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大阪府立大学博士（応用生命科学）学位論文

Enzymatic synthesis of water-soluble ferulic acid derivatives and their improving effects for Alzheimer's disease

水溶性フェルラ酸誘導体の酵素合成および アルツハイマー病改善効果に関する研究

菊川　昌希

２０１７年

Contents

Abbreviations

Aβ, amyloid-β; AD, Alzheimer's disease; BSA, bovine serum albumin; CA, caffeic acid; CMC, carboxymethylcellulose; CNS, central nervous system; DG, diglycerol; DIV, days *in vitro*; dmCA, 3,4-dimethoxycinnamic acid; DMEM, Dulbecco's modified Eagle's medium; FA, ferulic acid; FA-DG1, 1-feruloyl diglycerol; FA-G1, 1-feruloyl glycerol; FAE, ferulic acid esterase; FAE-PL, An *Aspergillus niger* FAE purified from Pectinase PL"AMANO"; FBS, fetal bovine serum; IκB-α, inhibitor of NF-κB-α; iNOS, inducible NO synthase; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NF-κB, nuclear factor (NF)-κB; NO, nitric oxide; PBS, phosphate buffered saline. pCA, *p*-coumaric acid; SA, sinapic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SE, standard error; TPI, triose-phosphate isomerase.

Introduction

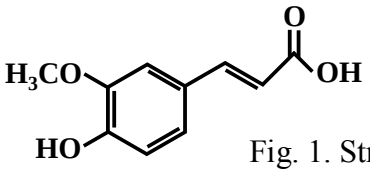

Fig. 1. Structure of FA

Location of ferulic acid (FA)

Ferulic acid (FA) (4-hydroxy-3-methoxycinnamic acid; Fig. 1) is a polyphenol located in the cell walls of a wide variety of plants, such as artichokes, eggplants, wheat bran, and maize bran (Smith and Hartley 1983; Saulnier et al. 1995). FA is esterified to the C-5 of α-L-arabinofuranosyl side chains of arabinoxylans in monocotyledons such as maize bran (Saulnier et al. 1995) and wheat bran (Smith and Hartley 1983). In pectins of dicotyledons such as sugar beet (Rombouts and Thibault 1986; Ralet et al. 1994; Colquhoun et al. 1994) and spinach (Fry 1982; Ishii and Tobita 1993), FA mainly attaches to the C-2 of α-1,5-linked arabinofuranose residues or the C-6 of β-1,4-linked galactopyranose residues. FA is also linked to the C-4 position of α-D-xylopyranosyl residues in xyloglucans (Ishii et al. 1990). The amounts of FA and *p*-coumaric acid (pCA) in various plants are summarized in Table 1. Some FAs occur in the form of dehydrodimers which function to cross-link and strengthen the cell walls, and decrease their digestibility by microorganisms (Eraso and Hartley 1990; Saulnier and Thibault 1999).

Table 1. The amounts of FA and pCA in various plants (Tsuchiyama 2007).

Class	Source	Hydroxy-cinnamic acid	Amount (%) in dry weight	Reference
Monocoty-ledoneae	Wheat bran	FA	0.66	Smith et al. 1983
		pCA	0.014	Lequart et al. 1999
	Wheat straw	FA	0.28	Lequart et al. 1999
		pCA	0.36	Lequart et al. 1999
	Rice end sperm	FA	0.9	Shibuya 1984
	Maize bran	FA	3.1	Saulnier et al. 1995
	Barley grain	FA	0.14	Nordkvist et al. 1984
Dicoty-ledoneae	Sugar beet pulp	FA	0.8	Micard et al. 1994
	Chinese water chestnut	FA	0.7	Parr et al. 1996

Ferulic acid esterase (FAE)

Some microorganisms, to defeat this defensive strategy in plants, secrete FAEs (EC 3.1.1.73) as well as cellulases, pectinases, and hemicellulases. FAEs are enzymes that catalyze the hydrolysis of ester bonds between FAs and sugars. FAE was first isolated by Faulds and Williamson (1991). Since then, many microbial FAEs have been isolated and classified based on their catalytic properties (Crepin et al. 2004).

Antioxidant action of FA

FA is a cinnamic acid derivative with antioxidant properties (Castelluccio et al. 1995; Kikuzaki et al. 2002) and protects molecules that are essential to life (*i.e.*, lipid, protein, and DNA) from oxidative stress (Rice-Evans et al. 1996). Its antioxidant action involves the up-regulation of heme-oxygenase-1, heat shock protein 70, and signal transduction kinase 1/2, and protects cells from stress (Barone et al. 2009). Antioxidants such as FA are commonly used to inhibit the oxidation of lipids in food, cosmetics, and medicine.

Anti-inflammatory effect of FA

FA has anti-inflammatory effects and can reduce the overexpression of prostaglandin E2, tumor necrosis factor-α (TNF-α), and inducible NO synthase (iNOS) induced by lipopolysaccharide (LPS) in cells (Ou et al. 2003; Tetsuka et al. 1996). Administration of FA for one month counteracted the overexpression of endothelial NO synthase induced by Aβ in mouse hippocampal astrocytes (Cho et al. 2005). Moreover, FA was shown to prevent iNOS induction in cerebral ischemia model rats (Koh 2012).

UV-absorbing action of FA

FA is a strong UV absorber and can reduce UV-induced skin damage. FA is efficient at absorbing the sunburn-inducing UVB (290–320 nm) and UVA (320–400 nm) radiation. Indeed, FA has been proved to be an effective protectant from UVB-induced skin erythema in healthy subjects (Saija et al. 2000). Importantly, FA prepared at an acidic or neutral pH is readily absorbed by the skin.

Clinical and statistical data of Alzheimer's disease (AD)

AD is a neurodegenerative disorder that mostly affects the elderly. Characteristics of AD include memory deficits and delusions. AD is a significant problem for the super-aged population in Japan, and many studies have aimed to produce drugs to treat AD. Figure 2 illustrates the increase in the number of dementia patients over time. In Japan, as of 2010, approximately 2 million people were considered senile, with some researchers estimating that 10% of people 65 years of age or older (about 2.4 millions) having dementia. Furthermore, the number of patients with dementia is expected to increase to 3.2 million by 2020, with the increasing number of people aged 65 years or above (Ministry of Health, Labour and Welfare in Japan).

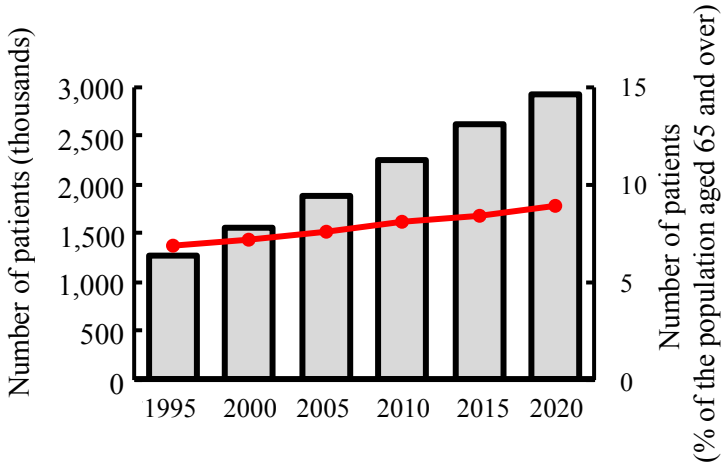

Fig. 2. Number of dementia patients

(Ministry of Health, Labour and Welfare in Japan 2010)

Treatment of AD in Japan

Four drugs have been approved in Japan for the treatment of AD (Table 2). Donepezil (Aricept) is used through it before, and binds and reversibly inactivates cholinesterases, thus inhibiting acetylcholine hydrolysis. This results in an increased acetylcholine concentration at cholinergic synapses. The three other drugs were given approval in 2011, and this suggests that the Japanese people suffering from AD are receiving increased attention.

Table 2. Licensed palliative treatments for AD in Japan.

General name	Action mechanisms	Year of sale (Japan)
Donepezil[*1] (Aricept)	Acetylcholinesterase inhibitor	1999
Galantamine[*2]	Acetylcholinesterase inhibitor Allosteric modulator of nicotinic receptors	2011
Memantine[*3]	Inhibitor of NMDA receptors	2011
Rivastigmine[*4]	Acetylcholinesterase inhibitor Butylcholinesterase inhibitor	2011

[*1] Eisai and Pfizer, [*2]Janssen Pharmaceutical K.K., [*2] Takeda Pharmaceutical Co., Ltd, [*3] Daiichi Sankyo Healthcare Co., Ltd., [*4] Novartis Pharmaceuticals, [*4] Ono Pharmaceutical Co., Ltd.

Pathology of AD

AD was first described in 1906 at a conference in Tubingen, Germany by Alois Alzheimer (1906) as a loss of memory and cognitive dysfunction. The clinicopathological features of AD are not completely understood. However, there are three possible mechanisms: deposition of senile plaque, deposition of tau proteins in the cytoplasm of neurons, and neuronal degeneration due to both of these components (Masliah et al. 1993). Senile plaques are extracellular plaques in the brain consisting mainly of amyloid-β (Aβ), a peptide thought to be a leading cause of neurotoxicity (Masters et al. 1985). Overproduction and aberrant self-assembly of Aβ into fibrillar aggregates constitute the first steps of the so-called amyloid cascade hypothesis, which is thought to trigger AD (Hardy and Higgins 1992). In fact, Aβ aggregation is known to cause neuronal cell death. Although it is not clear exactly how Aβ causes neuronal loss and tangle formation, the peptide disrupts calcium homeostasis and increases intraneuronal calcium concentrations (Estus et al. 1997; Loo et al. 1993). Inhibition of the aggregation of Aβ and destabilization of aggregated Aβ in the central nervous system (CNS) would be an attractive therapeutic target for treatment of AD (Antonella et al. 2015).

Other hypotheses for AD include the cholinergic hypothesis, Aβ oligomer hypothesis, tau protein, and oxidative stress, among others (Terry and Buccafusco 2003; Walsh et al. 2002). The cholinergic hypothesis is the first and most widely studied approach describing AD pathophysiology and suggests that dysfunction of acetylcholine-containing neurons in the brain contributes substantially to the cognitive decline observed in AD. The cholinergic hypothesis is also the basis of most drug development approaches (acetylcholinesterase inhibitors, allosteric cholinergic receptor potentiators, *N*-methyl-D-aspartate (NMDA) receptor blockers) (Doggrell and Evans 2003).

Astrocytes and AD

Astrocytes are the most abundant glial cells in the brain. They play important roles in neuronal support and maintenance, such as providing structural support, delivery of nutrients, maintaining the ionic environment inside the central CNS, and preserving the blood-brain barrier (Sofroniew and Vinters 2010). In addition, astrocytes play a crucial role in inflammatory and degenerative process in the CNS, conditions accompanied by a surge in the expression of inducible nitric oxide synthase (iNOS), the enzyme that synthesizes the inflammation mediator nitric oxide (NO) (Heneka et al. 2010; Hamby and Sofroniew 2010; Moncada and Bolaños 2006).

FA and AD

FA has been shown to have beneficial effects in AD (Barone et al. 2009). FA inhibits Aβ aggregation, destabilizes preformed Aβ fibrils *in vitro* (Ono et al. 2005) and FA ethyl ester (EFA) protects cultured neuronal cells against Aβ-induced cytotoxicity (Sultana et al. 2005). Moreover, long term administration of FA protects mice against Aβ (1–42) peptide-induced learning and memory deficit by suppressing the activation of astrocytes and microglia (Yan et al. 2001; Kim et al. 2004; Cho et al. 2005). A study by Yan et al. (2013) used a transgenic mouse model of AD which overexpressed a mutant form of amyloid precursor protein (APP) and a deletion mutant of presenilin-1 through removal of exon 9. Their results suggested that administration of FA for six months significantly reduces cortical levels of Aβ and interleukin-1β (IL-1β). Therefore, FA is expected to be a key molecule for the development of therapeutics for AD.

Synthesis of FA derivatives using enzymes

A problem is that FA is insoluble in water and oil, which limits its applications. Esterification is one way of modifying the physical properties of FA. Many feruloylated compounds are synthesized by lipases or FAEs to increase their hydrophobicity (Vafiadi et al. 2008; Thörn et al. 2011; Sun et al. 2007b, 2009). One such study by Antonopoulou et al. (2017) optimized synthesis of prenyl ferulate using an FAE from *Myceliophthora thermophile*, however, very little work has been done to synthesize feruloylated compounds with high solubility in water. The water solubility of FA can be increased by linking it to hydrophilic compounds such as glycerol and sugars. Kelle et al. (2016) synthesized feruloyl-saccharide esters using an FAE from *Pleurotus sapidus*. Esterases such as lipases can catalyze esterifications (reverse reactions) and transesterifications in non- or low-aqueous solvents. However, esterification reactions are more practical for industrial applications than transesterification reactions, because FA can be directly used as the donor in the reactions. Transesterification reactions are expensive and complicated because of an additional required step of preparing FA esters, such as EFA, to act as substrates.

We previously found that an FAE from *A niger* efficiently synthesized hydroxyl cinnamic acid glycerol esters including 1-feruloyl glycerol (FA-G1), 1-sinapoyl glycerol, and 1-*p*-coumaroyl glycerol through estesterification reaction for the first time (Tsuchimaya et al. 2006, 2007). Since then, some researches of FA-Gs were reported (Sun et al. 2007a, Compton et al. 2012). However, the water solubility of FA-G1 at 20 °C (1.20 mg/ml) was only slightly higher than that of FA (0.69 mg/ml).

Synthesis of derivatives for the purpose of water-soluble improvement

Some compounds regardless of FA were reported about enzymatic synthesis for the purpose of water solubility. For example, glucosyl rutin is more soluble 3,000 times than free form rutin (Suzuki and Suzuki, 1991). Neohesperidin monoglucoside is more soluble 1,500 times, and reduce bitter taste less than 10 times than free neohesperidin (Kometani et al. 1996). Esterification is also possible that not only is souble but also has other functions.

Aim of this study

As mentioned above, FA has multifunctions and can be applied in several industries such as foods, cosmetics, and medicines. However, the solubility in both water and oil is so low that the industrial applications have difficulties. The first aim of this study is to develop FA derivatives with high water solubility to overcome this limitation. I also evaluate the effects of the FA derivatives developed in improving AD. Because the elderly population is increasing worldwide, AD is quickly becoming a major universal healthcare problem (Ferri et al. 2005). Although FA has been shown to have beneficial effects in AD, it is insoluble and it is difficult for elderly patients who have a decreased ability to swallow to increase their FA intake. It would be easier to take FA by increasing the water solubility.

In chapter 1, I describe the enzymatic synthesis of the diglycerol (DG) esters of FA, one of which has a water solubility of at least two orders of magnitude higher than the water solubility of FA and FA-G1. I also describe the optimal conditions for esterification, the structures of the esters, and their seveal characteristics such as the ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. Moreover, I describe the biological activity of water-soluble FA derivatives against AD. In chapter 2, I examine whether FA and its derivatives inhibite NO production in primary astrocytes stimulated with LPS. In chapter 3, I examine the effect of these compounds on Aβ aggregation, Aβ-induced neurotoxicity in cortical neurons *in vitro*, and Aβ-induced learning and memory deficits i*n vivo*.

Chapter 1. Synthesis of water-soluble feruloyl diglycerols by esterification of an *Aspergillus niger* feruloyl esterase.

Summary

As noted above, one problem with FA is its insolubility in both water and oil, limiting its application. Here I describe the enzymatic synthesis of DG esters of FA with the goal of increasing its water solubility. I synthesized water-soluble FA derivatives by esterification of FA with DG using feruloyl esterase purified from a commercial enzyme preparation produced by *A niger*. The major reaction product, FA-DG1, is a sticky liquid whose water solubility (>980 mg/ml) is dramatically higher than that of FA (0.69 mg/ml). The structure of FA-DG1 was determined to be γ-feruloyl-α,α'-DG by spectroscopic methods. Under suitable conditions, 95% of FA converted into feruloyl DGs (FA-DG1, 2, and 3). I also developed a batch method which resulted in synthesis of 729 mg of feruloyl DGs and 168 mg of diferuloyl DGs from 600 mg of FA and 1 g of DG (corresponding to conversion of 69% of the FA to feruloyl DGs and 21% of the FA to diferuloyl DGs). As an anti-oxidant, feruloyl DGs were essentially equal to FA and butyl hydroxytoluene in scavenging 1,1-diphenyl-2-picrylhydrazyl radicals. In contrast, the scavenging abilities of diferuloyl DGs were twice those of feruloyl DGs.

Materials and Methods

Chemicals and reagents

FA and EFA were kindly donated by Tsuno Food Industrial Co., Ltd. (Wakayama, Japan). DG (Diglycerin S) was kindly gifted by Sakamoto Yakuhin Kogyo Co., Ltd. (Osaka, Japan). Diglycerin S mainly contains DG isomers of α,α′-DG (71%), α,β′-DG (24%), and β,β′-DG (1%). Pectinase PL "AMANO" was from Amano Enzyme Inc. (Nagoya, Japan). Sephadex LH-20 was from GE Healthcare UK Ltd. (Buckinghamshire, UK). Mightysil RP-18 GP 250-4.6 (5 μm) was from Kanto Chemical Co., Inc. (Tokyo,

Japan). Cosmosil C18-AR-2 (20×250 mm) and DPPH were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise stated and were of certified reagent grade.

Enzyme assay

An *A.niger* FAE, termed FAE-PL, was purified from Pectinase PL "AMANO" as described previously (Tsuchiyama et al. 2006). Hydrolytic activity of FAE was assayed by measuring the release of FA in a reaction mixture containing 200 μl of 0.04% EFA in 100 mM acetate buffer (pH 5.0) and 5 μl enzyme solution at 37°C for 5 min. After inactivation of the enzyme by boiling for 5 min, the FA released was quantified by high-performance liquid chromatography (HPLC). One unit was defined as the amount of enzyme that releases 1 μmol of FA in 1 min.

HPLC conditions

Reaction products were quantified by HPLC using Mightysil RP-18 GP 250-4.6. Elution was carried out with a binary gradient of solvent A (0.1% acetic acid) and solvent B (methanol containing 0.1% acetic acid) at 0.7 ml/min and 40°C. The elution sequence consisted of 40% B for 10 min, then a linear gradient from 40% B to 70% B over 15 min, followed by 100% B for 10 min. The effluent was monitored by measurement of absorbance at 320 nm.

Isolation of feruloyl DGs synthesized from FA and DG using FAE-PL

The reaction mixture (50 ml) contained 42 ml of DG, 2.5 ml of 20% FA in dimethylsulfoxide (DMSO), 2.5 ml of 1 M acetate buffer (pH 4.0), and 3 ml of FAE-PL (10 U). The mixture was incubated at 50°C for 12 h followed by boiling for 10 min to inactivate the enzyme. The mixture was added to an equal volume of water and centrifuged at 15,000 rpm for 10 min to remove the precipitates. The supernatant was loaded onto a Sephadex LH-20 column (4×50 cm) equilibrated with water. The esterification products were eluted with water at a flow rate of 5 ml/min. Ten-milliliter fractions were collected and the products in the fractions were analyzed by HPLC. The fractions containing esterification products were pooled and concentrated to 5 ml under reduced pressure. One milliliter of the concentrate was injected into a reversed-phase

preparative column of Cosmosil C18-AR-2 equilibrated with 35% methanol containing 0.1% acetic acid and chromatographed on a recycling preparative HPLC system (Shimadzu LC-6 AD; Shimadzu Corp., Kyoto, Japan). Elution was performed with the same solvent at a flow rate of 5 ml/min. The effluent was monitored by measurement of absorbance at 320 nm.

Structure elucidation of esterification products

^{1}H and ^{13}C NMR spectra were recorded on a JNM-AL 400 spectrometer (JEOL, Tokyo, Japan). Chemical shifts were referenced to the solvent peaks (DMSO, δ_H 2.49 and δ_C 39.5; methanol, δ_H 3.30 and δ_C 49.0) as an internal standard. Electrospray ionization mass spectrometry (ESI–MS) experiments were achieved on a NanoFrontier mass spectrometer (Hitachi High-Technologies, Tokyo, Japan) using semi-micro ESI ion source.

Effect of reaction conditions for esterification of FAE-PL

The standard reaction mixture contained 100 mg of FA powder, 1 g (0.8 ml) of DG, 0.1 ml of 1 M phosphate buffer (pH 6.0) corresponding to 10% (vol/vol) phosphate buffer, and 10 to 50 μl of FAE-PL (2 U). All the components excluding enzyme were mixed and heated in microwave oven to dissolve FA. After cooling the mixtures to 50°C, the enzyme was added to them followed by incubation at 50°C at the times shown. Aliquots (50 μl) were taken at intervals, boiled for 10 min to inactivate the enzyme, and diluted 1,000 times with water followed by quantification of FA and the reaction products with HPLC. In order to optimize reaction conditions for esterification of FAE-PL, several factors in the above standard condition were varied. In examining the effect of pH on esterification, acetate buffer (pH 4 and 5) and phosphate buffer (pH 6, 7, and 8) were used. The conversion rate of FA was calculated based on the molar concentration by the following formula: conversion rate of FA (%) = $(FA_{before} - FA_{after}) / FA_{before} \times 100$, where FA_{before} and FA_{after} are FA concentration (mM) before and after the enzyme reaction in the mixture, respectively.

Enzymatic ester synthesis by a fed-batch method

FA was esterified in a 30-h reaction at 50°C under reduced pressure (to remove water) as follows: a mixture containing 1 g of DG, 56 μl of 2.5 M phosphate buffer (pH 7.0), and FAE-PL (12 U) was evaporated in a 50-ml evaporator flask, incubated for 9 h with addition of 40 mg solid FA at the beginning of each hour, incubated for 4 h to catalyze esterification reaction efficiently, incubated for 4 h with addition of 40 mg solid FA at the beginning of each hour, incubated for 13 h to complete the esterification, and boiled for 10 min to inactivate the enzyme.

Isolation of diferuloyl DGs synthesized by a fed-batch method

The reaction mixture obtained from a fed-batch method was mixed with 200 ml of water and centrifuged at 15,000 rpm for 10 min. The supernatant was loaded onto a Sephadex LH-20 column (4×50 cm) equilibrated with water. Stepwise elution was carried out using water (1,000 ml), 10% ethanol (500 ml), 30% ethanol (500 ml), and 100% ethanol (500 ml). Ten-milliliter fractions were collected and the products in the fractions were analyzed by HPLC. Diferuloyl DGs-containing fractions were pooled and concentrated under reduced pressure. The concentrate was injected into a reversed-phase preparative column of Cosmosil C18-AR-2 equilibrated with 40% methanol containing 0.1% acetic acid. Diferuloyl DGs were eluted with the same solvent at a flow rate of 5 ml/min. The effluent was monitored by measurement of absorbance at 320 nm.

Ability to scavenge DPPH radicals

The reaction mixture consisting of 100 μl of 250 μM DPPH in methanol and 100 μl of sample (0 to 200 μM) in methanol was incubated at room temperature for 20 min. Activity was expressed as the decrease in absorbance of the reaction mixture at 490 nm.

Results

Synthesis and isolation of feruloyl DGs

Direct esterification reaction was performed using FA and DG with FAE-PL at pH 4.0 and 50°C for 12 h. A major esterification product (FA-DG1) was detected by HPLC together with a small amount of two other products (FA-DG2 and FA-DG3) as shown in Fig. 1-1a. The feruloyl DG products were comprised of 82% FA-DG1, 11% FA-DG2, and 7% FA-DG3. Although the chemical equilibrium of hydrolases favors the hydrolysis rather than the synthesis of esters, approximately 70% of the initial FA was converted to feruloyl DGs even in the presence of 11% water. A high esterification yield seems to be obtained because high concentration of DG (84 %), which is an acceptor of FA, is present in the reaction mixture. We previously obtained an 80% yield in the synthesis of FA-Gs through a direct esterification reaction by PAE-PL in a 10% aqueous solution (Tsuchiyama et al. 2006). To determine their structures, the three esterification products were isolated from the above reaction mixture.

After inactivation of the enzyme, the esters were purified using two column chromatographies. On the first column, a Sephadex LH-20 column, the three esters (FA-DG1, 2, and 3) eluted together at around 600 ml with water. Hardly any residual FA eluted from the column with water. DG did not adsorb to the column. The second chromatography was performed using a preparative Cosmosil C18-AR-2 reversed phase column on a recycling HPLC system, which was used to separate FA-DG1, 2, and 3. Under reduced pressure at room temperature, the purified esters dried down to sticky liquids. Like glycerol, they were miscible in all proportions with water. The water solubility of FA-DG1 was more than 980 mg/ml at 20°C which is dramatically higher than the solubilities of FA (0.69 mg/ml) and FA-G1 (1.20 mg/ml) at 20°C (Tsuchiyama et al. 2006).

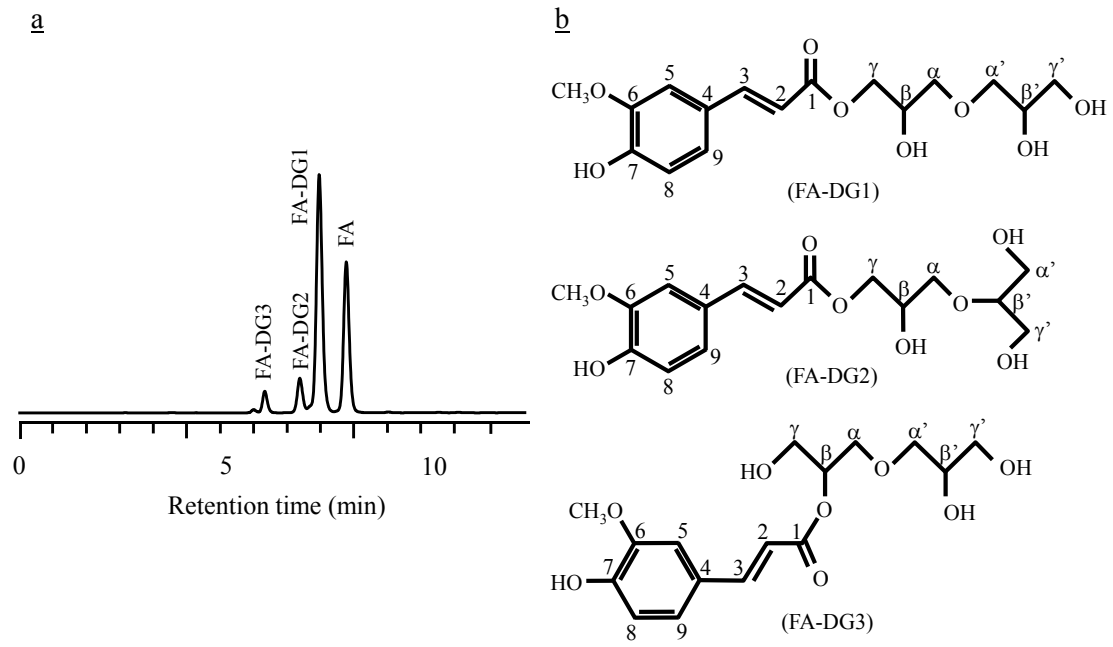

Fig. 1-1. HPLC chromatogram of compounds synthesized by treating FA and DG with FAE-PL (a) and structures of FA-DG1, FA-DG2, and FA-DG3 (b).

The reaction products were analyzed by HPLC using a Mightysil RP-18 reversed-phase column.

Structural determination of FA-DG1, 2, and 3

The structures of the isolated products were examined by spectroscopic methods. ESI-MS of FA-DG1, 2, and 3 detected protonated molecules $[M+H]^+$ at *m/z* 343, indicating that their molecular masses are 342 and they are conjugated products of DG with FA. The ^1H, ^{13}C NMR, ^1H-^1H COSY, and HMQC spectra of FA-DG1 confirmed the presence of FA and α,α′-DG units (Table 1-1). The HMBC correlation of γ-methylene protons (δ_H 4.07–4.18 ppm) to carbonyl carbon at C-1 (δ_C 169.0 ppm) revealed that γ-hydroxy group of α,α′-DG was feruloylated (Fig. 1-1b). Thus, the structure of FA-DG1 was determined to be γ-feruloyl-α,α′-DG. Similarly, the structures of FA-DG2 and FA-DG3 were identified as γ-feruloyl-α,β′-DG and β-feruloyl-α,α′-DG (Fig. 1-1b), respectively. Spectral data used for the structural determination of FA-DG2 and FA-DG3 are presented in Tables 1-2 and 1-3, respectively.

Table 1-1. Spectral data for FA-DG1.

Position	δ_C	δ_H (integral, mult, *J* Hz)	HMBC (H to C)
α	73.6	3.38-3.51 (2H, m)	β, γ, α’
β	69.7	3.92 (1H, m)	α, γ
γ	66.6	4.07-4.18 (2H, m)	α, β, 1
α’	73.9	3.38-3.51 (2H, m)	α, γ’
β’	72.2	3.68 (1H, m)	α’, γ’
γ’	64.2	3.38-3.51 (2H, m)	α’, β’
1	169	-	
2	115.1	6.29 (1H, d, 16.1)	1, 4
3	147.1	7.55 (1H, d, 16.1)	1, 2, 4, 5, 9
4	127.7	-	
5	111.7	7.10 (1H, d, 1.7)	3, 6, 7, 9
6	149.4	-	
7	150.6	-	
8	116.5	6.71 (1H, d, 8.3)	4, 6, 7
9	124.1	6.98 (1H, dd, 8.3, 1.7)	3, 5, 7, 8
OMe	56.4	3.80 (3H, s)	6

Table 1-2. Spectral data for FA-DG2.

Position	δ_C	δ_H (integral, mult, *J* Hz)	HMBC (H to C)
α	72.5	3.47-3.65 (2H, m)	β, γ, α’
β	70.1	3.93 (1H, m)	α, γ
γ	66.6	4.05-4.20 (2H, m)	α, β, 1
α’	62.6	3.47-3.65 (2H, m)	β’, γ’
β’	83.4	3.35 (1H, m)	α, α’, γ’
γ’	62.6	3.47-3.65 (2H, m)	α’, β’
1	169.1	-	
2	115.1	6.29 (1H, d, 16.1)	1, 4
3	147.1	7.55 (1H, d, 16.1)	1, 2, 4, 5, 9
4	127.7	-	
5	111.7	7.10 (1H, d, 2.0)	3, 6, 7, 9
6	149.3	-	
7	150.7	-	
8	116.5	6.71 (1H, d, 8.3)	4, 6, 7
9	124.1	6.98 (1H, dd, 8.3, 2.0)	3, 5, 7, 8
OMe	56.4	3.80 (3H, s)	6

Table 1-3. Spectral data for FA-DG3.

Position	δ_C	δ_H (integral, mult, *J* Hz)	HMBC (H to C)
α	69.6	3.50-3.62 (2H, m)	β, γ, α’
β	73.2	4.96 (1H, m)	α, γ, 1
γ	60.0	3.50-3.62 (2H, m)	β
α’	72.8	3.26-3.35 (1H, m)	α, β’, γ’
		3.38-3.45 (1H, m)	
β’	70.5	3.50-3.62 (1H, m)	α’
γ’	63.0	3.26-3.35 (2H, m)	β’, γ’
1	166.3	-	
2	114.6	6.46 (1H, d, 15.6)	1, 4
3	145.0	7.54 (1H, d, 15.6)	1, 2, 4, 5, 9
4	125.5	-	
5	111.1	7.31 (1H, d, 2.0)	3, 6, 7, 9
6	147.9	-	
7	149.4	-	
8	115.5	6.78 (1H, d, 8.4)	4, 6, 7
9	123.1	7.10 (1H, dd, 8.4, 2.0)	3, 5, 6
OMe	55.7	3.80 (3H, s)	6

Effects of substrate concentration, pH, temperature, and water concentration on feruloyl DGs synthesis

Starting with 1 g DG, the yield of feruloyl DGs increased as the concentration of FA increased from 11 to 142 mg/ml (Table 1-4). However, at a concentration of 142 mg/ml, the conversion rate of FA decreased, probably because some of the FA precipitated. Therefore, I determined the optimal FA concentration for 1 g of DG to be 100 mg/ml. FAE-PL is stable from pH 4.0 to 9.5, and, in the presence of 0.02% FA, has its highest esterification activity at pH 4.0 (Tsuchiyama et al. 2006). In the present study, the conversion rate of FA (initial concentration 10%) varied little over the pH range 4-7, but was slightly decreased at pH 8 (Fig. 1-2). FAE-PL had the highest esterification activity at 50°C to 60°C and no activity at 70°C (Fig. 1-3a). After incubation of the enzyme in the presence of 90% DG at 50°C for 4 days, more than 80% of the initial activity remained (Fig. 1-3b). Feruloyl DG synthesis decreased with increasing concentration of water (Fig. 1-4). Taking into account all the above results, I determined the suitable conditions for esterification of FA with DG to be as follows: 100 mg of FA in the presence of 1 g (0.8 ml) of DG and 0.1 ml of 1 M phosphate buffer (pH 6.0) at 50°C. When the reaction was conducted for 24 h with the optimized reaction mixture under atmospheric pressure, the conversion rate of FA at 24 h was 70%. However, when the reaction was conducted at reduced pressure in an evaporator flask, the conversion rate increased to 95% (Fig. 1-5). About 168 mg of feruloyl DGs could be obtained from 100 mg of FA and 1 g of DG.

Table 1-4. Effect of FA concentration on feruloyl DG synthesis with FAE-PL.

FA concentration (mg/ml)	Amount of feruloyl DGs (mg) after incubation for			Conversion rate of FA after 12 h (%)
	1 h	6 h	12 h	
11	2 ± 1	8 ± 1	10 ± 0	54 ± 2
53	35 ± 3	56 ± 1	59 ± 0	67 ± 0
100	48 ± 5	99 ± 8	121 ± 0	69 ± 0
142	31 ± 2	112 ± 2	146 ± 5	56 ± 2

The reaction mixtures consisted of the indicated concentrations of FA, 1 g (0.8 ml) of DG, 10 μl of FAE-PL (2 U), and 10% (vol/vol) 1 M phosphate buffer (pH 6.0), and were incubated at 50°C for the indicated times. All experiments were performed in triplicate.

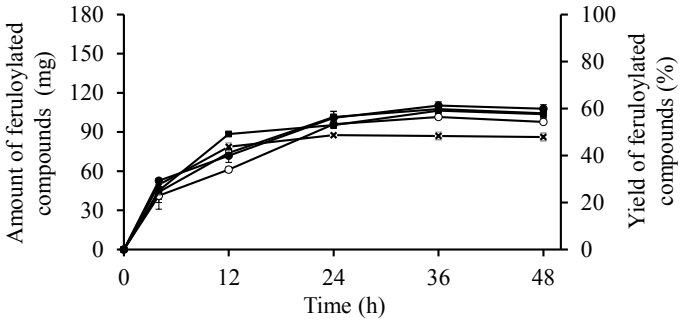

Fig. 1-2. Effects of pH of enzyme reaction on yield of feruloylated compounds (%).

The reaction mixtures consisted of 100 mg of FA, 1 g of DG, 50 μl of FAE-PL (2 U), and 0.1 ml of 1 M acetate buffer pH 4.0 (○), 5.0 (□), phophate buffer pH 6.0 (●), 7.0 (■), or 8.0 (×), respectively, and were incubated at 50°C. All experiments were performed in triplicate.

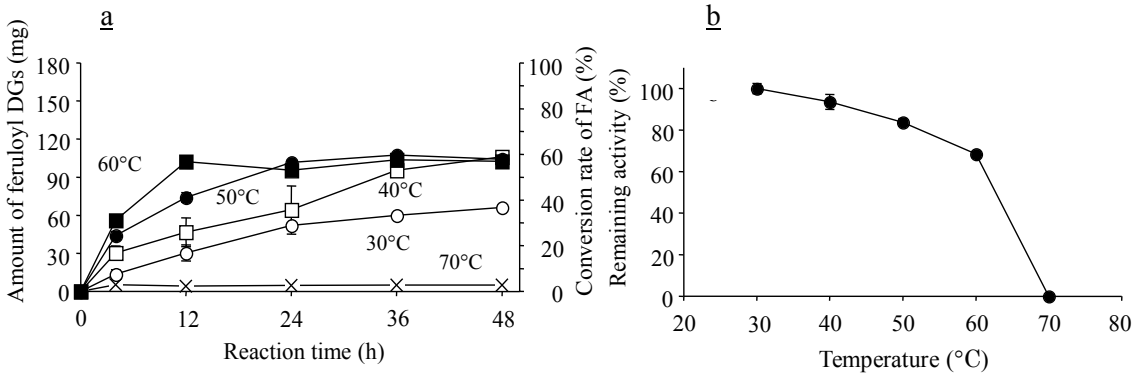

Fig. 1-3. Effects of temperature on feruloyl DG synthesis (a) and the stability of FAE-PL (b).

a Reaction mixtures consisted of 100 mg of FA, 1 g of DG, 50 μl of FAE-PL (0.5 U), and 0.1 ml of 1 M phosphate buffer (pH 6.0), and incubated at various temperatures.

b Stability was evaluated by measuring the residual hydrolytic activity after 4 days pre-incubation of the enzyme at temperatures between 30°C and 70°C in 100 mM phosphate buffer (pH 6.0) containing 90% DG. All experiments were performed in triplicate.

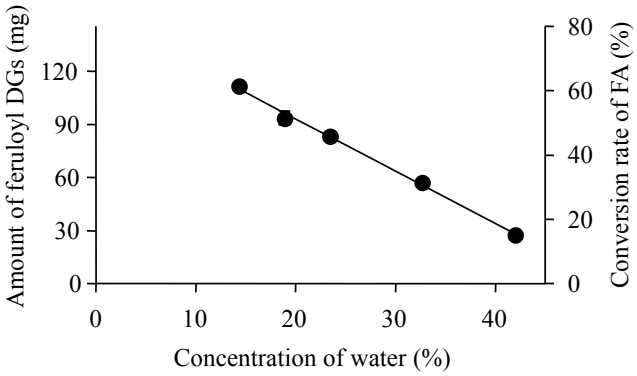

Fig. 1-4. Effect of water concentration on feruloyl DG synthesis with FAE-PL.

The reaction mixtures consisted of 100 mg of FA, 1 g of DG, 50 μl of FAE-PL (2 U), 0.1 ml of 1 M phosphate buffer (pH 6.0), and different volumes of water, and incubated at 50°C for 12 h. All experiments were performed in triplicate.

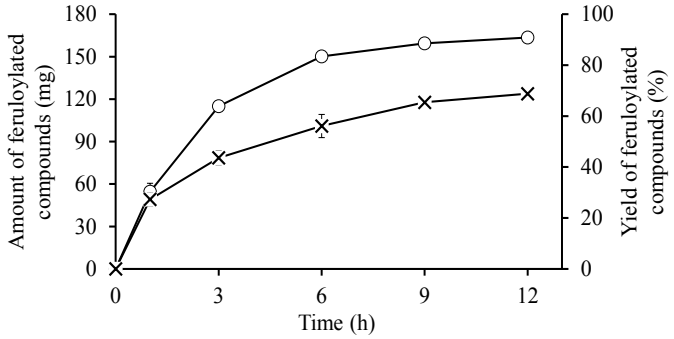

Fig. 1-5. Effects of enzyme reaction using vacuum-rotary evaporation on yield of feruloylated compounds (%).

The reaction mixtures consisted of 100 mg of FA, 1 g of DG, 50 μl of FAE-PL (2 U), and 0.1 ml of 1 M phosphate buffer (pH 6.0), and were incubated at 50°C under reduced pressure (○) or atmospheric pressure (×). All experiments were performed in triplicate.

Feruloyl DG synthesis with FAE-PL by a fed-batch method

Under the optimized conditions, only 8% of the DG was converted to feruloyl DGs. To obtain higher yields, I tried using a fed-batch method, in which 40 mg of FA was added to the reaction mixture 15 times every hour. At first, the fed-batch reaction was carried out in pH 6.0 phosphate buffer. However, feruloyl DG synthesis soon stopped, probably because the addition of FA decreased the pH. Therefore, I performed the fed-batch reaction in pH 7.0 phosphate buffer. The mixture was incubated in an evaporator flask at 50°C under reduced pressure throughout the reaction. FA was added to the mixture as a cloudy suspension, but as the reaction proceeded, the mixture became clear. Three feruloyl DGs (FA-DG1, 2, and 3) and five other unidentified products, which were subsequently identified as diferuloyl DGs by NMR and ESI-MS analyses, were detected (Fig. 1-6a). The production of both feruloyl DGs and diferuloyl DGs increased with time (Fig. 1-7). After the 30-h reaction, 729 mg of feruloyl DGs and 168 mg of diferuloyl DGs were obtained from 600 mg of FA and 1 g of DG (corresponding to conversion of 69% of the FA to feruloyl DGs and 21 % of the FA to diferuloyl DGs). The conversion rates of FA and DG to the esters was 90% and 41% in the fed-batch method, respectively.

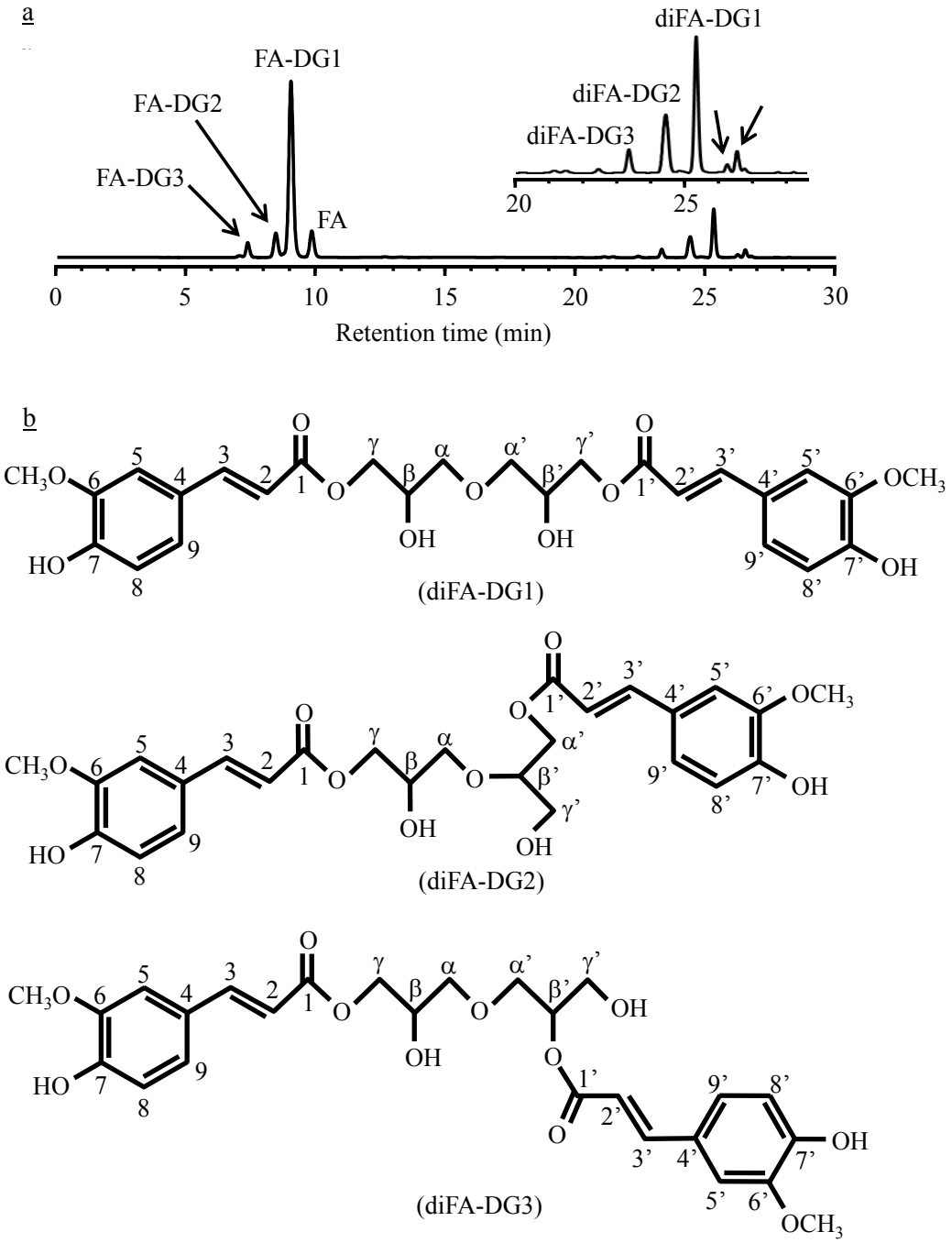

Fig. 1-6. HPLC chromatogram of the enzymatic products obtained from FA and DG using a fed-batch method (a) and structures of diFA-DG1, diFA-DG2, and diFA-DG3 (b). Composition of the reaction mixture is described in the text. The mixture was incubated at 50°C for 30 h followed by boiling for 10 min to inactivate the enzyme. The mixture was diluted 5,000 times with water and analyzed by HPLC using a Mightysil RP-18 reversed-phase column. The upper figure shows the part from 20 to 29 min of the lower figure.

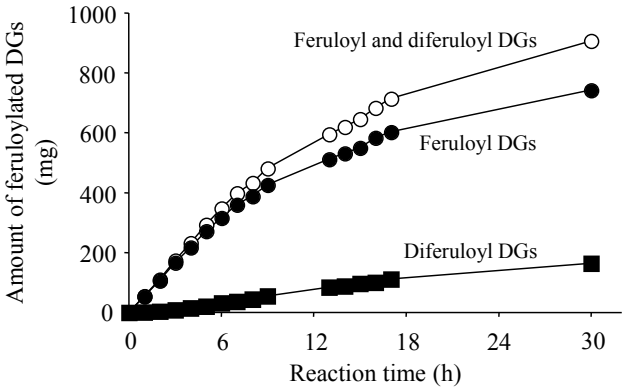

Fig. 1-7. Production of feruloyl and diferuloyl DGs by the fed-batch method.
Details are described in the legend of Fig. 1-6.

Isolation and identification of diferuloyl DGs

A mixture containing the feruloyl DGs and diferuloyl DGs (whose exact structures were unknown) were separated by Sephadex chromatography as described above. The feruloyl DGs (FA-DG1, 2, and 3) were eluted from the Sephadex column with water and the remaining diferuloyl DGs were eluted with 30% (vol/vol) ethanol. diFA-DG1, 2, and 3 in the latter fraction were separated on the reversed-phase column as described above and dried under reduced pressure, yielding white powders. The water solubility of diFA-DG1 (0.20 mg/ml) was lower than that of FA (0.69 mg/ml). The three isolated products were expected to be diferuloyl DGs due to their increased hydrophobicity on reversed-phase HPLC and to their increased masses ($[M + H]^+$ at *m/z* 519) detected by ESI–MS. The NMR experiments (^1H, ^{13}C, ^1H-^1H COSY, HMQC, and HMBC) showed that the structures of diFA-DG1, diFA-DG2, and diFA-DG3 were γ,γ′-diferuloyl-α,α′-DG, γ,α′-diferuloyl-α,β′-DG, and γ,β′-diferuloyl-α,α′-DG, respectively (Fig. 1-6b). Spectral data used for the structural determination of diFA-DG1, diFA-DG2, and diFA-DG3 are presented in Tables 1-5, 1-6, and 1-7, respectively.

Table 1-5. Spectral data for diFA-DG1.[a]

Position	δ_C	δ_H (integral, mult, *J* Hz)
α, α’	65.4	3.46 (4 H, m)
β, β’	72.5	3.87 (2H, m)
γ, γ’	67.4	4.03-4.15 (4 H, m)
1, 1’	166.6	-
2, 2’	114.3	6.45 (2H, d, 16.0)
3, 3’	145.1	7.55 (2H, d, 16.0)
4, 4’	125.5	-
5, 5’	111.2	7.30 (2H, d, 2.0)
6, 6’	147.9	-
7, 7’	149.4	-
8, 8’	115.5	6.77 (2H, d, 8.4)
9, 9’	123.1	7.09 (2H, dd, 8.4, 2.0)
OMe	55.6	3.80 (2H, s)

[a] diFA-DG1 has a symmetry plane.

Table 1-6. Spectral data for diFA-DG2.

Position	δ_C	δ_H (integral, mult, *J* Hz)	HMBC (H to C)
α	71	3.54-3.61 (2H, m)	β, γ, α’
β	67.5	3.84 (1H, m)	α, γ
γ	65.5	4.04-4.29 (2H, m)	1, α, β
α’	63	4.04-4.19 (1H, m)	1’, α, β’, γ’
		4.25-4.29 (1H, m)	
β’	79	3.54-3.61 (1H, m)	α’, γ’
γ’	60.5	3.50 (2H, m)	α’, β’
1	166.6	-	
2	114.3	6.44 (1H, dd, 15.6, 1.6)	1, 4
3	145.1	7.54 (1H, m)	1, 2, 4, 5, 9
4	125.5	-	
5	111.2	7.29 (1H, m)	3, 6, 7, 9
6	147.9	-	
7	149.4	-	
8	115.5	6.77 (1H, d, 8.4)	4, 6, 7
9	123.2	7.08 (1H, d, 8.4)	3, 5
OMe	55.5	3.80 (3H, s)	6
1’	166.6	-	
2’	114.3	6.44 (1H, dd, 15.6, 1.6)	1, 4
3’	145.1	7.54 (1H, m)	1, 2, 4, 5, 9
4’	125.5	-	
5’	111.2	7.29 (1H, m)	3, 6, 7, 9
6’	147.9	-	
7’	149.4	-	
8’	115.5	6.77 (1H, d, 8.4)	4, 6, 7
9’	123.2	7.08 (1H, d, 8.4)	3, 5
OMe’	55.5	3.80 (3H, s)	6

Table 1-7. Spectral data for diFA-DG3.

Position	δ_C	δ_H (integral, mult, *J* Hz)	HMBC (H to C)
α	72.3	3.52-3.62 (2H, m)	α, γ, α’
β	67.4	3.83-3.86 (1H, m)	β
γ	65.4	4.00-4.12 (2H, m)	α, β, 1
α’	69.7	3.52-3.62 (2H, m)	β, β’
β’	73.1	4.98 (1H, m)	α’, γ’, 1’
γ’	59.9	3.52-3.62 (2H, m)	α’, β’
1	166.3-166.6	-	
2	114.1-114.4	6.41-6.46 (1H, d, 16.0)	1, 4
3	145.1	7.53 (1H, d, 15.6)	1, 2, 4, 5, 9
4	125.3	-	
5	111.1	7.28 (1H, m)	3, 6, 7, 9
6	148.0	-	
7	148.0	-	
8	115.5	6.77 (1H, d, 8.4)	4, 6, 7
9	123.1	7.07 (1H, d, 8.4)	3, 5
OMe	55.6	3.79 (3H, s)	6
1’	166.3-166.6	-	
2’	114.1-114.4	6.41-6.46 (1H, d, 16.0)	1, 4
3’	145.1	7.53 (1H, d, 15.6)	1, 2, 4, 5, 9
4’	125.3	-	
5’	111.1	7.28 (1H, m)	3, 6, 7, 9
6’	148.0	-	
7’	148.0	-	
8’	115.5	6.77 (1H, d, 8.4)	4, 6, 7
9’	123.1	7.07 (1H, d, 8.4)	3, 5
OMe’	55.6	3.79 (3H, s)	6

Anti-oxidant activity and UV properties of feruloylated DGs

The abilities of feruloyl DGs to scavenge DPPH radicals were similar to the scavenging abilities of FA and butyl hydroxytoluene, a synthetic anti-oxidant. The scavenging abilities of three diferuloyl DGs were twice those of feruloyl DGs (Table 1-8). FA has an absorption peak at 321 nm. The UV spectra of feruloyl DGs and diferuloyl DGs were very similar to the UV spectrum of FA (data not shown). The molar absorption coefficients (ε) of the feruloyl DGs were very similar to the ε of FA, but those of the diferuloyl DGs were twice the ε of FA (Table 1-8).

Table 1-8. DPPH scavenging ability and molar absorption coefficient of feruloylated DGs.

Compound	DPPH scavenging ability (mmol)[a]	λ_{max} (nm)	ε ($M^{-1}cm^{-1}$)[b]
FA-DG1	0.76 ± 0.02	325	20,000 ± 300
FA-DG2	0.79 ± 0.00	325	20,000 ± 100
FA-DG3	0.78 ± 0.01	325	21,000 ± 900
diFA-DG1	0.40 ± 0.00	325	39,000 ± 1,500
diFA-DG2	0.41 ± 0.00	325	39,000 ± 1,700
diFA-DG3	0.40 ± 0.00	325	38,000 ± 400
FA	0.73 ± 0.01	321	19,000 ± 300
Butyl hydroxytoluene	0.79 ± 0.00	-	-

[a] Concentration that scavenges 1 mmol of DPPH radicals. The values are the means ± standard errors of four experiments. [b] Molar absorption coefficients of the compounds were determined at 320 nm. Experiments were performed in duplicate.

Discussion

Because FA has several beneficial functions, there have been many attempts in the past decade to synthesize feruloylated compounds by esterification or transesterification reactions with lipases or FAEs (reviewed by Figueroa-Espinoza and Villeneuve 2005; Faulds 2010). The goal of many of these studies was to create more hydrophobic feruloylated compounds such as butyl ferulate (Vafiadi et al. 2008; Thörn et al. 2011) and feruloylated monoacyl- and diacyl-glycerol (Sun et al. 2007b, 2009). On the other hand, Topakas et al. (2005) succeeded in synthesizing feruloylated L-arabinose with *Sporotrichum thermophile* FAE (StFaeC), which is the first demonstration of enzymatic feruloylation of hydrophilic compounds. Since then, several feruloyl polyols such as sugars (Vafiadi et al. 2006, 2007) and glycerol (Tsuchiyama et al. 2006; Sun et al. 2007a; Sun and Chen 2015) have been enzymatically synthesized. As described in the Introduction, esterification reactions are more practical than transesterification reactions for industrial applications. Tsuchiyama et al. (2006) first succeeded the esterification of FA using an FAE. Since then, synthesis of FA derivatives through esterification reactions have been reported (Ishihara et al 2010; Couto et al 2011; Zeng et al. 2014).

The goal of chapter 1 was to obtain feruloylated compounds that have high water solubility and retain the beneficial functions of FA. We previously synthesized FA-G1, whose water solubility was higher than that of FA. However, the solubility of the product in water at 20°C was only 1.20 mg/ml (Tsuchiyama et al. 2006). Therefore, I replaced glycerol with DG, which is liquid and has more hydroxyl groups than glycerol, as the acceptor of the feruloylation. The water solubility of FA-DG1 (γ-feruloyl-α,α′-DG; Fig. 1-1b), which is the major reaction product obtained from FA and DG by FAE-PL, was more than 980 mg/ml (corresponding to an FA concentration of 556 mg/ml) which was many times more than that of FA-G1. No other feruloylated compound has been reported to have such a high water solubility.

Suitable conditions for esterification of FA with DG were 100 mg of FA in the presence of 1 g of DG and 0.1 ml of 1 M phosphate buffer (pH 6.0) at 50°C under reduced pressure. The conversion rate of FA was inversely related to water content in the

reaction mixture. Surprisingly, the conversion rate of FA was increased from 70% to 95% when the reaction was conducted under reduced pressure, indicating that continuous removal of water from the reaction mixture using evaporation diminish the hydrolysis of the synthesized esters by FAE-PL. The fed-batch reaction resulted in higher yields of feruloylated DGs than the conventional reaction. This method made it possible to add large amounts of FA to the reaction mixture, increasing the yield of feruloylated DGs by more than five fold. In the fed-batch reaction, a considerable amount of the FA (21%) was converted to diferuloyl DGs, probably because large amounts of feruloyl DGs synthesized in the reaction mixture were used as acceptors of feruloylation. On the other hand, diferuloyl DGs were not synthesized in the standard reaction, probably because the amount of DG is much higher than that of feruloyl DGs.

Feruloylated DGs may have some applications. The DPPH scavenging abilities of feruloyl DGs were comparable with those of FA and butyl hydroxytoluene, which is a synthetic anti-oxidant. These results suggest that differences in the chemical structures of feruloyl DGs did not affect their anti-oxidant activities. The scavenging abilities of three diferuloyl DGs were twice those of feruloyl DGs, possibly because diferuloyl DGs have two molecules of FA. Feruloyl and diferuloyl DGs also retained the UV absorption properties of FA. Thus, the esterification of FA did not appear to alter its properties. Based on these properties, feruloylated DGs might have applications in beverages, food, and cosmetics.

Chapter 2. Ferulic acid and its water-soluble derivatives inhibit nitric oxide production and inducible nitric oxide synthase expression in rat primary astrocytes.

Summary

In the previous chapter, I described the synthesis of 1-feruloyl diglycerol (FA-DG1), which exhibited much higher solubility in water than FA. There is insufficient published evidence regarding the properties of FA derivatives and the protective effects of FA itself on activated astrocytes. Excessive iNOS expression in astrocytes is one of the factors involved in the development of neurodegenerative diseases such as Parkinson's disease and AD. To evaluate the possible application of water-soluble FA derivatives as neuroprotective agents, this chapter examines the ability of FA and FA derivatives to suppress abnormal activation of astrocytes, an important event in the later stages of disease progression and further neurodegeneration.

I investigated the effects of FA derivatives on NO production and iNOS expression in rat primary astrocytes. The results showed that these compounds inhibited NO production and iNOS expression in a concentration-dependent manner and that the mechanism underlying these effects was the suppression of the nuclear factor-κB (NF-κB) pathway. This evidence suggests that FA and its derivatives may be effective neuroprotective agents.

Materials and Methods

Chemicals and reagents

FA was kindly gifted by Tsuno Food Industrial Co., Ltd. FA-DG1 and FA-G1 were prepared as described in chapter 1 and previous paper (Tsuchiyama et al. 2006),

respectively. Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycin solutions were purchased from Wako Pure Chemical Industries. Sinapic acid (SA), pCA, caffeic acid (CA), 3,4-dimethoxycinnamic acid (dmCA), LPS (from *Escherichia coli*; L8274), protease inhibitor cocktail, deoxyribonuclease (DNase), and anti-actin antibody were obtained from Sigma–Aldrich (St. Louis, Missouri, USA). Fetal bovine serum (FBS) was purchased from Biowest (Nuaille, France). Mouse monoclonal antibody against iNOS was prepared as previously described (Nakamura et al. 2006). Anti-NF-κB p65 antibody, anti-histone H2B antibody, and anti-NF-κB inhibitor α (IκB-α) antibody were from Cell Signaling Technology (Danvers, Massachusetts, USA). Anti-triose-phosphate isomerase (TPI) antibody was kindly provided by Dr. R. Yamaji (Osaka prefecture University, Japan). Peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were from GE Healthcare Life Science (Piscataway, New Jersey, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was from Dojindo Laboratories (Kumamoto, Japan). The rest of the reagents were of analytical grade or higher and were obtained from standard sources.

Cell culture

Preparation of astrocytes was carried out in compliance with the Guidelines for Animal Experimentation of Osaka Prefecture University. Primary astrocytes were harvested from Wistar rats using a modification of a technique previously described (Nakamura et al. 2006). Briefly, brain cortices from 20-day-old rat embryos were deprived of their meninges, cut into blocks, and dissociated with 0.25% trypsin. An equal volume of horse serum supplemented with 0.1 mg/ml DNase I was added to the medium to inactivate the trypsin, and the tissues were centrifuged at 300 *g* for 5 min. The tissue sediments were suspended in DMEM containing 10% FBS and 1% penicillin–streptomycin. The cells were plated on 100-mm diameter polyethyleneimine-coated plastic dishes at a density of 0.8–1.3 × 10^5 cells/cm^2. Cultures were maintained at 37°C in 5% CO_2 and 95% air, and the medium was changed every 3 days. After one week, astrocytes were replated to remove the neurons by using a standard trypsin treatment technique.

Cell treatment

To determine the effects of the tested compounds on NO production, iNOS protein expression, NF-κB translocation, and IκB-α degradation, the cells were pre-incubated in culture medium containing the compounds for 30 min. After pre-incubation, the astrocytes were treated with LPS (1 μg/ml) and incubated at 37°C for 24 h.

Nitrite assay

NO synthesis was determined by quantification of nitrite, a stable product from the reaction between NO and molecular oxygen, in the supernatant. A 100 μl of culture supernatant was dispensed into the wells of a 96-well plate and mixed with 50 μl Griess reagent A (1% sulfanilamide in 5% H_3PO_4) for 5 min before adding 50 μl Griess reagent B [0.1% N-(1-naphthyl)-ethylenediamine] to the mixture. After incubation for 5 min at room temperature, absorbance was measured spectrophotometrically at 540 nm. Fresh culture media served as the blank in all experiments. Nitrite concentration was calculated by extrapolating from a standard curve obtained from the reaction of $NaNO_2$ in the assay.

Cytotoxicity assay

Cytotoxicity of the compounds was estimated using the MTT assay (Mosmann 1983). MTT solution of DMEM (50 μl, 0.5 mg/ml) was added to cells that had been cultured for 24 h in the presence or absence of the compounds tested. After incubation for 3 h at 37°C, the suspension was mixed with 50 μl of an MTT stop solution (10% sodium dodecyl sulfate (SDS) in 10 mM HCl) and further incubated for 24 h. The optical density of the assay samples was measured at 540 nm.

Western blotting analysis

To obtain total proteins for immunoblot analysis of iNOS levels, astrocytes were washed with PBS and then lysed with 1% Triton-X and protease inhibitor cocktail in PBS. To prepare the nuclear fraction for an immunoblot analysis of NF-κB p65 and the cytosolic fraction for immunoblot analysis of IκB-α, the cell pellets were processed using Nuclear Extraction Kit I (EpiQuik; Epigentek, New York, USA) according to the

manufacturer's instructions. Soluble proteins from each sample were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane (GE Healthcare). The membrane was blocked with Blocking One (Nacalai Tesque) and incubated at 4°C overnight with the antibodies against the following proteins: iNOS, β-actin, NF-κB p65, histone H2B, IκB-α, and TPI. After washing with PBS, the membranes were incubated at room temperature for 1 h with peroxidase-conjucated anti-rabbit or anti-mouse IgG secondary antibody. Immunoreactive bands were detected using a chemiluminescence reagent (Millipore, Bedford, Massachusetts, USA) with a luminescent image analyzer (LAS-1000: Fujifilm, Tokyo).

Protein concentrations

Protein concentrations were determined via the Bradford method with bovine serum albumin (BSA) as the standard (Bradford 1976).

Statistical analysis

All the experiments were performed at least three times. Mean values for individual experiments are presented as means ± standard error (SE) and were analyzed using one-way analysis of variance (ANOVA) with the Newman-Keuls post-hoc test. Statistical analysis was determined with a using the Prism program (GraphPad, San Diego, CA, USA). A p value of less than 0.05 was considered significant.

Results

Inhibitory effects of cinnamic acid derivatives on LPS-induced NO production in primary astrocytes

Excessive NO production plays a key role in neuropathological conditions such as AD and Parkinson's disease (Hamby and Sofroniew 2010). First, I evaluated the effects of the following cinnamic acid derivatives: FA, SA, pCA, CA, and dmCA on LPS-induced NO production in astrocytes. After pre-incubation with these compounds (1.5 mM), the astrocytes were treated with LPS (1 μg/ml) and incubated for 24 h. The production of

NO was determined by measuring the nitrite content of the culture medium. As shown in Fig. 2-1, stimulation with LPS induced NO production in primary astrocyte cultures, whereas pretreatment with FA and SA significantly inhibited NO production ($p < 0.05$).

However, pCA, CA, and dmCA did not significantly inhibit NO production. According to the MTT assay results, none of the concentrations used during the experiments were cytotoxic to the astrocytes (data not shown). These results suggest that FA and SA may be useful as neuroprotective agents. Based on these findings, I examined the effects of FA and its water-soluble derivatives: FA-G1 and FA-DG1.

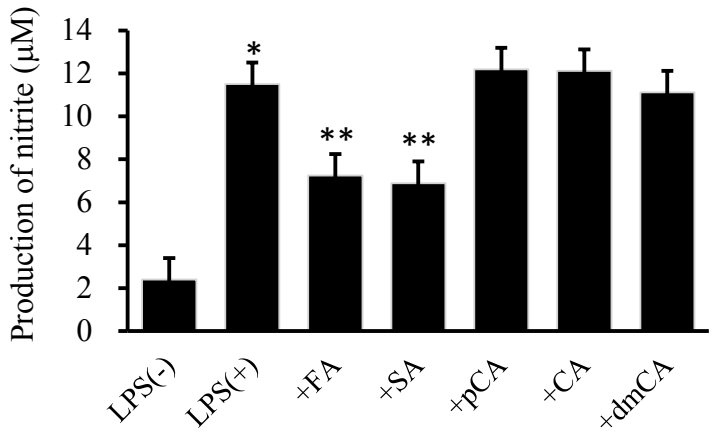

Fig. 2-1. Inhibitory effects of various cinnamic acid derivatives on LPS-induced NO production in primary astrocytes.

Astrocytes were pre-incubated in the presence of absence of FA, SA, pCA, CA, and dmCA (1.5 mM) for 30 min, followed by treatment with LPS (1 μg/ml) and incubated for 24 h. The cells were plated on polyethyleneimine-coated 96-well plate at a density of 1.3×10^5 cells/cm^2. The production of NO was determined by measuring the nitrite content of the culture medium. Results are expressed as concentrations (μM) of nitrite content. Data are presented as the mean ± SE (n = 6). One-way ANOVA with the Newman-Keuls post-hoc test was used for statistical analysis. * $p < 0.05$ vs. LPS (－). ** $p < 0.05$ vs. LPS (＋).

Inhibitory effects of FA and its water-soluble derivatives on LPS-induced NO production in primary astrocytes

Astrocytes were pre-incubated with FA and its water-soluble derivatives at several concentrations (0.25, 0.5, 1.0, and 1.5 mM), treated with LPS (1 μg/ml), and incubated for 24 h. As shown in Fig. 2-2a, pretreatment with FA, FA-G1, and FA-DG1, significantly inhibited NO production at concentration of 1.0 mM, 0.25 mM, and 0.25 mM or higher, respectively ($p < 0.05$). Cytotoxicity of the compounds at the above concentrations to the astrocytes was not observed via the MTT assay (Fig. 2-2b), indicating that FA, FA-G1, and FA-DG1 show potential as neuroprotective agents.

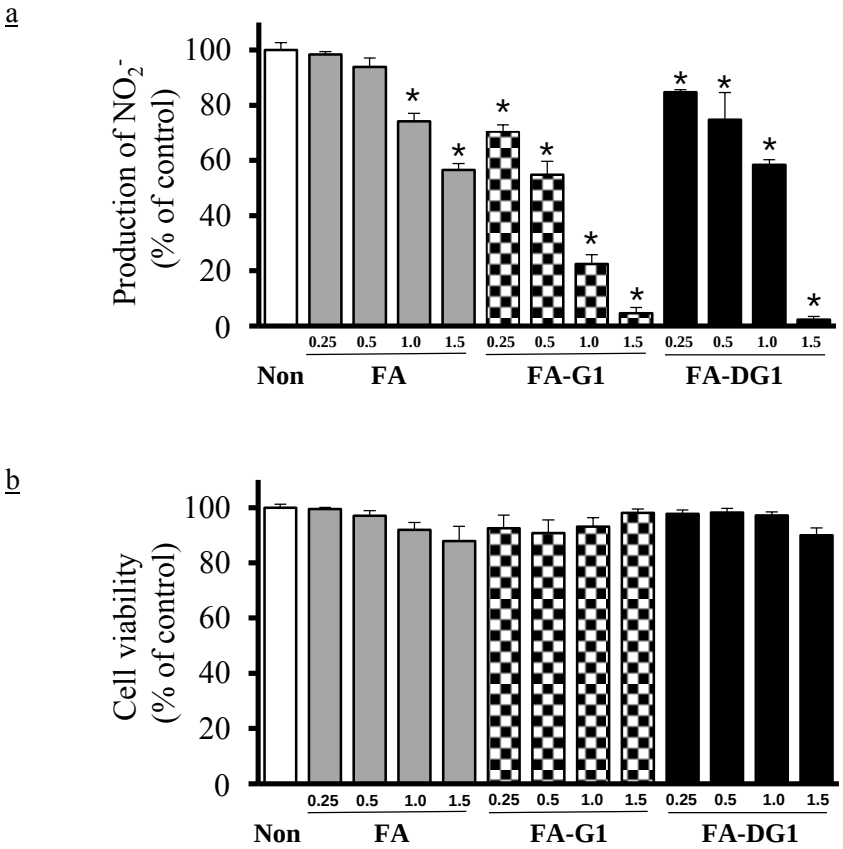

Fig. 2-2. Inhibitory effects of FA and its water-soluble derivatives on LPS-induced NO production in primary astrocytes.

Astrocytes were prepared as described in the legend for Figure 2-1, except that they were pre-incubated with different concentrations (from 0.25 to 1.5 mM) of FA or its water-soluble derivatives before addition of LPS. The cells were plated on polyethyleneimine-coated 96-well plate at a density of 1.3×10^5 cells/cm^2. (a) The production of NO was determined by measuring the nitrite content of the culture medium. (b) Cell viability was determined by MTT assay. Data are presented as the mean ± SE (n = 6). One-way ANOVA with the Newman-Keuls post-hoc test was used for statistical analysis. * $p < 0.05$ vs. control.

Inhibitory effect of FA and its water-soluble derivatives on LPS-induced iNOS expression in primary astrocytes

LPS induces iNOS expression, and iNOS synthesizes NO in astrocytes (Hamby and Sofroniew 2010). To determine whether the inhibition of NO production by FA and its water-soluble derivatives was a result of the inhibition of iNOS protein expression, I assessed the latter by immunoblotting using anti-iNOS antibodies. As shown in Fig. 2-3a, LPS stimulation increased iNOS protein expression in primary astrocyte cultures, whereas pretreatment with FA, FA-G1, and FA-DG1 (1.0 mM) significantly inhibited such an increase in iNOS protein expression. These results suggest that FA, FA-G1, and FA-DG1 repress NO production in astrocytes through inhibition of LPS-induced iNOS expression.

Inhibitory effect of FA and its water-soluble derivatives on intranuclear transport of NF-κB and degradation of IκB-α in primary astrocytes

LPS-induced iNOS expression in astrocytes is mediated through NF-κB signaling (Nomura 2001; Saha and Pahan 2006). To determine whether inhibition of iNOS expression by FA and its water-soluble derivatives is mediated through NF-κB signaling, I examined the effects of FA and its derivatives on LPS-induced NF-κB nuclear translocation and IκB-α degradation in astrocytes. As shown in Fig. 2-3b and Fig. 2-3c, LPS stimulation increased NF-κB nuclear translocation and IκB-α degradation in primary astrocyte cultures. Pretreatment with FA, FA-G1, and FA-DG1 (1.0 mM) significantly inhibited the increase in NF-κB nuclear translocation and IκB-α degradation. These results indicate that FA, FA-G1, and FA-DG1 inhibit LPS-induced activation of NF-κB signaling.

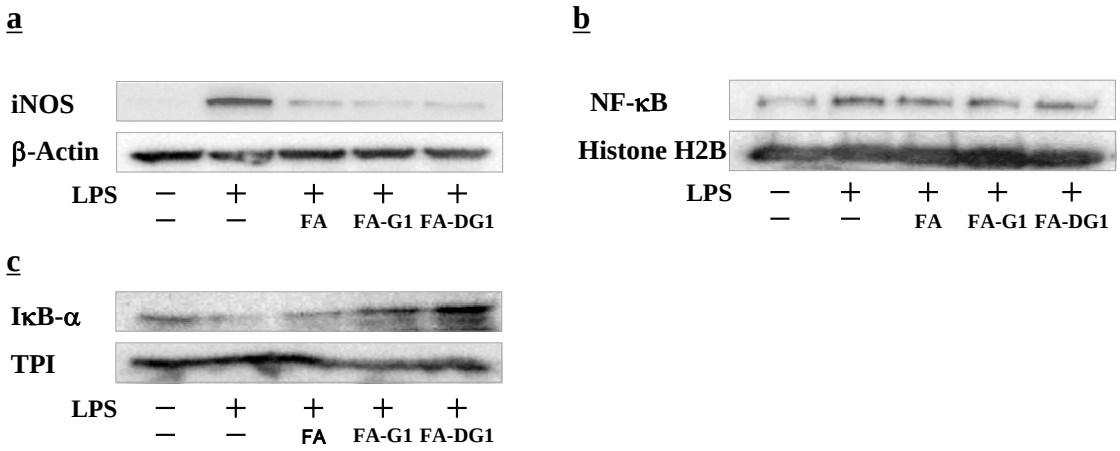

Fig. 2-3. Effect of FA and its water-soluble derivatives on iNOS protein expression, intranuclear transport of NF-κB, and degradation of IκB-α.

Astrocytes were prepared as described in the legend for Figure 2-1, except that they were pre-incubated with FA or its water-soluble derivatives (1.0 mM) before addition of LPS. The cells were plated on 35-mm diameter polyethyleneimine-coated plastic dishes at a density of 0.8×10^5 cells/cm^2. (a) iNOS protein levels were determined by immunoblotting with anti-iNOS antibody. β-Actin served as a loading control. (b) Nuclear translocation of NF-κB was examined by immunoblotting with anti-NF-κB p65 antibody. Histone was used as a nuclear marker. (c) Degradation of IκB-α was assessed by immunoblotting with anti-IκB-α antibody. TPI was used as a cytosolic maker.

Discussion

In chapter 2, to expand the utility of FA, FA-G1, and FA-DG1, I examined the effects of these compounds on NO production and iNOS expression in primary astrocytes. Excessive iNOS expression in astrocytes is one of the factors involved in the development of neurodegenerative diseases such as Parkinson's disease and AD.

Prior to the experiments carried out with FA and its derivatives, I conducted screening tests with various cinnamic acid derivatives. These experiments yielded FA and SA as potential neuroprotective agents. Both FA and SA have a methoxy and a hydroxy group in their benzene ring. The balance between hydrophilic and hydrophobic groups makes possible for these compounds to bind to and stimulate cell membrane proteins.

I found that FA inhibited NO production and iNOS expression in primary astrocytes. Furthermore, inhibition of iNOS expression was mediated by a suppression of the NF-κB pathway. The water-soluble FA derivatives developed possess neuroprotective effects similar to FA, suggesting that the neuroprotective effect is attributable to the phenol groups rather than the ester groups.

In addition, in astrocytes, Aβ induces expression of iNOS and other inflammatory cytokines through the NF-κB pathway (Kaltschmidt et al. 1997). Our results suggest that treatment with FA, FA-G1, and FA-DG1 may improve AD through the inhibition of Aβ aggregation and the excessive activation of the NF-κB pathway in astrocytes. The NF-κB pathway induces inflammatory cytokines that cause neuronal death (Nomura 2001). FA, FA-G1, and FA-DG1 may inhibit the overexpression of inflammatory cytokines originating through the NF-κB pathway and therefore exert a neuroprotective effect.

Some polyphenols are known to exhibit neuroprotective properties, including a therapeutic effect in AD (Granzotto and Zatta 2014; Malar and Devi 2014). Recent research on plant-derived polyphenols has focused on their safe pharmacological profile and their potential to positively affect human health. Water-soluble FA derivatives showed antioxidant activity similar to that of FA, suggesting that they may be effective

as neuroprotective agents. The ability of certain compounds to inhibit the excessive activation of astrocytes has been previously reported. For example, polymethoxyflavone, extracted from young fruits of *Citrus unshiu,* was found to inhibit NO production and iNOS expression in rat primary astrocytes (Ihara et al. 2012). In addition, resveratrol was found to strongly prevent H_2O_2-induced increase in reactive nitrogen species (RNS) production and iNOS expression in a C6 astrocyte cell line (Quincozes-Santos et al. 2013). These compounds are considered to be neuroprotective agents and the same could be true for FA, FA-G1, and FA-DG1, based on our latest results.

Chapter 3. Water-soluble ferulic acid derivatives improve amyloid-β induced neuronal cell death and dysmnesia through inhibition of amyloid-β aggregation.

Summary

In the previous chapters, I describe the successful synthesis of water-soluble FA derivatives (FA-DG1) and investigation of the inhibitory effects of these derivatives and FA on LPS-induced NO production in primary astrocytes. The effects of FA derivatives on Aβ-induced neurodegeneration have not been reported previously. It is generally accepted that aggregation of Aβ is one of the crucial pathogenic events in AD.

Here in, I detail the neuroprotective effects of these water-soluble FA derivatives on Aβ-induced neurodegeneration in both *in vitro* and *in vivo* experiments. FA and water-soluble FA derivatives inhibited Aβ aggregation and destabilized pre-aggregated Aβ to a similar extent. Furthermore, water-soluble FA derivatives, as well as FA, inhibited Aβ-induced neuronal cell death in cultured neuronal cells. In *in vivo* experiments, oral administration of water-soluble FA derivatives to mice improved Aβ-induced dysmnesia assessed by contextual fear conditioning test and protected hippocampal neurons against Aβ-induced neurotoxicity. This study provides useful evidence suggesting that water-soluble FA derivatives are expected to be effective neuroprotective agents.

Materials and Methods

Chemicals and reagents

FA was kindly gifted by Tsuno Food Industrial Co., Ltd. FA-DG1 and FA-G1 were prepared as described in chapter 1 and previous paper (Tsuchiyama et al. 2006), respectively. DMEM and penicillin-streptomycin solution were purchased from Wako

Pure Chemical Industries. FBS was from Biowest; horse serum, from Gibco (Carlsbad, CA, USA); MTT, from Dojindo Laboratories; Aβ (25–35), from AnaSpec (San Jose, CA, USA); and Aβ (1–40) and Aβ (1–42), from Peptide Institute (Osaka, Japan). RPMI-1640 medium, thioflavin-T, trypsin, DNase I and cytosine arabinofuranoside (Ara-C) were bought from Sigma-Aldrich. PC12 cells and NeuroTrace 500/525 green-fluorescent Nissl stain were from Invitrogen (Carlsbad, CA, USA). The rest of the reagents were of analytical grade or higher and were obtained from standard sources.

Preparation of Aβ

Aβ (25–35) was dissolved in ultrapure water at a concentration of 1 mM and stored at −80°C until use. Aβ (1–40) and Aβ (1–42) were dissolved in DMSO at a concentration of 500 and 250 μM, respectively, and stored at −80°C until use.

Thioflavin-T fluorescence assay

In order to determine the formation of aggregated Aβ, reaction mixtures containing fresh 200 μM Aβ (25–35), 25 μM Aβ (1–40), or 25 μM Aβ (1–42) with or without FA, FA-G1, or FA-DG1 were incubated at 37°C for 7, 3, or 1 days, respectively. To determine the destabilization of aggregated Aβ, 400 μM Aβ (25–35), 100 μM Aβ (1–40), or 50 μM Aβ (1–42) were incubated at 37°C for 3, 3, and 1 day, respectively. After the aggregation reaction, the mixtures were incubated with or without FA, FA-G1, or FA-DG1 at 37°C for 6 h. Ten microliters of the reaction mixtures were added to 700 μl of 50 mM glycine–NaOH buffer (pH 8.0), containing 10 μM thioflavin-T. Fluorescence (excitation 450 nm and emission 485 nm) was monitored with a spectrofluorometer FP-6200 (JASCO, Tokyo, Japan).

Cell culture

Primary cortical neurons were prepared from 20-day-old fetal Wistar rats as described previously (Janssens and Lesage 2001) with minor modifications. Briefly, brain cortices from embryos were cleaned of their meninges, cut into blocks, and dissociated with 0.25% trypsin. An equal volume of horse serum supplemented with

0.1 mg/ml DNase I was added to the medium to inactivate the trypsin, and the tissues were centrifuged at 300 × *g* for 5 min. The tissue sediments were resuspended in DMEM containing 10% FBS and 1% penicillin-streptomycin. The cells were plated on polyethyleneimine-coated 96 well plates at a density of 1.5×10^6 cells/cm^2. After culturing for 2 to 4 days *in vitro* (DIV), the medium was replaced with fresh medium containing 5 μM Ara-C to inhibit proliferation of non-neuronal cells; thereafter, no further medium change was carried out before the experiments. Cells were maintained at 37°C in 5% CO_2 and 95% air.

PC12 cells were plated on polyethyleneimine-coated 96 well plates at a density of 3.0×10^7 cells/cm^2 in RPMI-1640 containing 10% FBS and 1% penicillin-streptomycin. Cells were maintained at 37°C in 5% CO_2 and 95% air.

Cytotoxicity assay

Reaction mixtures containing fresh 2 mM Aβ (25–35) with or without 2 mM FA, FA-G1, or FA-DG1 were incubated at 37°C for 72 h. Primary cortical neurons (7 DIV) or PC12 cells were treated with the reaction mixtures diluted in culture medium for 24 h at a final concentration of 20 μM Aβ (25–35). Cell viability was evaluated by MTT assay as described in chapter 2. Briefly, a 0.5 mg/mL solution of MTT in DMEM or RPMI-1640 was added to the culture medium (10 μl/well). Cultures were incubated at 37°C for 3 h. After incubation, the suspension was mixed with 110 μl of MTT stop solution (10% SDS in 10 mM HCl) and further incubated for 24 h. Absorbance was measured at 540 nm.

Animals

Male C57BL/6J mice were obtained from Kiwa Laboratory Animal Co., Ltd. The mice were allowed free access to food and water and maintained in a 12-hour dark-light cycle for 1 week. Animal care and experimental procedures were carried out in compliance with the Guide for Animal Experimentation at Osaka Prefecture University.

Intracerebroventricular injection of Aβ

The mice were divided into five groups (n=6 each). FA, FA-G1, and FA-DG1 were dissolved in 0.5% carboxymethylcellulose (CMC) at a concentration of 10 mM. After oral administration of FA, FA-G1, or FA-DG1 (0.1 μmol/g/day) for 42 days, mice were injected 4 nmol Aβ (25–35) diluted in 2 μl of ultrapure water by intracerebroventricular injection, as described previously (Tsunekawa et al. 2008). Briefly, using a 25-μl Hamilton microsyringe fitted with a 27-gauge needle, each mouse was injected at a point 0.8 mm lateral from bregma at a depth of 2.5 mm in the lateral cerebral ventricle.

Contextual fear conditioning test

The contextual fear conditioning test was carried out on days 8–9 after Aβ (25–35) injection, according to a previous report (Tsunekawa et al. 2008) with a minor modification. For training (conditioning phase), mice were placed in the conditioning cage for 90 sec, and then a 15-sec tone (75 dB) was delivered as a conditioned stimulus. During the last 2 sec of the tone stimulus, a foot shock of 0.15 mA (unconditioned stimulus) was delivered through a shock generator (O'Hara & Co., LTD, Tokyo, Japan). This procedure was repeated four times at 15-sec intervals. One day after fear conditioning, mice were placed in the conditioning cage, and the freezing response for tone stimulus was continuously measured for 2 min (retention session). The freezing response was defined as no movement by any of the mice.

Nissl stain

After fear conditioning test, the mice were killed with a lethal dose of pentobarbitone sodium (0.1 ml, 60 mg/ml) and perfused through the left ventricle with 60 ml of PBS, followed by 80 ml of PBS containing 4% paraformaldehyde. The brain was removed and fixed in 4% paraformaldehyde for 2 h at 4°C, followed by cryoprotection in 30% sucrose overnight at 4°C. Ten-micrometer thick serial coronal sections were cut using a Leica cryostat CM 1950 (Leica Microsystems, Wetzlar, Germany). Nissl staining was performed using NeuroTrace 500/525 green-fluorescent Nissl stain,

according to the manufacturer's instructions. The sections were coverslipped and observed under a fluorescence microscope (Nikon, Tokyo, Japany). Further image processing and quantification were performed by using Adobe Photoshop v. 7.0 (Adobe Systems, Waltham, MA, USA).

Statistical analysis

All results are represented as means ± S.E of three to five determinations and were analyzed using one-way analysis of variance (ANOVA) with the Newman-Keuls post-hoc test. Statistical analysis was determined with a using the Prism program (GraphPad, San Diego, CA, USA). A p value of less than 0.05 was considered significant.

Results

Effects of water-soluble FA derivatives on Aβ aggregation

FA has been shown to inhibit Aβ aggregation *in vitro* (Ono et al. 2005). Therefore, we examined whether water-soluble FA derivatives, FA-G1 and FA-DG1, showed similar inhibitory effects on Aβ aggregation. When fresh Aβ (25–35), Aβ (1–40), or Aβ (1–42) was incubated at 37°C for 5 days, thioflavin-T fluorescence was significantly enhanced, indicating that aggregated Aβ fibrils bound to thioflavin-T (data not shown). As shown in Fig. 3-1, FA inhibited aggregation of Aβ (25–35), Aβ (1–42) in a concentration of 10 μM, 1 μM or higher, respectively ($p < 0.05$), consistent with previous data. FA-G1 and FA-DG1 also prevented Aβ aggregation in a each concentration (Fig. 3-1).

These results show that, *in vitro*, FA-G1 and FA-DG1 as well as FA inhibit Aβ aggregation to a similar extent. Aggregabilities of Aβs differ by differences in their peptide lengths. The aggregability of Aβ (1–42) is considerably high compared with that of Aβ (25–35). Therefore, the amounts of Aβ or the amounts of FA derivatives in the reaction mixtures were varied depending on the types of Aβs used for the experiments.

Effects of water-soluble FA derivatives on destabilization of Aβ aggregates

Since FA has been also shown to destabilize Aβ aggregates *in vitro* (Ono et al. 2005), we next tested whether the water-soluble FA derivatives also destabilize Aβ aggregates. As shown in Fig. 3-2, when the aggregated Aβ (25–35), Aβ (1–40), or Aβ (1–42) were incubated with FA, thioflavin-T fluorescence decreased, indicating that FA destabilized the Aβ aggregates. Similarly, FA-G1 and FA-DG1 also decreased the fluorescence intensity of thioflavin-T; this reduction depended on FA-G1 and FA-DG1 concentration (Fig. 3-2). These results indicate that water-soluble FA derivatives are able to destabilize pre-aggregated Aβ *in vitro*.

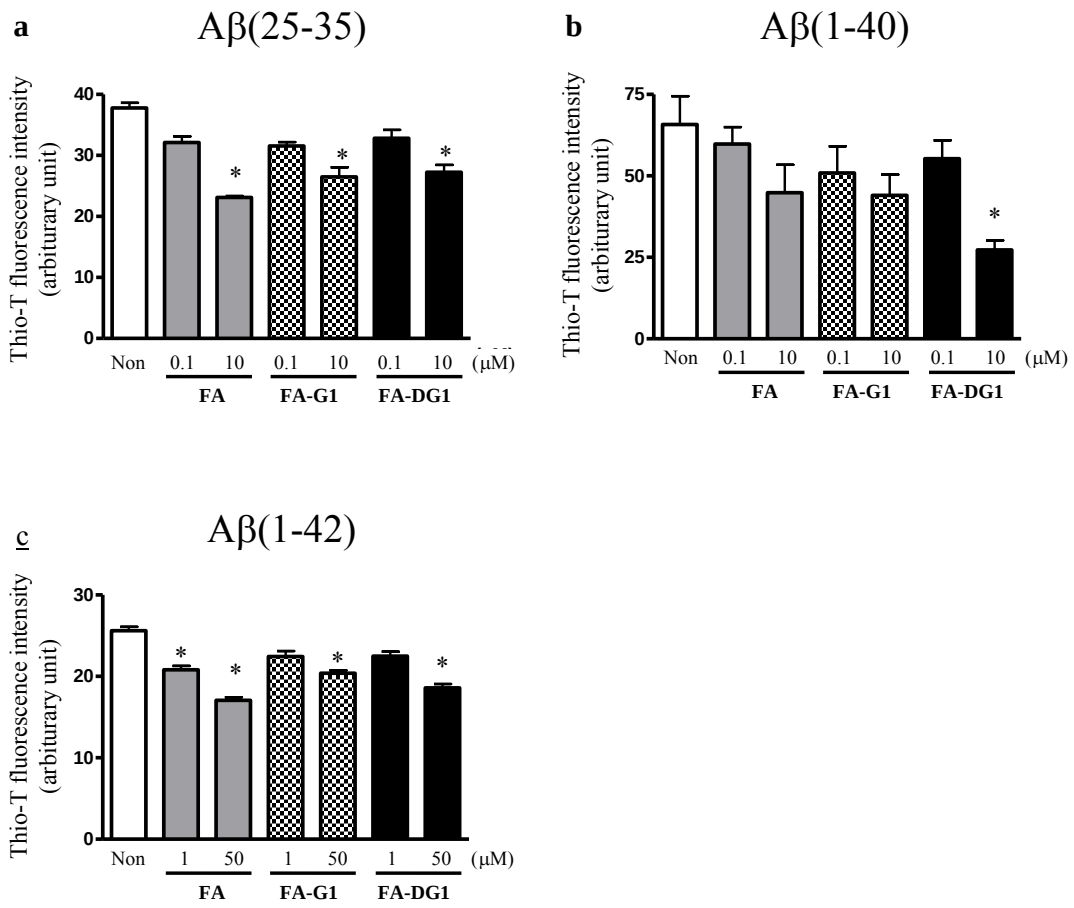

Fig. 3-1. Effects of FA and water-soluble FA derivatives on Aβ aggregation.

The reaction mixtures containing 200 μM fresh Aβ (25–35) (a), 25 μM Aβ (1–40) (b), or 25 μM Aβ (1–42) (c) were incubated at 37°C in the presence or absence of FA, FA-G1, or FA-DG1. Aβ aggregation was determined by thioflavin-T fluorescence assay, as described in Materials and Methods. Data for all experiments are represented as means ± S.E. of values from three independent assays in triplicate experiments. One-way ANOVA with the Newman-Kleus post-hoc test was used for statistical analysis. **P* < 0.05 vs. values for control.

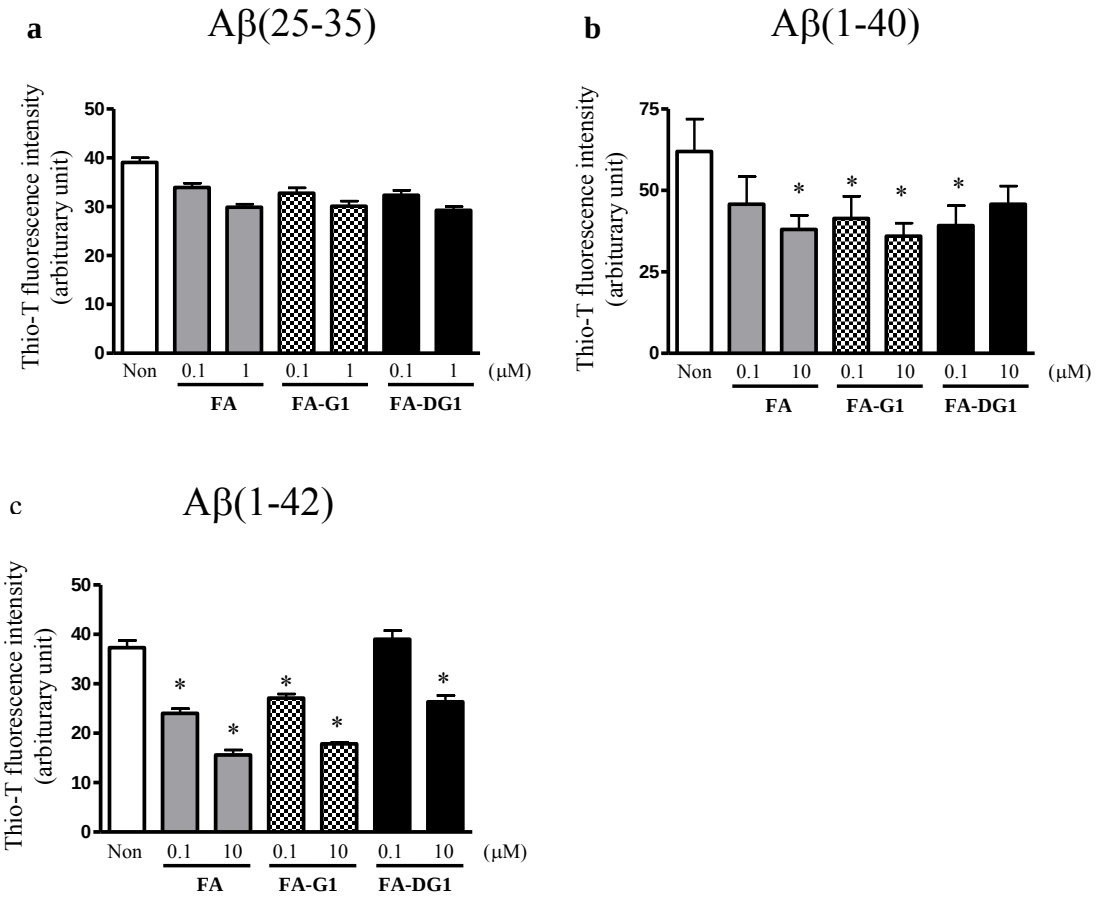

Fig. 3-2. Effects of FA and water-soluble FA derivatives on destabilization of aggregated Aβ.

The reaction mixtures containing 400 μM aggregated Aβ (25–35) (a), 100 μM aggregated Aβ (1–40) (b), or 50 μM aggregated Aβ (1–42) (c) were incubated at 37°C with or without FA, FA-G1, or FA-DG1. Aβ aggregation was determined by thioflavin-T fluorescence assay as described in Materials and Methods. Data for all experiments are represented as means ± S.E. of values from three independent assays in triplicate experiments. One-way ANOVA with the Newman-Kleus post-hoc test was used for statistical analysis. **P* < 0.05 vs. values for control.

Effects of water-soluble FA derivatives on Aβ-induced cell death in cultured neurons or PC12 cells

Previous reports have indicated that EFA, whose structure is similar to FA, inhibited Aβ-induced cytotoxicity (Sultana et al 2005). Here, I examined whether water-soluble FA derivatives have an inhibitory effects on Aβ (25–35)-induced cytotoxicity in neuronal cells. PC12 cells are rat pheochromocytoma cells that are a well-known model for studying neuronal signaling pathways and other neurobiochemical events (Fujita et al. 1989). Many neurobiological investigations were studied using PC12 cells rather than primary neurons. As shown in Fig. 3-3, aggregated-Aβ (25–35) induced cytotoxicity in primary cerebral cortical neuron cultures and PC12 cells, whereas pretreatment of Aβ (25–35) with FA significantly inhibited this cytotoxicity.

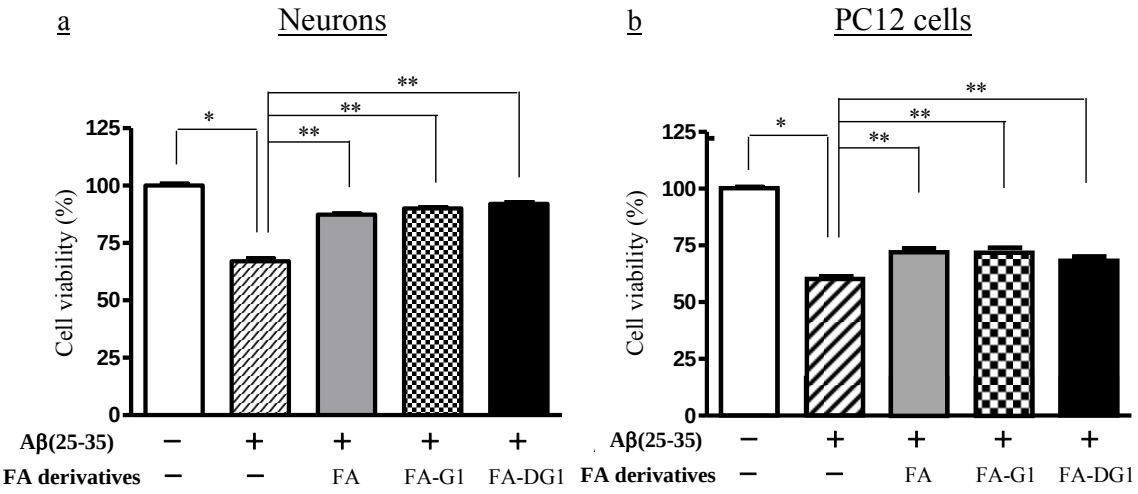

Fig. 3-3. Inhibitory effects of FA and water-soluble FA derivatives on Aβ-induced neuronal cell death in cultured neurons or PC12 cells.

Aβ (25–35) (2 mM) was aggregated in the presence or absence of 2 mM FA, FA-G1, or FA-DG1 for 3 days. Primary cortical neurons (7 DIV) (a) or PC12 cells (b) were treated with reaction mixtures for 24 h at a final concentration of 20 μM Aβ (25–35). Cell viability was assessed by MTT assay as described in Materials and Methods. Data are represented as means ± S. E. of values from triplicate experiments. One-way ANOVA with the Newman-Kleus post-hoc test was used for statistical analysis. $*P < 0.05$ vs. values for control, $**P < 0.05$ vs. values for Aβ (25–35) alone.

Similarly, pre-incubation of Aβ (25–35) with FA-G1 or FA-DG1 also inhibited Aβ (25–35)-induced neuronal cell death. These results indicate that water-soluble FA derivatives, in addition FA, have a neuroprotective effect against Aβ-induced neuronal cell death.

Effects of water-soluble FA derivatives on Aβ-induced dysmnesia

Previous *in vivo* studies have demonstrated that long-term administration of FA protected mice against Aβ-induced learning and memory deficits (Yan et al. 2001). Here, I examined whether water-soluble FA derivatives improve Aβ-induced dysmnesia using a contextual fear conditioning test. No significant change in body-weight was observed in FA, FA-G1, and FA-DG1-injected mice (Table 3-1). After administration of FA, FA-G1, or FA-DG1 for 42 days, followed by injection of Aβ (25–35), a fear-conditioning test was performed (Fig. 3-4a).

Table 3-1. Effects of FA and water-soluble FA derivatives administration on the body weight of mice.

Experimental group	Initial (g)	Final (g)
0.5% CMC + UPW	19.7±0.8	23.5±0.9
0.5% CMC + Ab(25–35)	16.3±0.9	23.6±0.8
FA + Ab(25–35)	19.5±0.7	25.1±0.7
FA-G1 + Ab(25–35)	16.7±0.9	24.6±0.6
FA-DG1 + Ab(25–35)	17.2±0.9	22.7±0.9

After orally administered FA or water-soluble FA derivatives (0.1 μmol/g/day) for 42 days, mice were injected with Aβ (25–35) as described in Fig. 3-4a. Body weight was measured before the administration (initial) and after the injection (final). Data are means ± S.E. (n = 6). UPW = ultra-pure water

Contextual-dependent tests were performed 24 h after conditioning. Aβ (25–35)-injected mice exhibited a lower contextual-dependent freezing response than distilled water-injected mice, indicating an impairment of associative memory (Fig. 3-4b). FA-

G1 and FA-DG1 as well as FA attenuated the Aβ (25–35)-induced impairment of contextual freezing responses. However, there were no significicant differences between FA and its derivatives. These results suggest that water-soluble FA derivatives, in addition to FA, improve contextual freezing response impairment in mice injected with Aβ (25–35).

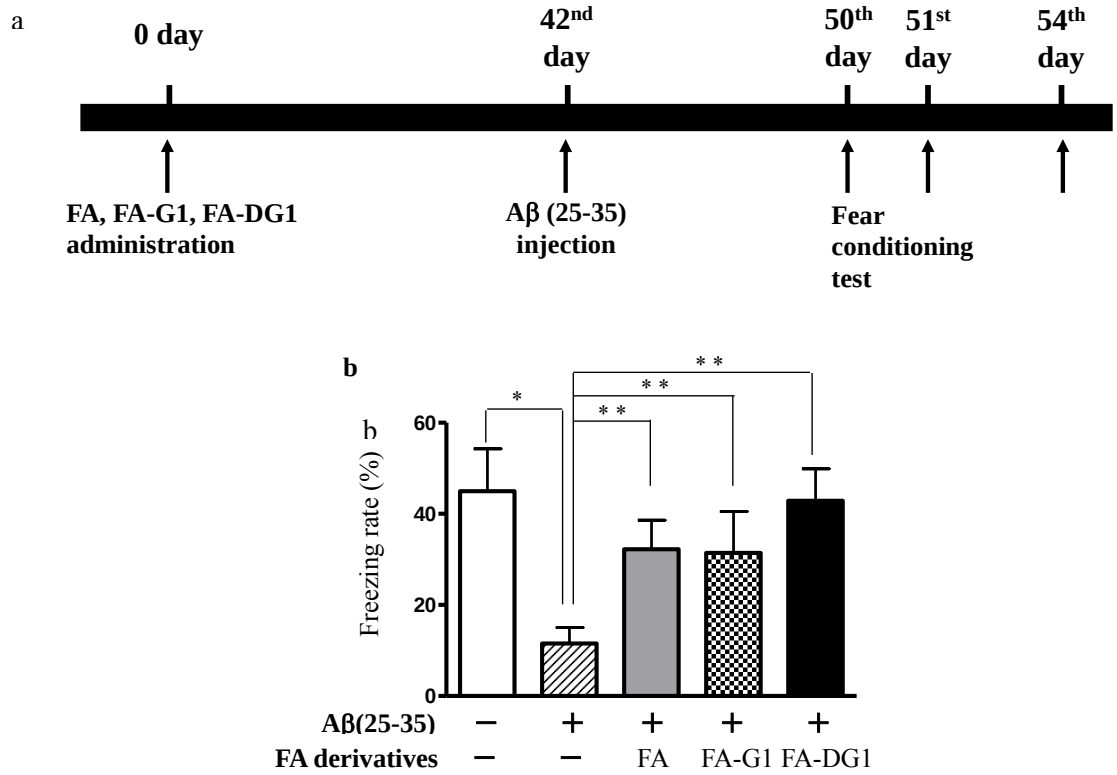

Fig. 3-4. Effects of FA and water-soluble FA derivatives on cognitive functions in Aβ (25–35)-injected mice.

(a) Behavioral experiments schedule. After orally administration of FA or water-soluble FA derivatives (0.1 μmol/g/day) for 42 days, mice were injected with Aβ (25–35). Each behavioral test was conducted on the indicated time point. (b) The freezing rate in fear conditioning test was measured. Data for all experiments are represented as means ± S.E. One-way ANOVA with the Newman-Kleus post-hoc test was used for statistical analysis. **P* < 0.05 vs. 0.5% CMC-administrated, vehicle-injected mice. ***P* < 0.05 vs. 0.5% CMC-administrated, Aβ (25–35)-injected mice.

In vivo effects of water-soluble FA derivatives on Aβ-induced neuronal dysfunction

In order to examine the protective effects of FA and water-soluble FA derivatives on Aβ-induced neuronal cell death *in vivo*, Nissl staining was performed in the hippocampus of mice treated with Aβ (25–35). As shown in Figs. 3-5a and b, the number of Nissl-positive neuronal cells in the hippocampus decreased in mice treated with Aβ (25–35). Positive staining was recovered when mice were pretreated with FA. In addition, FA-G1 and FA-DG1 dramatically suppressed Aβ (25–35)-mediated neuronal cell death. These results demonstrate that FA and water-soluble FA derivatives protect neuronal cells against Aβ (25–35)-induced neurotoxicity *in vivo*. The *in vivo* neuroprotective effects were consistent with the results from *in vitro* experiments using culture neuron (Fig. 3-3).

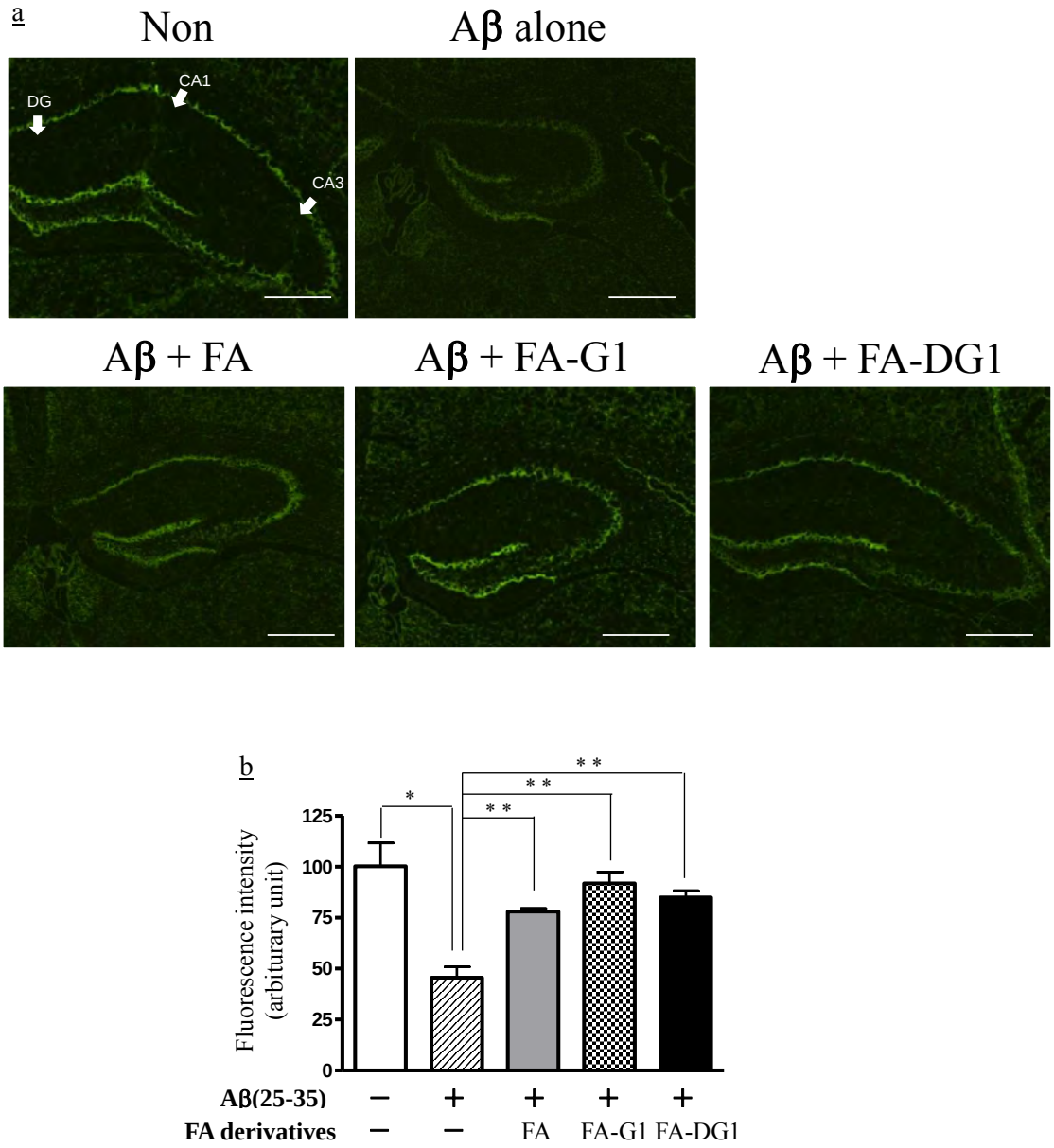

Fig. 3-5. Effects of FA and water-soluble FA derivatives on the nerve function in Aβ (25–35)-injected mice.

After each behavioral test described in Fig. 3-4a, the brain was harvested and fixed in 4% paraformaldehyde, followed by cryoprotection in 30% sucrose. Serial coronal sections were stained with NeuroTrace 500/525 green-fluorescent Nissl stain, according to manufacturer's instructions. (a) Fluorescence microscopic observation of Nissl stain in Aβ (25–35)-injected mice. Cornu Ammonis 1 (CA1), CA3, and Dentate Gyrus (DG) are indicated by arrows, respectively. Scale bars, 100 μm. (b) Fluorescence intensity of Nissl stain in Aβ (25–35)-injected mice. Data are represented as means ± S.E. of

values from triplicate experiments. One-way ANOVA with the Newman-Kleus post-hoc test was used for statistical analysis. *$P < 0.05$ vs. 0.5% CMC-administrated, vehicle-injected mice. **$P < 0.05$ vs. 0.5% CMC-administrated, Aβ (25–35)-injected mice.

Discussion

FA exerts neuroprotective effects against Aβ-induced neurotoxicity *in vitro* (Sultana et al. 2005) and *in vivo* (Yan et al. 2001; Kim et al. 2004). In chapter 3, I compared the neuroprotective effects of two water-soluble FA derivatives, FA-G1 and FA-DG1, with that of FA *in vitro* and *in vivo.*

Aβ is a 40-42 amino acid peptide derived from the cleavage of the membrane protein APP by β- and γ-secretases. The overproduction and aberrant self-assembly of Aβ into fibrillar aggregates constitutes the first steps of the so-called amyloid cascade hypothesis that is thought to trigger AD. In AD, neurodegeneration may be caused by Aβ aggregation in senile plaques in the brain, and this is generally regarded as a critical pathogenic event in AD (Selkoe 2004; Lleo et al. 2006). Aβ is also found in the brain in healthy individuals; it is a secondary product of APP metabolism and may have some physiological function. However, Aβ is generally degraded and discharged in healthy individuals, resulting in little Aβ deposition. Aβ accumulation likely stems from an abnormality in metabolism, possibly as the result of aging, where an imbalance occurs between the production and degradation of Aβ.

The mechanism by which Aβ becomes aggregated is complex and is composed of several steps. These aggregates are characterized by having transitional aggregation species as initial seeds, soluble small oligomers, protofibrils, and insoluble amyloid fibrils, with a β-sheet conformation. Ono et al. (2005) demonstrated that FA inhibited Aβ aggregation *in vitro*. Hence, I first confirmed whether water-soluble FA derivatives also show this beneficial effect despite their structural modifications. Water-soluble FA derivatives not only prevented Aβ aggregation, but also destabilized aggregated Aβ fibrils. Therefore, differences in the chemical structures of these derivatives did not affect their

disaggregation activities (Fig. 3-1 and 3-2). This suggests that water-soluble FA derivatives could be protective against Aβ-induced neurotoxicity. Indeed, I show evidence that the derivatives prevented Aβ-induced cytotoxicity in cultured neuronal cells (Fig. 3-3).

FA is also a well-known antioxidant. Since Aβ-induced neurotoxicity is at least partially due to oxidative stress, FA may exert its neuroprotective effect through its antioxidant activity, besides its inhibitory effect on Aβ aggregation. Sultana et al. characterized the protective effect of EFA, which is more hydrophobic than FA, on Aβ-induced oxidative stress in rat primary neuronal cell cultures (Sultana et al. 2005). Indeed, the water-soluble FA derivatives showed similar antioxidant activity compared to FA, suggesting that the antioxidant activity of these derivatives may also be involved in their neuroprotective effect. It is reported that FA binds Aβ and induces cooperative rupture of the backbone hydrogen bonds and of the Asp23-Lys28 salt bridge (Sgarbossa et al. 2013). Cui et al. (2013) used thioflavin T fluorescence, CD spectroscopy, and transmission electron microscopy and showed that FA inhibited the Aβ aggregation process by blocking the formation of β-sheets, interacting with Aβ by hydrogen bonding with His14 and Glu22. Thus, it may be reasonable to speculate that water-soluble FA derivatives could prevent AD development by directly inhibiting Aβ aggregation in the brain rather than scavenging reactive oxygen species. As shown previously, intracerebroventricular administration of Aβ increases Aβ deposits, decreases choline acetyltransferase activity, and causes a marked memory deficit in mice, whereas long-term oral administration of FA significantly reverses neuroinflammation in mouse hippocampus and improves memory loss (Yan et al. 2001; Kim et al. 2010). In this study, I also evaluated the neuroprotective effects of water-soluble FA derivatives *in vivo* by contextual fear conditioning test and measuring neuronal cell death in the hippocampus. Our results showed that oral administration of FA and water-soluble FA derivatives improved Aβ-induced dysmnesia in parallel with exerting neuroprotective effects in the hippocampus. Previous study shows that FA is cell-permeable and is absorbed via stomach mucosal cells (Mori et al. 2013). FA is also reported to reach the brain and protect neurons against Aβ-induced oxidative stress and neurotoxicity (Sultana

et al. 2005; Qin et al. 2007). These observations suggest that water-soluble FA derivatives could also reach the hippocampus, leading to neuroprotection against Aβ-induced cytotoxicity.

Polyphenols from green tea and grapes protected primary rat cortical neurons against Aβ-induced cytotoxicity and improved cognitive functions in a mouse model of AD (Wang et al. 2012; Qin et al. 2012). The ability of plant-derived polyphenols to reduce Aβ toxicity suggests their therapeutic utility for age-related disorders such as AD and dementia. In the present study, FA derivatives were orally administered to mice with the dose level of 0.1 μmol/g body weight/day, which is equivalent to about 1 g/day in humans. It is difficult to ingest this quantity of FA or the derivatives from foods in daily life. However, it is possible to take in large amounts of FA derivatives as supplements, because they have high solubilities in water. It is worth noting that I did not detect any adverse events including occurrence of atypical behavior, altered food/water intake, or mortality associated with long-term administration in mice treated with FA derivatives (data not shown). Moreover, no evidence of pathological changes in body weight was observed (Table 3-1). These findings support the notion that the dose of FA derivatives is safe, although toxicology analyses in humans should be conducted. Collectively, our results provide insights on dietary polyphenolic components that may benefit brain functions and clarify some mechanisms underlying neurodegenerative disorders.

Conclusion

Because the elderly population is increasing worldwide, including in Japan, AD is quickly becoming a major universal healthcare problem. Thus, I evaluated FA because of its many biological activities such as its ability to improve AD symptoms. However, its insolubility in water and oil limits its application in the food, health, cosmetic, and pharmaceutical industries. The aim of this study was to develop FA derivatives with high water solubility. In chapter 1, I succeeded in synthesizing highly water-soluble feruloyl diglycerols by esterification using an *A. niger* feruloyl esterase. No other feruloylated compound has been reported to have such high water solubility. Next, I examined the neuroprotective effects of these water-soluble FA derivatives *in vitro* and *in vivo*. In chapter 2, I found that FA and its derivatives inhibited NO production and iNOS expression in primary astrocytes. Furthermore, inhibition of iNOS expression was mediated by suppression of the NF-κB pathway. In chapter 3, I found that FA and its derivatives inhibited Aβ aggregation, Aβ-induced neurotoxicity in cortical neurons *in vitro*, and Aβ-induced learning and memory deficits i*n vivo*. Figure 3 illustrates some of the possible mechanisms by which water-soluble FA may improve the AD phenotype. Although FA has been expected to be an effective and naturally occurring neuroprotective agent, its use for a wide variety of applications is limited owing to its poor water solubility.

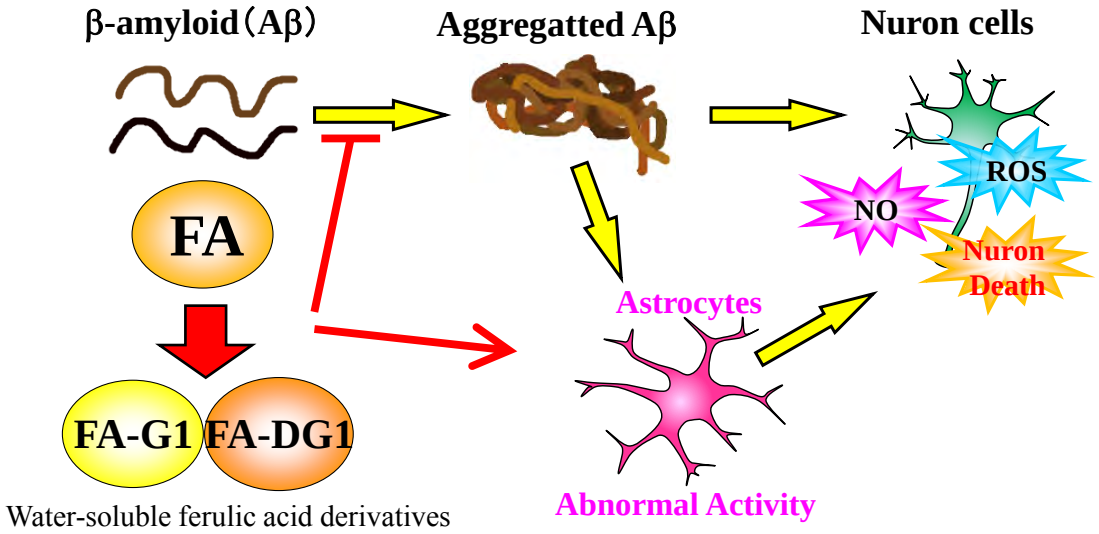

Fig. 3. Improvement mechanisms for AD of water-soluble FA derivatives

This study indicates that water-soluble FA derivatives not only overcome this problem but also exhibit the same neuroprotective effects as observed with FA, and thus, they may be more effective and useful molecules for development of AD therapeutics. I thought that one of the ways to prevent AD was to consume phytochemical compounds like FA, which occur naturally and are not expensive, as it is now which has only basic remedy of AD. Additionally, the water-soluble FA derivatives might have novel applications in the food, beverage, and cosmetics industries. I am presently attempting to optimize the dose of these FA derivatives for their practical use.

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