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Inhibition of Porcine Pancreatic Elastase (PPE) by Korean Mistletoe (*Viscum album* var. *coloratum*) Fractions

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Abstract – The serine proteases such as human leukocyte elastase (HLE) and porcine pancreatic elastase (PPE) are classified in the chymotrypsin family, and possibly the most destructive enzymes having the ability to degrade virtually all of the connective components in the body. In the present study, the extracts of water and methanol of Korean mistletoe (*Viscum album* var. *coloratum*) inhibited significantly the PPE activity. The fractions eluated on Amberlite XAD-2 from methanol extract were further purified on the repeated SiO_2 column chromatography and the fractions A, B and C were eluated. The fractions A, B and C at 3 mg/ml inhibited significantly the PPE activity up to 66%, 95% and 85%, respectively. In conclusion, the fraction A assumed as lignans or phenylpropanes, and fraction B and C assumed as triterpenoids showed the PPE inhibitory effects on the PPE and that these compounds in mistletoe may be used for treatment of pathological processes such as age-dependent tissue loss or inflammation.
Key words – Mistletoe, Elastase, PPE, Inhibition

Introduction

The tight regulation of proteolytic activity is important for tissue homeostasis. Its disturbances play important roles in a number of pathological processes. The age-dependent tissue loss is, partially at least, the result of the imbalance between activation and inhibition of proteolytic activity (Khorramizadeh and Tredget, 1999). Among the proteases involved in this age-dependent tissue loss, endopeptidases of elastase type play an important role (Homsy *et al.*, 1989). This class of enzymes comprises serine proteases (Vogelmeier *et al.*, 1991) and matrix metalloproteases (MMPs) such as fibroblastic elastase (Shapiro 1998).

Uncontrolled proteolytic degradation by elastase has been implicated in a number of pathological conditions. Elastase also has been implicated as a destructive protease that impedes wound healing. The presence of elevated levels of elastase in non-healing wounds has been associated with the degradation of important growth factors (Yager *et al.*, 1997) and fibronectin (Grinell and Zhu, 1994) necessary for wound healing. Under normal circumstances, however, the proteolytic activity of HLE is tightly controlled by its natural inhibitors such as α_1-protease inhibitor (α_1-PI) and the secretory leukocyte protease inhibitor (Cregge *et al.*, 1998).

Elastase inhibitors have been reported to demonstrate protective effects in animal models of viral myocarditis (Lee *et al.*, 1998), pulmonary hypertension (Cowan *et al.*, 2000), repetitive cardiac ischemia and infarction (Tiefenbacher *et al.*, 1997) or hyperoxic lung injury (Yamamoto *et al.*, 2000). Several natural and synthetic inhibitors were shown to modulate the activity of these enzymes, comprising both natural serine-protease inhibitors, such as α_1Pi (Travis, 1994) or SLPI and sesquiterpene lactones (Siedle *et al.*, 2002). A number of synthetic inhibitors were described for both of these families of proteases (Gérard *et al.*, 2002a, Robert and Robert, 1992, Gérard *et al.*, 2002b).

European mistletoe (*Viscum album*) and, a subspecies of European mistletoe, Korean mistletoe (*Viscum album* var. *coloratum*) have long been recognized as therapeutic herbs. Mistletoe contains high molecular weight components such as lectins (MW > 60 kDa), viscotoxins (MW = 5 kDa) and polysaccharides, and low molecular weight components such as phenypropanes and lignans (Wagner *et al*, 1986), triterpenoids (Ahn, 1996) and flavonoids (Fukunaga *et al.*, 1989, Kong *et al.*, 1990). Mistletoes are traditionally used as sedative, analgesic and cardiac. The herbs are also used to strengthen tendons and bones, expel pathogens associated with rheumatism and, even more important, anticancer agent

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(Büssing, 2000). Among several components in mistletoe, the anticancer properties are considered to be related to glycoprotein, lectins (Büssing, 2000, Lyu *et al.*, 2001, 2002). Although mistletoes have been used as therapeutic herbs for such various purposes and a number of compounds have been isolated from mistletoe, we have little knowledge about mistletoe as elastase inhibitor. In this study, we screened the inhibition of porcine pancreatic elastase (PPE) by mistletoe fractions and obtained three fractions showing inhibitory effect of PPE.

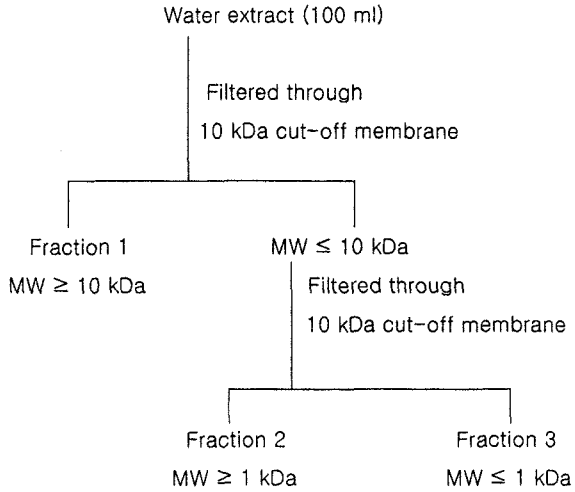
Experimental

Plant material – Korean mistletoe (*Viscum album* L. var. *coloratum*) growing on oak tree was collected in winter in Kangwon province, Korea. The botanical identity was established by Prof. Jong Suk Lee, College of Natural Sciences, Seoul Womens University. Leaves, berries and 1- to 5- year old stems of the plants were sorted and chopped in slices.

Preparation of water extract and fractionation by ultrafiltration – To prepare mistletoe extract, the whole plant slices were crushed with 10 vols of saline between two rollers going in opposite directions in a vegetable juice miller (Angel Life Co., Korea). The mixture was separated by filtration through a cheese cloth, and centrifuged at 12,000 rpm for 30 min. Thereafter, the supernatant was filtered in stages with 60, 20, 7.2 and 0.45 μm pore sizes. The concentration (mg/ml) of water extract of fresh plant was expressed as the amount of the fresh plant to prepare 1 ml of solution.

To fractionate the different molecular weight components of mistletoe by ultrafiltration, 100 ml of water extract was filtered through 10 kDa cut-off membrane with nitrogen gassing, and the filtrate was refiltered through 1 kDa cut-off membrane (Diaflo, Amicon Co., Danvers, MA, USA) using Amicon Standard UF-Cell 8050 (Amicon Co., Danvers, MA, USA). To eliminate the lower molecular weight components of each step, the remainder of filtration was washed with saline by filtering twice and adjusted the volume of each fraction to 100 ml with saline. The volume of filtrate through 1 kDa cut-off membrane was also adjusted to 100 ml with saline (Scheme 1).

Isolation of VCA – The Korean mistletoe lectin (*Viscum album* var. *coloratum* agglutinin, VCA) was purified from the Korean mistletoe by affinity chromatography on asialofetuin-Sepharose 4B as described previously (Lyu *et al.*, 2000). The purity check and the molecular weight were determined by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) as described previously (Lyu *et al.*, 2000). The concentration of total protein was determined by micro BCA assay (Pierce, Rockford, IL). The hamagglutination and sugar specificity of lectin were measured as described previously (Lyu *et al.*, 2000).

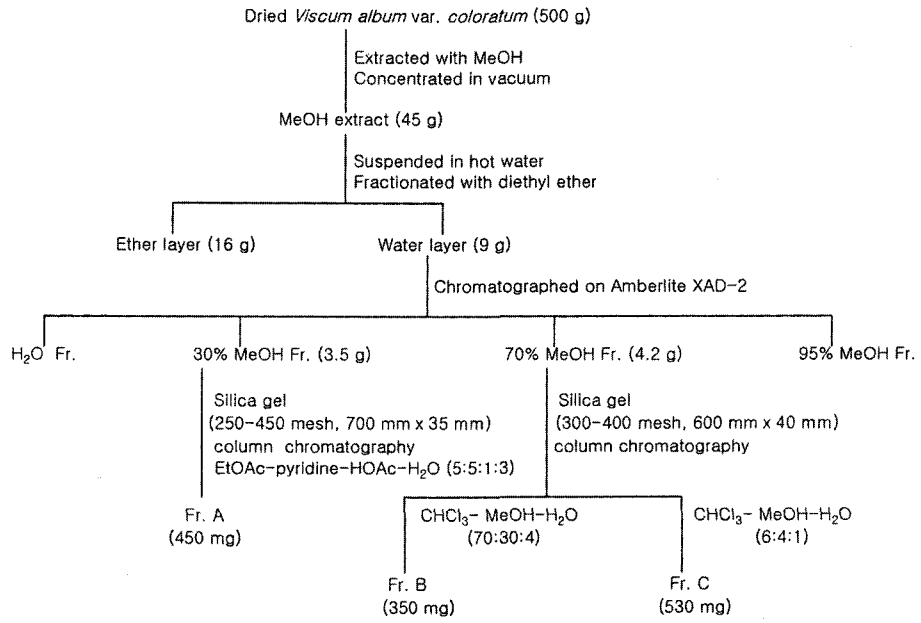


Scheme 1. Fractionation process of water extract of Korean mistletoe. To eliminate the lower molecular weight components of each step, the remainder of filtration was washed with saline by filtering twice and adjusted the volume of each fraction to 100 ml with saline. The volume of filtrate through 1 kDa cut-off membrane was also adjusted to 100 ml with saline.

Preparation of methanol extract and partial fractionation by solvent (Scheme 2) – For the preparation of methanol (MeOH) extract, dried samples were extracted with 4 volumes of hot methanol for 3 h three times at room temperature and the extracts were concentrated to dryness *in vacuo*. The MeOH extract was suspended with two volumes of hot water and fractionated with diethyl ether. The water layer was chromatographed on highly porous polymer (Amberlite XAD-2, Sigma) using H_2O, 30% MeOH, 70% MeOH and 95% MeOH.

Fraction A was obtained from the 30% MeOH fraction on the SiO_2 column chromatography with the solvent system, EtOAc-pyridine-HOAc-H_2O (5:5:1:3, v/v). Fraction A gave a positive reaction by vanillin-phosphate. Two fractions B and C were obtained from the 70% MeH fraction by SiO_2 column chromatography with the solvent system, $CHCl_3$-MeOH-H_2O (70:30:4) for fraction B and $CHCl_3$-MeOH-H_2O (6:4:1) for fracion C. Fr. B gave a positive reaction in Liebermann-Burchard and Molish test.

Enzyme assays – The elastase-catalyzed hydrolysis of substrate was measured in the presence of various concentrations of mistletoe extracts and its fractions. The measurement of enzyme activity was demonstrated by spectrophotometric monitoring of the release of *p*-nitroaniline at 410 nm from the enzymatic hydrolysis of the substrate: *N*-succinyl-



Scheme 2. Fractionation process of MeOH extract of Korean mistletoe.

trialanine-*p*-nitroanilide (*N*-Suc-$(Ala)_3$-*P*NA; Sigma). The mixture of 0.5 ml of the sample and 2 ml of substrate (0.4 mM in Tris buffer, pH 8.0) was preincubated at 25°C for 20 min and 0.5 ml of porcine pancreatic elastase (PPE, 50 mM, pH 8.0) were added and incubated at 25°C for indicated time period. The appearance of the substrate hydrolysis product (*p*-nitroaniline) was measured at 410 nm. The percentage of inhibition of enzyme activity was expressed as {1-(A-B)/C}x100 (A; absorbance at 410 nm in the presence of test sample and enzyme, B; absorbance at 410 nm in the absence of test sample and enzyme, C; absorbance at 410 nm in the absence of test sample and in the presence of enzyme).

Results

Inhibitory activities of PPE by water extract and its fractions by ultrafiltration – To determine whether mistletoe inhibits PPE, we first investigated the activity of PPE inhibition of water extract (WE) and different molecular size fractions of WE. The WE was prepared with fresh plant and fractionated by ultrafiltration with 10 kDa and 1 kDa cut-off membrane. The inhibition of PPE-catalyzed hydrolysis of *N*-succinyltrialanine*p*-nitroanilide (substrate) by WE and each fraction was measured. The solution of enzyme reaction was incubated for 60 min at indicated doses. The WE inhibited significantly the PPE activity and the inhibition increased proportionally to the concentration of WE. IC_{50} value obtained is 76 mg/ml. There is no significant difference of enzyme inhibition by three fractions at lower concentrations (< 50 mg/ml), but treatment of higher concentration (100 mg/ml) shows distinct inhibition pattern. The fraction 1 shows the highest inhibition while fraction 3 shows the lowest inhibition (Fig. 1A).

Next, the inhibition of elastase by WE and each fraction was measured as a function of time. The enzyme inhibition was measured by treating 100 mg/ml of whole extract and fraction for each test at each time point. The degree of PPE inhibition by WE approaches to almost maximum at 60 min. The fraction 1 shows the highest enzyme inhibition among three fractions tested while the fraction 3 shows the lowest inhibition (Fig. 1B).

Considering the fact that the fraction 1 containing mistletoe lectin (VCA) shows the highest enzyme inhibition activity, we expected that the purified VCA may inhibit strongly the PPE activity. Hence, the VCA was purified and the PPE inhibition was investigated. However, the VCA did not inhibit the PPE activity even at high concentration (1 mg/ml) (data are not shown.). These results indicated that the activity of PPE inhibition is to be related on low molecular compounds. Alternatively, we prepared methanol extract to evaluate further the activity of PPE inhibition of low molecular weight components (<1 kDa).

Inhibition of PPE by methanol extract and its fractions – The methanol (MeOH) extract was prepared, suspended with hot water and fractionated with diethyl ether. The solution of enzyme reaction was incubated for 20 min at the indicated doses and the rates of PPE-catalyzed hydrolysis of substrate were measured. Fig. 2 shows the inhibition of PPE by MeOH extract and fractions of water

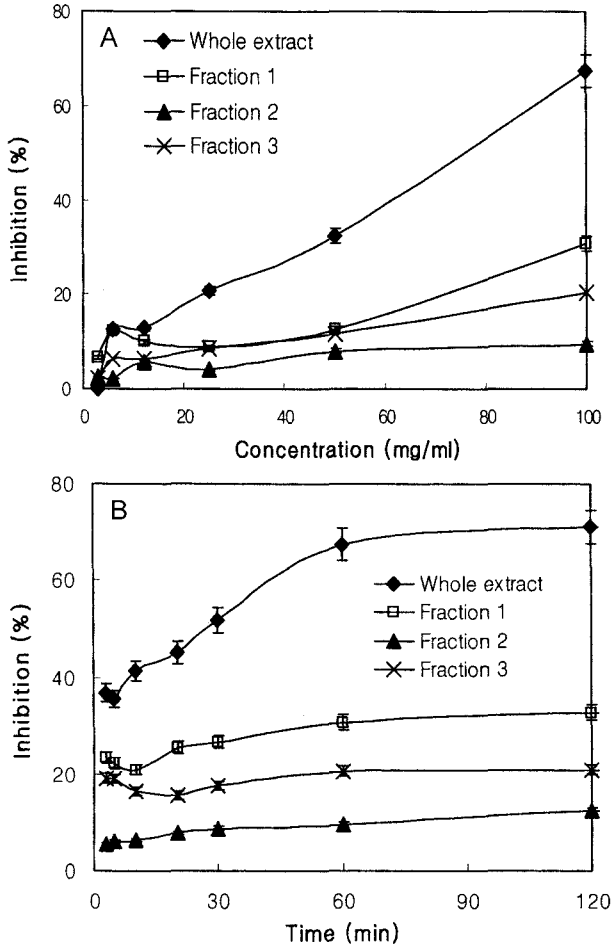


Fig. 1. Dose- (A) and time-dependent (B) inhibition of elastase by whole extract and fractions with different molecules of water extract of Korean mistletoe. Fraction 1; MW°Ð10 kDa, fraction 2; MW = 1~10 kDa, fraction 3; MW°¬1 kDa. The solution of enzyme reaction was incubated for 60 min (A) and the concentration of whole extract and fraction for each test was 100 mg/ml (B). The absorbance at 410 nm was measured.

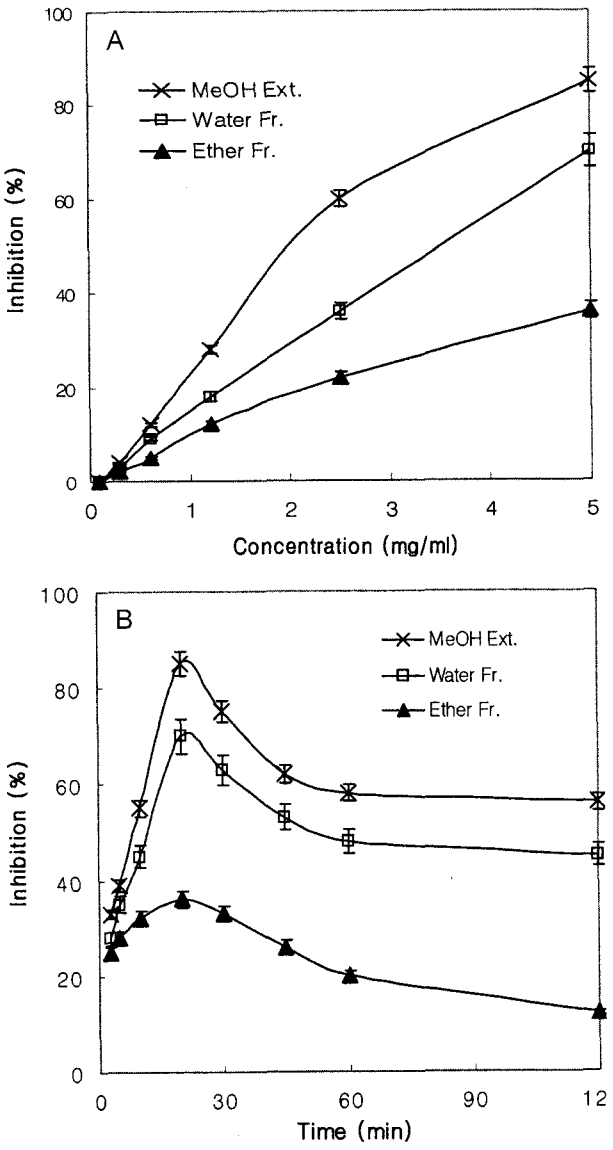


Fig. 2. Dose- (A) and time- (B) dependent inhibition of elastase by extract of MeOH and its fractions of water and diethylether. The solution of enzyme reaction was incubated for 20 min (A) and the concentration of extract for each test was 5 mg/ml. The absorbance at 410 nm was measured.

and diethylether. Each fraction inhibits the PPE activity significantly in dose-dependent manner showing that the inhibition by water extract was higher than that of highly hydrophobic ether fraction (Fig. 2A). And the inhibition by MeOH extract and by water fraction reaches to the maximum at 20 min and afterward decreases abruptly (Fig. 2B).

The water fraction of MeOH extract was further fractionated by column chromatography on Amberlite XAD-2 using H_2O, 30% MeOH, 70% MeOH and 95% MeOH, respectively. The inhibition of PPE activity by each fraction shows dose-dependent manner (Fig. 3A). The fractions eluated with 30% MeOH and 95% MeOH show a moderate degree of PPE inhibition, but the fraction eluated with water shows the lowest inhibition. Importantly, the 70% MeOH eluate inhibits the PPE activity most significantly among fractions tested. On further increasing the concentration of 70% MeOH fraction up to 5 mg/ml, the fraction inhibits the PPE activity completey (98%) showing that IC_{50} value is 0.8 mg/ml (Table 1). The inhibition pattern of each fraction as a function of time is similar to Fig. 2B showing maximum inhibition at 20 min (Fig. 3B). Fraction A further purified from the 30% MeOH fraction showed a positive reaction by vanillin-phosphate. Both fraction B and C further purified from the 70% MeOH fraction showed a positive reaction in Liebermann-Burchard and Molish tests. The PPE inhibition

Table 1. The IC_{50} values and degree of inhibition at 3 mg of each fraction of MeOH extract of mistletoe against porcine pancreatic elastase

Fraction	IC_{50} (mg/ml)	Inhibition (%)
MeOH ext.	1.9	65
H_2O Fr	>20.0	32
30% MeOH Fr.	1.8	57
70% MeOH Fr.	0.8	83
95% MeOH Fr.	3.8	47
Fr. A	1.2	66
Fr. B	0.3	95
Fr. C	0.4	85

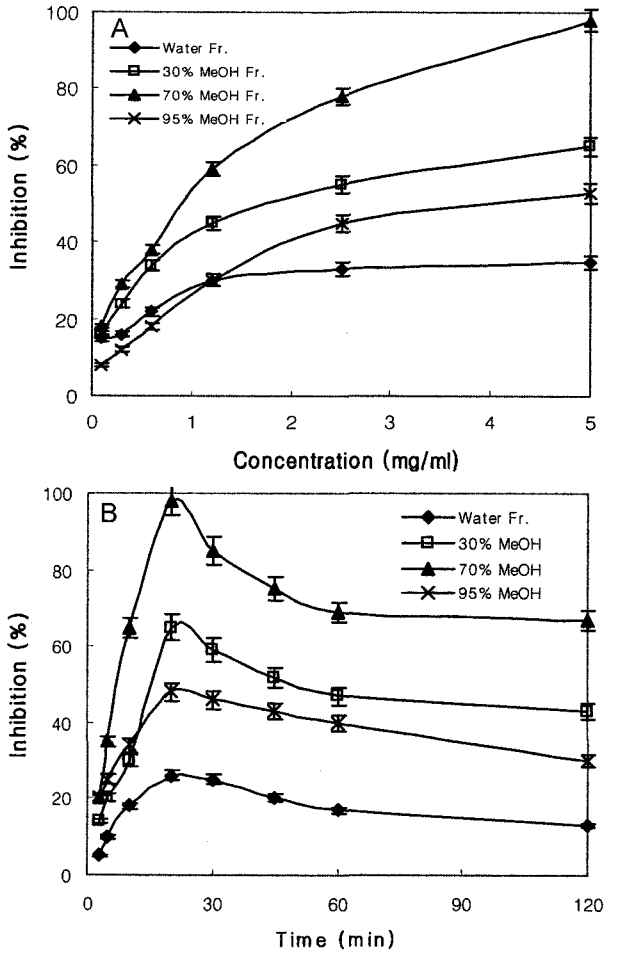


Fig. 3. Dose- (A) and time-dependent (B) inhibition of elastase by fractions eluted with different concentrations of MeOH on Amberlite XAD-2. The solution of enzyme reaction was incubated for 20 min (A) and the concentration of fraction for each test was 5 mg/ml (B). The absorbance at 410 nm was measured.

by three fractions was improved by further purification process. The fraction A at 3 mg/ml inhibits the enzyme activity up to 66% and IC_{50} was 1.2 mg/ml. The fraction B and C at 3 mg/ml inhibits significantly the enzyme activity up to 95%, 85% respectively. The IC_{50} values of fraction B and C were 0.3 mg/ml and 0.4 mg/ml repectively (Fig. 4, Table 1).

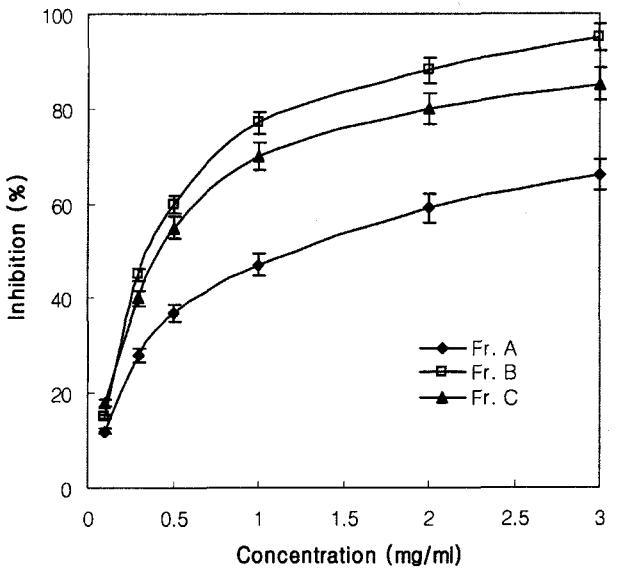


Fig. 4. Dose-dependent inhibition of elastase by fractions purified from 30% MeOH and 70% MeOH fractions on silica gel column chromatography. Fraction A was obtained from the fraction of 30% MeOH on the repeated SiO_2 column chromatography with the solvent system, EtOAc-pyridine-HOAc-H_2O (5:5:1:3, v/v). Fraction B and C was obtained from the fraction of 70% MeOH by repeated SiO_2 column chromatograph with the solvent system, $CHCl_3$-MeOH-H_2O (70:30:4) for fraction B and $CHCl_3$-MeOH-H_2O (6:4:1) for fraction C. The solution of enzyme reaction was incubated for 20 min and the absorbance at 410 nm was measured.

Discussion

Elastase is capable of degrading many structural proteins notably elastin, collagens, fibrinogen and is thought to participate in endothelial cell, vascular and cardiac damage encountered in various pathologies. The serine proteases such as human leukocyte elastase (HLE) and porcine pancreatic elastase (PPE) (Scheele *et al.*, 1981) are classified in the chymotrypsin family, and possibly the most destructive enzymes having the ability to degrade virtually all of the connective components in the body. Human leukocyte elastase (HLE) participates in a number of inflammatory diseases through the destruction of keratin, elastin and different types of collagen. The readily available enzyme, PPE, considered as a good model of HLE is well-described as a serine protease secreted by exocrine pancreas (Scheele *et al.*, 1981). The amino acid sequence homology of PPE and HLE is only 37%, but the backbone architecture of both elastases is conserved and the structures of the active sites near the cleavage site are very similar (Edwards and Bernstein, 1994). The PPE capable of binding mannose

may play a significant role in inducing an inflammatory response to infection (Zhang *et al.*, 2003). The PPE has the ability to hydrolyze elastin, a fibrous and insoluble protein in connective tissue and causes the fatal disease pancreatitis (Rinderknecht, 1993).

Various reactive chemical structures have been considered to interact with the essential serine nucleophile of the enzymic cavity as, for instance, α-fluoroketones (Cregge *et al.*, 1998), halo-enol-lactones (Rai and Katzenellenbogen, 1992), phthalimides (Kerrigan *et al.*, 2000), thiadiazolidinones (Kuang *et al.*, 2000) and coumarinic derivatives (Cabaret *et al.*, 2001, Pochet *et al.*, 2000). The elastase has been reported to be inhibited by a naturally occurring glycoprotein, serumalpha-1-antitrypsin (AAT) (Wiedow *et al.*, 1992). In the present study, the inhibitory effect of porcine pancreatic elastase (PPE) by mistletoe extract and its fractions was screened.

Taking into account the fact that mistletoe contains lectins (MW > 60 kDa), we have considered whether inhibition of the PPE by mistletoe extract might be feasible. Hence, we first investigated the activity of PPE inhibition of water extract (WE) and different molecular weight fractions of WE. The WE inhibited significantly the PPE activity (IC_{50} = 76 mg/ml) and the fraction of high molecular weight components showed the highest inhibition. The whole extract showed much higher inhibition than each fraction, assuming that the components in the whole extract of mistletoe exert synergistic activity of inhibition of PPE. We expected that the purified lectin may inhibit strongly the PPE activity because of the highest enzyme inhibition activity of fraction containing mistletoe lectin. Hence, the VCA was purified and the PPE inhibition was investigated. However, the VCA did not inhibit the PPE activity even at high concentration (1 mg/ml). It is assumed from the results that the PPE inhibition by fraction 1 is probably due to some other components such as polysaccharide.

Alternatively, we prepared methanol extract to evaluate further the PPE inhibition by low molecular weight components (<1 kDa). Although the concentration of the sample was not able to compare directly, the inhibition of the PPE activity by MeOH extract was much higher than the inhibition by fraction 3 extracted with water. This is probably due to the process of more efficient extraction of low molecular weight components with MeOH. The inhibition by water fraction was higher than that of highly hydrophobic ether fraction.

The fractions of 70% MeOH fractionated on Amberlite XAD-2 inhibited the PPE activity most significantly among fractions tested. On further increasing the concentration up to 5 mg/ml, the fraction inhibited the PPE activity completey (98%) showing that IC_{50} value was 0.8 mg/ml (Table 1). The PPE inhibition by fraction A, B and C was improved by further purification process. The fraction A, B and C at 3 mg/ml inhibited significantly the enzyme activity up to 66%, 95% and 85% respectively. Fraction A further purified from the 30% MeOH fraction showed a positive reaction by vanillin-phosphate, which indicated it may contain lignans or phenylpropanes. Both fraction B and C further purified from the 70% MeOH fraction showed a positive reaction in Liebermann-Burchard and Molish tests, which indicated may contain triterpenoid saponin. It has been reported that phenypropanes and lignans such as syringin (Wagner *et al*, 1986), triterpenoids such as oleanolic acid and β-amyirin (Ahn, 1995) and flavonoids such as viscumneoside and viscoside (Fukunaga *et al.*, 1989, Kong *et al.*, 1990) were isolated from mistletoe. The PPE inhibition by three fractions was improved by further purification process. The fraction A, B and C at 3 mg/ml inhibits significantly the PPE activity up to 66%, 95% and 85% respectively.

In conclusion, these results suggest that Korean mistletoe can be used for treatment of pathological processes such as age-dependent tissue loss or inflammation. To confirm the active compounds, further identification process would be necessary.

Acknowledgements

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