

Effects of Aloe Vera Raw Gel on Human Toxin Absorption/Excretion and ACE Inhibition Activity

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Abstract: *Aloe barbadensis miller* raw gel (KJM) was extracted and purified to homogeneity and then identified as a single band with a molecular weight greater than 250 kDa by toluidine blue staining on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel treated with 2-mercaptoethanol. The purified KJM was shown to inhibit ACE in a dose-dependent manner (28.7–59.8% ACE inhibition by 102.46–409.84 µg/mL KJM, respectively) using (N-(3-[2-furyl]acryloyl)-Phe-Gly-Gly) (FAPGG) as a substrate by spectrophotometry. The concentration of KJM required for 50% inhibition (IC₅₀) of ACE activity was 256.2 µg/mL, whereas that of captopril was 0.00781 µM (0.0095 nmole). Commercially available polysaccharide pectin (102.46–307.38 µg/mL) did not exhibit any inhibitory activity against ACE. Detection of FAPGG and FAP using fluorescent silica TLC or C 18 reversed-phase HPLC showed that KJM inhibited ACE. KJM exhibited mixed inhibition of ACE, and the Michaelis constant was 0.33 mM in the presence of KJM. Consumption of *Aloe barbadensis* Miller raw gel may be beneficial for human blood and vascular health.

Keywords: *Aloe vera*, Raw gel, mucopolysaccharide, Angiotensin converting enzyme (ACE), HPLC, (N-(3-[2-furyl]acryloyl)-Phe-Gly-Gly) (FAPGG), Raw gel, TLC

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I. 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 (peptidyl dipeptide 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, b-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 a-lactalbumin and b-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).

Aloe vera species is a succulent plant belonging to the family *Asphodelaceae*, native to Egypt, Greece, West Africa, Southeast Asia, and the Caribbean (Amar Surjushe, 2008). In oriental medicine, *Aloe* is known as a medicinal herb because the dried whole leaves are often used as traditional medicine. The anthraquinone

component, a storage polysaccharide of Aloe raw gel, has been shown to exhibit carbonic anhydrase and trypsin inhibitor activities (Nuray et al., 2014), dehydratase and monohydratase reductase activities (Hou et al., 1999b), and antioxidant activities (Hamid, 2015). Aloe raw gel contains mucilage, which has been reported as acemannan-D-isomer mucopolysaccharide (Zhang et al., 1996; Kim et al., 2023). Recently, KimJungMoon Aloe Jeju Aloe vera raw gel (KJM) was reported to exhibit antioxidant activity (Kim et al., 2024). In this study, we report for the first time that purified KJM exhibits a novel dose-dependent ACE inhibitory activity. Captopril was used as a positive control, and commercial pectin was used as a negative control. KJM showed mixed inhibition on ACE, and the Michaelis constant in the presence of KJM was also determined.

II. EXPERIMENTAL PROCEDURE

Materials

Tris, electrophoretic reagents, and silica gel 60 F254 were purchased from E. Merck Inc. (Darmstadt, Germany); captopril was purchased from Calbiochem Co. (CA, USA); Seebblue 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%), oluidine blue, coomassie brilliant blue R-250, and other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

KJM extraction and purification

Fresh *Aloe barbadensis* Miller leaves were directly donated by KimJeongMoon Aloe Jeju Agricultural Factory. After washing and peeling, the aloe raw gel was cut into strips for KJM (KimJungMoon Aloe Jeju Aloe vera raw gel) extraction and purification according to the method of Kim et al. (2024). 1 kg of Aloe raw gel 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 supernatant was saved and isopropanol was added to a final concentration of 70%. The solution was stirred rapidly and left overnight at 4°C. The sediment was filtered, dehydrated with 100% isopropanol, and washed with acetone. After drying in an oven at 40°C, the crude KJM was ground and collected for further purification by SDS and heating procedures. 1.0 g of crude KJM powder was dissolved in 200 mL of deionized water and kept warm in a water bath at 50°C. 40 mL of 5% SDS solution (dissolved in 45% ethanol) was added to the crude KJM solution and kept at 50°C with gentle stirring for 20 min, and then stirred for 2 h at room temperature. The solution was then placed in an ice bath to lower the temperature and precipitate the SDS-protein complex. After centrifugation at 14,000 g for 30 min at 0°C, the supernatant was retained and the SDS-protein complex was precipitated with isopropanol as mentioned above. After drying in an oven at 40°C, the semi-purified KJM was ground, dissolved, and heated in boiling water for 20 min. After centrifugation at 14,000 g for 30 minutes at 0°C, the supernatant was collected and isopropanol was added to a final concentration of 70%. The purified KJM was filtered, dehydrated, rinsed with acetone, dried, and collected for further use.

Mucopolysacchride 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 mucopolysacchride staining (Neuhoff et al., 1985). The 0.2% toluidine blue solution was used for proteoglycan staining (Carney, 1986).

Determination of ACE Inhibitory Activity of KJM 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 KJM (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 ($\Delta A_{\text{inhibitor}}$) were recorded over 5 min at room temperature. Deionized water was used instead of sample solution for blank reading (ΔA_{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: $[1 - (\Delta A_{\text{inhibitor}} + \Delta A_{\text{control}})] \times 100\%$. IC₅₀ was defined as the concentration of samples required to inhibit 50% of ACE activity under these conditions.

Determination of ACE Inhibitory Activity of KJM by TLC

The ACE inhibitory activity of KJM was determined by TLC method (Holmquist et al., 1979). The reactions of commercial ACE with purified KJM 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 F254 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 the Kinetic Properties of ACE Inhibition by KJM

The kinetic properties of ACE (20 mU) without or with purified KJM (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 K_m (without KJM) and K_m' (with KJM) were calculated from Lineweaver-Burk plots, where K_m' was the Michaelis constant in the presence of 204.92 $\mu\text{g}/\text{mL}$ KJM.

III. RESULTS AND DISCUSSIONS

Extraction and Purification of KJM

The crude KJM was obtained by isopropanol precipitation. KJM was purified by SDS treatment in the first step, during which mucopolysaccharides and proteins could be bound and then removed by centrifugation (Mariana et al., 2023, Kim et al., 2024). The average recovery was $52.0 \pm 0.28\%$ by SDS treatment in two independent trials. This semipurified KJM was further purified by heating in boiling water for 20 min in the second step. The recovery of the second treatment was $92.4 \pm 0.52\%$. The total recovery of purified KJM after two purification steps was about 48% starting from crude samples. Figure 1 shows the proteoglycan (Figure 1A), mucopolysaccharide and protein (Figure 1B) stainings of YTM on SDS-PAGE gels after 2-mercaptoethanol treatment. Lanes 1 and 2 represent crude and purified KJM, 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).

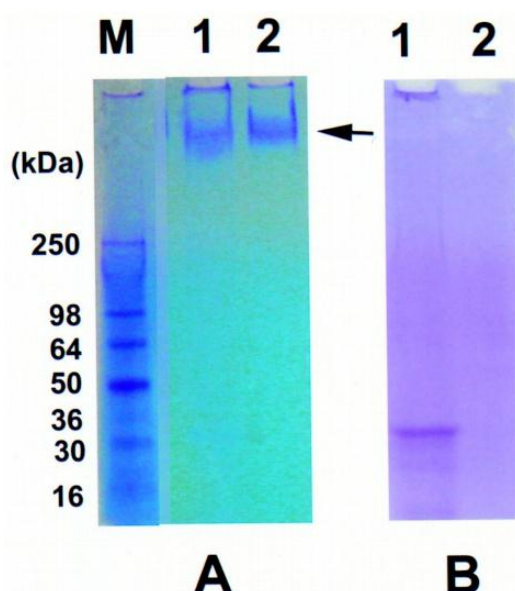


Figure 1. The proteoglycan (A) and protein (B) stainings of crude (lane 1) and purified (lane 2) Aloe vera raw gel on SDS-PAGE gels after 2-mercaptoethanol treatment. The gel system contained 2.5 cm, 4% stacking gel and 4.5 cm, 15% separating gel. M indicates the Seebule prestained markers of SDS-PAGE.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

From our previous report (Marzanna et al., 2019, Kim et al., 2024) the Aloe vera raw gel storage mucopolysaccharide, protein exhibited molecular mass of 32 kDa after 2-mercaptoethanol treatment. The 32 kDa mucopolysaccharide and protein in crude KJM (lane 1, Figure 1B) could be dioscorins, and the purified KJM did not contain them.

Inhibitory Activity of KJM by Spectrophotometry

The purified KJM 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 KJM 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 KJM, the $\Delta A/\text{min}$ of ACE activity was -0.0087, meaning the change in absorbance ($\Delta A_{\text{control}}$) was 0.044 after 5 min. However, in the presence of different amounts of purified KJM, the $\Delta A/\text{min}$ was changed to -0.0062, -0.0046, -0.0042, and -0.0035 for 102.46, 204.92, 307.38, and 409.84 $\mu\text{g/mL}$, respectively. The ACE inhibition (%) was calculated according to the equation $[1 - (\Delta A_{\text{inhibitor}} + \Delta A_{\text{control}})] \times 100\%$, as shown in Figure 2A.

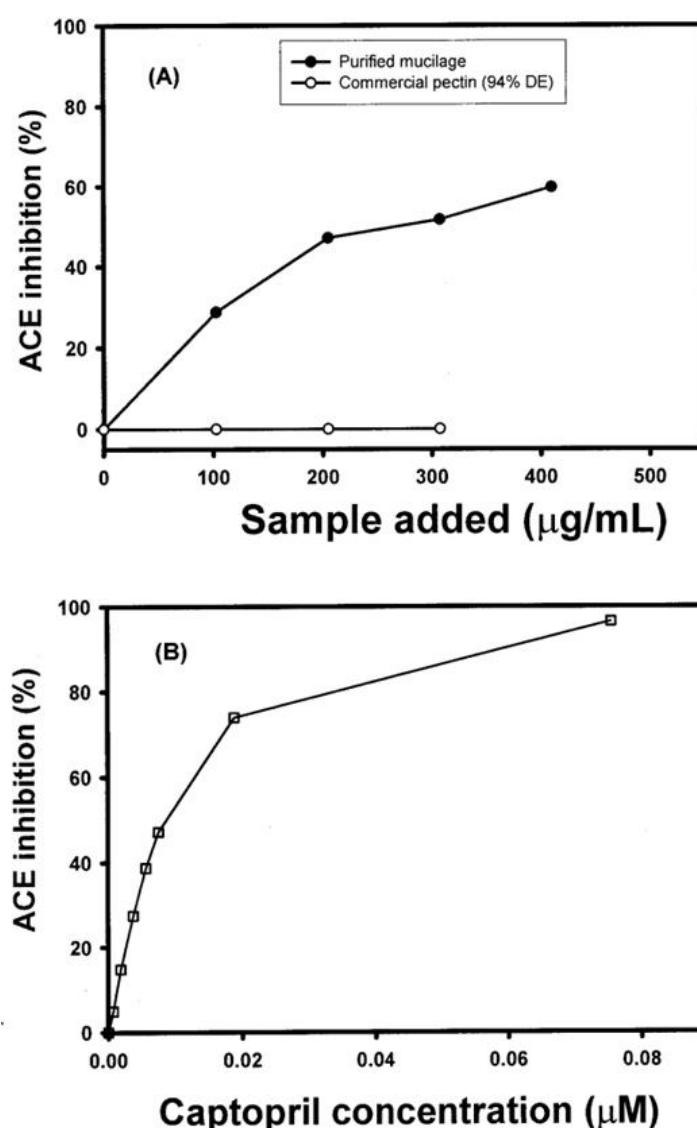


Figure 2. The effects of purified KJM, pectin, and captopril on ACE activity determined by spectrophotometry. (A) Purified KJM (102.46, 204.92, 307.38, and 409.84 $\mu\text{g/mL}$) or commercial pectin (102.46, 204.92, and 307.38 $\mu\text{g/mL}$); (B) Captopril (0.75, 1.89, 3.77, 5.66, 7.54, 18.8, and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

Purified KJM was found to show dose-dependent ACE inhibitory activities (102.46 to 409.84 $\mu\text{g/mL}$, respectively, 28.7 to 59.8% ACE inhibition). The IC_{50} of KJM in inhibiting ACE activity was 256.2 $\mu\text{g/mL}$. 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 $\mu\text{g/mL}$) 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 KJM as an interference of enzyme-substrate interaction by macromolecules. Both pectin and purified KJM were macromolecules; however, only the purified KJM showed special dose-dependent ACE inhibitory activity. In the literature, the mucopolysacchrides and 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; Kim et al., 2024), and the KJM was the first reported Aloe vera raw gel to exhibit ACE inhibition. The KJM was reported to be a acemannan-protein complex (Misaki et al., 1972; Tsai and Tsai, 1984, Kim et al., 2024). Whether the ACE inhibitory activity of KJM was due to mucopolysacchrides, acemannan, or protein portion, or the acemannan-protein complex must be investigated further. From calculations, the IC_{50} of KJM in inhibiting ACE activity was 256.2 $\mu\text{g/mL}$, less than that of the synthetic peptides α -lactorphin (YGLF, 322.7 $\mu\text{g/mL}$) and β -lactotensin (HIRL, 507.4 $\mu\text{g/mL}$) (Mullally et al., 1996, Kim et al., 2024). Several identified peptide fragments (Pihlanto-Leppälä et al., 1998) also exhibited much higher IC_{50} values than that of purified KJM; for example, LAHKAL of α -lactalbunin hydrolysates, 406 $\mu\text{g/mL}$; GLDIQK of β -lactoglobulin hydrolysates, 391 $\mu\text{g/mL}$; and VAGTWY of β -lactoglobulin hydrolysates, 1171 $\mu\text{g/mL}$.

Determinations of ACE Inhibitory Activity of KJM 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 F₂₅₄ for the effects of commercial pectin and purified KJM (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 KJM (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 KJM exhibited ACE inhibitory activity.

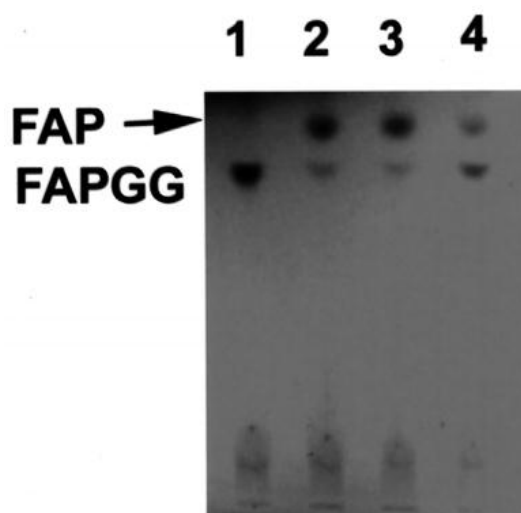


Figure 3. The TLC chromatograms of a silica gel 60 F₂₅₄ for the effects of Aloe vera raw gel or commercial pectin on ACE activity. Lane 1, blank test (FAPGG only); lane 2, control test (ACE reacted with FAPGG to produce FAP); lane 3, 250 μg commercial pectin added; lane 4, 250 μg Aloe vera raw gel added. Each solution was dried under reduced pressure and redissolved with 400 μL methanol. Each 50 μL was spotted on a silica gel 60 F₂₅₄ 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.

Determinations of ACE Inhibitory Activity of KJM 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 column for the inhibitory activity of Aloe vera raw gel 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 μ g commercial pectin; (D) is 250 μ g Aloe vera raw gel against 15 mU ACE. Compared with the control test (Figure 4B), it was found that purified KJM (Figure 4D, 250 μ g) 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 μ g, Figure 4C) showed no effect on FAP production compared with control (Figure 4B). This meant that purified KJM 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 KJM exhibited ACE inhibitory activity.

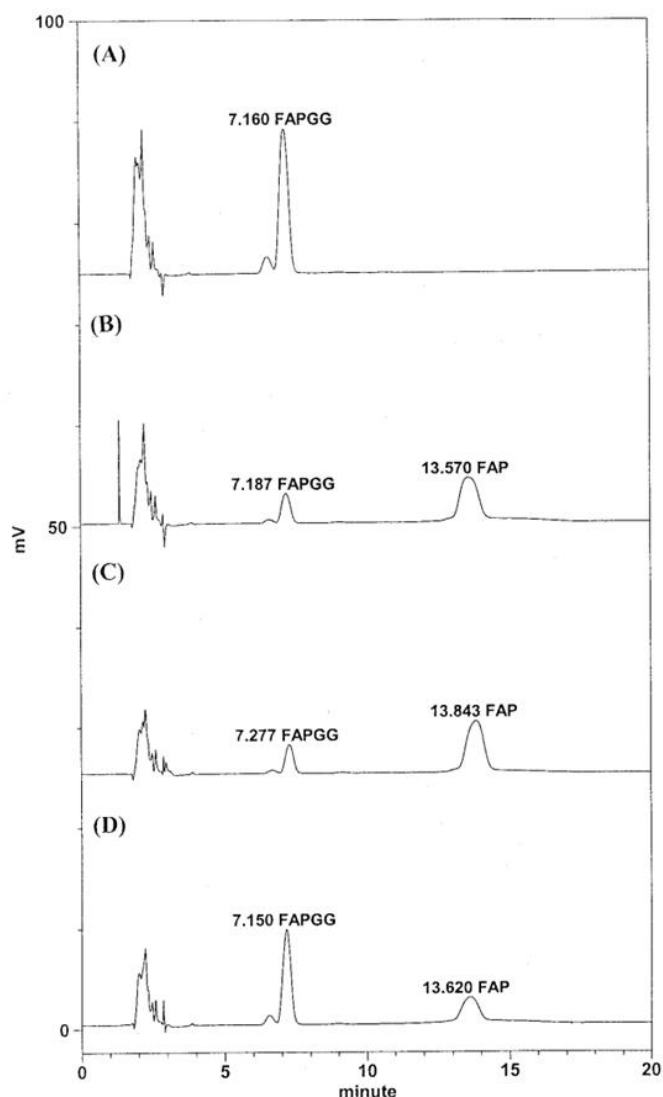


Figure 4. The HPLC chromatograms of Lichrospher 100 RP-18 endcapped column for the effects of Aloe vera raw gel or commercial pectin on ACE inhibitory activity. (A) blank test (FAPGG only with a retention time of 7.16 min); (B) control test (ACE reacted with FAPGG to produce FAP which had a retention time of 13.57 min); (C) 250 μ g commercial pectin; (D) 250 μ g Aloe vera raw gel 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 μ L; and the eluted fractions were detected at 345 nm.

Determinations of the Kinetic Properties of ACE Inhibition by KJM

The Lineweaver-Burk plots of ACE (20 mU) without or with purified KJM (204.92 $\mu\text{g/mL}$) in different concentrations of FAPGG are shown in Figure 5. The results indicated that purified KJM acted as a mixed type inhibitor with respect to the substrate (FAPGG). Without the purified KJM, the calculated K_m 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 $\mu\text{g/mL}$), the calculated K_m' was 0.3304 mM. In conclusion, the KJM 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 $\mu\text{g/mL}$, less than several peptides acting as ACE inhibitors. It might be beneficial to blood and vascular health when people consume Aloe.

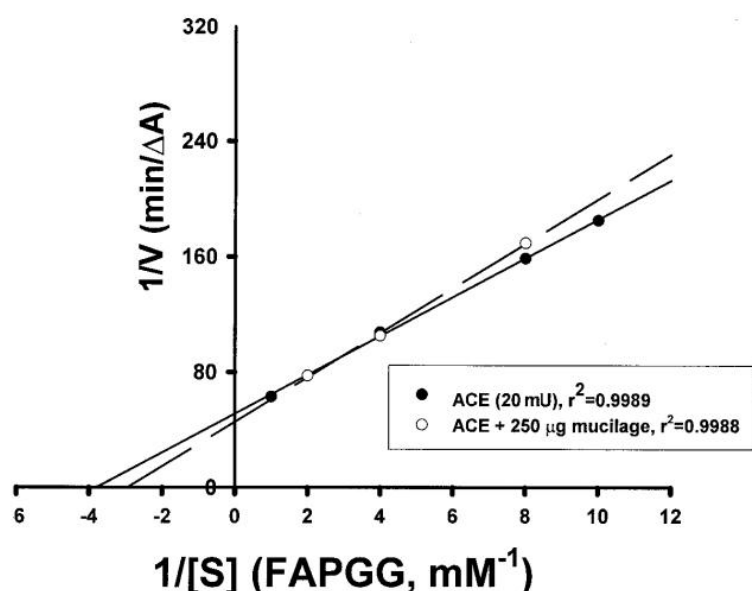


Figure 5. The Lineweaver-Burk plots of ACE (20 mU) without or with purified KJM mucilage (204.92 $\mu\text{g/mL}$) in different concentrations of FAPGG (0.1 to 0.5 mM)..

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