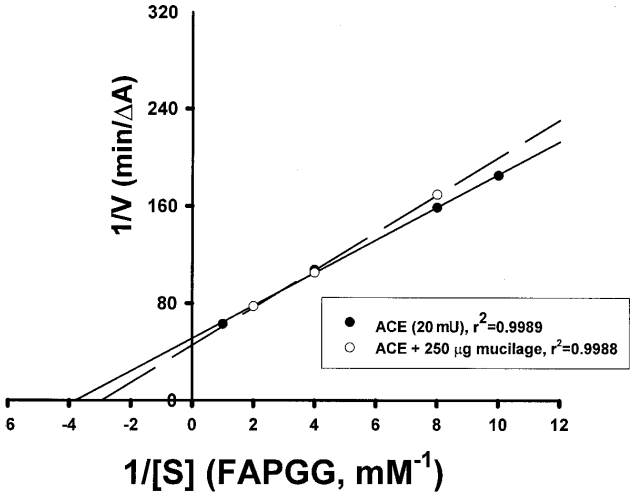


**Figure 5.** The Lineweaver-Burk plots of ACE (20 mU) without or with purified yam tuber mucilage (204.92 µg/mL) in different concentrations of FAPGG (0.1 to 0.5 mM).

### *Determinations of the Kinetic Properties of ACE Inhibition by YTM*

The Lineweaver-Burk plots of ACE (20 mU) without or with purified YTM (204.92 µg/mL) in different concentrations of FAPGG are shown in Figure 5. The results indicated that purified YTM acted as a mixed type inhibitor with respect to the substrate (FAPGG). Without the purified YTM, the calculated Km was 0.255 mM FAPGG for ACE, which was close to the result (0.3 mM) of Holmquist et al. (1979). In the presence of purified YTM (204.92 µg/mL), the calculated Km´ was 0.3304 mM.

In conclusion, the YTM exhibited dose-dependent ACE inhibitory activity and acted as a mixed type inhibitor with respect to the substrate (FAPGG). The $IC_{50}$ of YTM in inhibiting ACE activity was 256.2 µg/mL, less than several peptides acting as ACE inhibitors. It might be beneficial to health when people consume yam.

**Acknowledgment.** The authors want to thank the National Science Council, Republic of China, for its financial support (NSC 92-2313-B-038-001).

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# 山藥黏質多醣具有血管收縮素轉化酶抑制活性

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山藥（*Dioscorea batatas* Decne）黏質多醣經由抽取與純化步驟，以 SDS 電泳膠片顯示達到均質，以 toluidine blue 染色發現其分子量大於 250 kDa。以（*N*-(3-[2-furyl]acryloyl)-Phe-Gly-Gly）（FAPGG）為酵素反應基質，此一純化的山藥黏質多醣能抑制血管收縮素轉化酶（102.46 到 409.84 μg/mL，分別抑制28.7% 到 59.8% 血管收縮素轉化酶活性）其效果隨劑量增加而增加。山藥黏質多醣對於血管收縮素轉化酶 50% 抑制濃度為 256.2 μg/mL［對照組 captopril 為 7.81 nM (9.5 pmole)］。多醣類的果膠質（102.46，204.92 和 307.38 μg/mL）對血管收縮素轉化酶並沒有抑制的效果。利用螢光 silica TLC 或是逆相 $C_{18}$ HPLC 檢測 FAPGG 及其水解產物 FAP，結果顯示山藥黏質多醣可以抑制血管收縮素轉化酶。山藥黏質多醣對於血管收縮素轉化酶為混合型抑制，而 Michaelis constant 為 0.3304 mM。

**關鍵詞：**山藥；黏質多醣；血管收縮素轉化酶；混合型抑制。