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Inhibition of Df-Protease Associated with Allergic Diseases by Polyphenol

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It was reported that Df-protease from house dust mite (*Dermatophagoides farinae*) catalyzes the activation of the kallikrein−kinin system in human plasma and is closely associated with mite-induced allergy. Therefore, to prevent the release of kinin by Df-protease, the inhibitory activity of polyphenols including catechins and flavonols was tested in vitro and in vivo. Among them, myricetin and epigallocatechin gallate (EGCg) effectively inhibited the amidase activity of Df-protease with K_i values of 1×10^{-8} and 6×10^{-4} M, respectively. The kinin release in human plasma was extensively inhibited by the addition of EGCg in comparison with myricetin. Enhancement of vascular permeability in guinea pigs caused by Df-protease was markedly suppressed by EGCg.

Keywords: *Mite protease; kallikrein−kinin system; catechin; allergic disease; protease inhibitor; epigallocatechin gallate*

INTRODUCTION

During the course of investigations on allergy induced by house dust, Takahashi et al. (1990) isolated a serine protease, Df-protease, in a homogeneous state from mite (*Dermatophagoides farinae*), which has the molecular weight of 30000 and a substrate specificity similar to blood coagulation factor XIIa. Therefore, Df-protease catalyzed not only the activation of blood coagulation cascade (Matsushima et al., 1993a) together with fibrinolytic system (Matsushima et al., 1993b) but also the kallikrein−kinin system (Takahashi et al., 1990). Df-Protease gave rise to the activation of a kinin-generating cascade composed of the Hageman factor, prekallikrein, and high molecular weight kininogen (Maruo et al., 1991) followed by the rhythmic contraction of rat uterine horns (Kohmoto et al., 1991). For the purpose of treatment of mite-induced inflammation, it was reported that Df-protease is inhibited by synthetic amidine and guanidine derivatives with K_i values ranging between 10^{-6} and 10^{-9} M which suppress the enhancement of vascular permeability in guinea pig skin caused by kinin release (Matsushima et al., 1992; Noguchi et al., 1995).

The present paper deals with the inhibition of Df-protease-induced kinin release by polyphenol including catechin such as epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECg), and epigallocatechin gallate (EGCg) with its derivatives and flavonol such as myricetin, quercetin, rutin, and kaempferol, in vitro and in vivo. This study may lead to the treatment of mite-induced allergy-like disease.

MATERIALS AND METHODS

EC, EGC, ECg, and EGCg, were purchased from Kurita Kogyo Co. (Tokyo, Japan). Epigallocatechin gallate monoglucopyranoside (EGCgG-1) and epigallocatechin gallate diglucopyranoside (EGCgG-2) were gifts from Kikkoman Corp. (Chiba, Japan). Quercetin and kaempferol were purchased from Extrasynthese S.A. (Genay, France). Rutin and myricetin were purchased from Kanto Chemical Co. (Tokyo, Japan) and Sigma Chemical Co. (St. Louis, MO), respectively. The syn-

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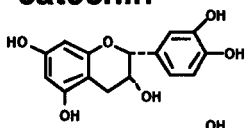
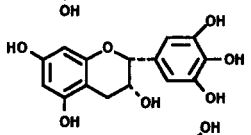
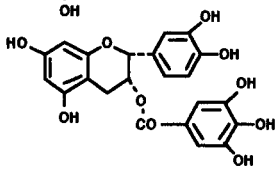
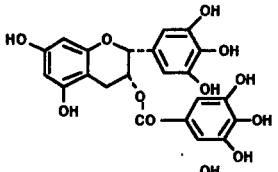
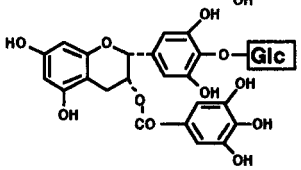
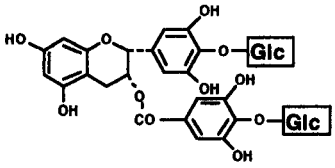
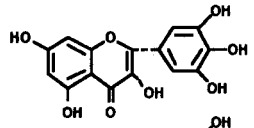
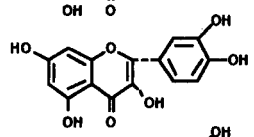
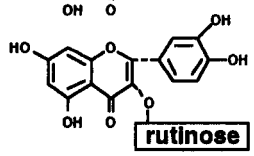
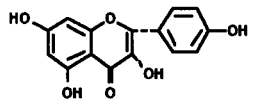
Table 1. Inhibition of Df-Protease Associated with Allergy by Polyphenol[a]

polyphenol		Ki (M)
catechin		
	(-)-epicatechin (EC)	4×10^{-3}
	(-)-epigallocatechin (EGC)	2×10^{-3}
	(-)-epicatechin gallate (ECg)	9×10^{-4}
	(-)-epigallocatechin gallate (EGCg)	6×10^{-4}
	(-)-epigallocatechin gallate monoglucopyranoside (EGCgG-1)	9×10^{-4}
	(-)-epigallocatechin gallate diglucopyranoside (EGCgG-2)	2×10^{-3}
flavonol		
	myricetin	1×10^{-8} 2×10^{-8}*
	quercetin	1×10^{-7}*
	rutin	5×10^{-6}*
	kaempferol	9×10^{-6}*

[a] (*) K_i value obtained by using benzoyl-L-arginine-*p*-nitroanilide as a substrate in place of Boc-Gin-Gly-Arg-MCA.

thetic substrate for blood coagulation factor XIIa, *tert*-butyloxycarbonyl-L-glutaminylglycyl-L-arginine 4-methylcoumaryl-7-amide (Boc-Gln-Gly-Arg-MCA), was purchased from Peptide Institute (Osaka, Japan). The kinin determination kit, MARKIT-A bradykinin, was purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Protease inhibitor of *N*-allyl-*N*-[4-(4-amidinophenoxycarbonyl)-α-methylcinnamoyl]-glycine mesylate (FO-349) was donated from Ono Pharmaceutical Co. Ltd. (Osaka, Japan). Normal human plasma was prepared from citrated blood of volunteers.

The protease from mite (*D. farinae*), Df-protease, was purified according to the method described previously, which is a serine protease with the molecular weight of 30000 (Takahashi et al., 1990). Its enzymic activity was determined using a synthetic substrate of blood coagulation factor XIIa, Boc-Gln-Gly-Arg-MCA. The amount of 7-amino-4-methylcoumarin (AMC) released by the hydrolysis of the substrate was detected with excitation at 380 nm and emission at 460 nm with a Hitachi F-3000 fluorescence spectrophotometer (Tokyo, Japan) in the presence and absence of inhibitors (Noguchi et

al., 1995; Matsushima et al., 1994) according to the modified method of Kawabata et al. (1988). The kinetic parameter, K_i value, was obtained from Lineweaver−Burk plots.

Release of kinin in human plasma induced by Df-protease was tested by using the method described previously (Noguchi et al., 1995). To normal human plasma (30 mL) was added 245 mL of 0.2% EDTA containing 0.7% NaCl, and the mixture was kept standing at 37 °C for 10 min. Then, to the EDTA-treated plasma (275 mL) was added 5 mL of Df-protease (9×10^{-6} M) in the presence and absence of 20 mL of EGCg and myricetin. The sample was incubated at 37 °C for 40 min followed by boiling for 5 min, and the amount of kinin in plasma was determined using MARKIT-A bradykinin.

The enhancement of vascular permeability in guinea pigs induced by injecting Df-protease was tested in the presence and absence of inhibitors (Matsushima et al., 1994) as follows: immediately after the intravenous injection of 1% Evans blue in saline (1.0 mL) to a guinea pig (250−300 g), a mixture (100 mL) of Df-protease (0.03 nmol) and EGCg (0−1.0 mmol) was injected intracutaneously. After 30 min, vascular permeability of the guinea pig was observed as the blue permeation spot on the inside surface of its skin.

RESULTS AND DISCUSSION

With the purpose of the treatment of mite allergy, inhibition of Df-protease with synthetic inhibitors has been tested; among the inhibitors including amidine and guanidine derivatives, *N*-allyl-*N*-[4-(4-amidinophenoxycarbonyl)-α-methylcinnamoyl]glycine mesylate (FO-349) effectively inhibited Df-protease with $K_i = 1 \times 10^{-8}$ M (Noguchi et al., 1995). Furthermore, in vivo effectiveness of the inhibitor was preliminarily checked: to dogs suffering from atopic dermatitis caused by mites was given orally FO-349 (10−30 mg/kg) twice a day for 2 weeks. Seven of the total 10 dogs showed effective improvement of their symptoms, but three generated side effects in the digestive system to some extent (T. Iwasaki, personal communication, 1998).

With this circumstantial evidence, the next experiment was focused on polyphenol, in place of synthetic inhibitors, which inhibits Df-protease activity. Because Df-protease has high enzymic activity toward the synthetic substrate of blood coagulation factor XIIa, Boc-Gln-Gly-Arg-MCA, the inhibitory effect of various kinds of polyphenols such as catechin and flavonol on the enzymic activity of Df-protease was tested. Catechin and flavonol used in this study were found to be noncompetitive inhibitors by the kinetic study on Lineweaver−Burk plots. Table 1 shows the chemical formulas of the polyphenols and their K_i values ranging between 10^{-3} and 10^{-8} M. EGCg in catechin and myricetin in flavonol were the most effective inhibitor toward Df-protease activity in comparison with others. Masking of the *p*-hydroxyl group in catechin with saccharide seems to increase the K_i value, as shown in Table 1.

To test whether polyphenols such as EGCg and myricetin inhibits kinin-release in normal human plasma by Df-protease, the amount of kinin released from the human plasma was measured by adding various amounts of polyphenol. The results are shown in Figure 1. The amount of kinin in human plasma induced by the catalytic action of Df-protease is markedly suppressed by increasing the amount EGCg. At 0.2 mM EGCg, kinin release is suppressed by 90%, which is shown by curve B in Figure 1. Although myricetin had $K_i = 1 \times 10^{-8}$ M, the amount of kinin released from kininogen in the presence of Df-protease was not suppressed by myricetin (curve A). The inhibitory effect of myricetin on kinin release may be due to the disturbance with a protein in human plasma. In fact, the inhibitory effect of myricetin using the synthetic substrate was lost by addition of bovine serum albumin (10 mg/mL).

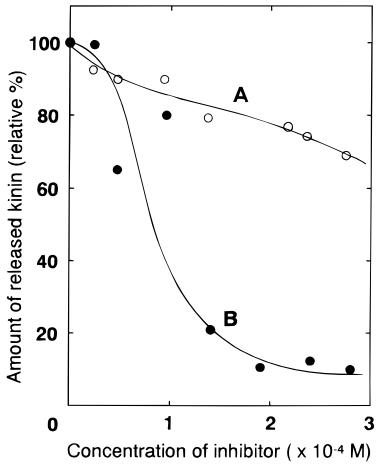


Figure 1. Inhibition of Df-protease-induced kinin release from human plasma by EGCg and myricetin: (A) myricetin; (B) EGCg. Df-protease concentration = 0.1 μM.

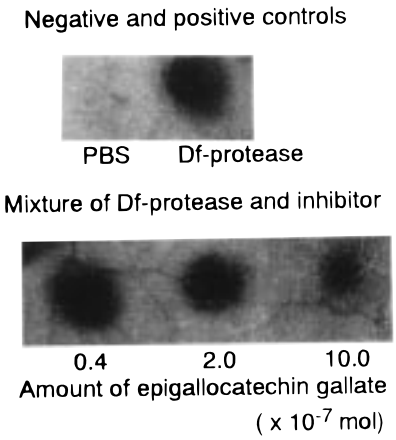


Figure 2. Suppression of vascular permeability in a guinea pig skin induced by Df-protease with EGCg: (top) Evans blue permeation spot induced by injecting PBS or Df-protease (0.03 nmol) as a negative or a positive control, respectively; (bottom) that by a mixture of Df-protease (0.03 nmol) and EGCg (0.4, 2.0 or 10.0×10^{-7} mol).

To test whether the production of kinin induced by Df-protease is suppressed in vivo with the inhibitors mentioned above, the following experiment was conducted using guinea pigs. Figure 2 shows the extravasation of Evans blue spots on guinea pig skin caused by activation of the kallikrein−kinin system with Df-protease, in the presence and absence of inhibitors. In the absence of catechins, intracutaneous injection of Df-protease greatly enhanced vascular permeability as assessed by intradermal dye leakage, which was the same result obtained previously (Matsushima et al., 1994). On the other hand, vascular permeability was decreased in a dose-dependent manner by the intracutaneous injection of a mixture of Df-protease and various amounts of EGCg. This suggests that EGCg has an inhibitory effect of kinin release in blood induced by Df-protease.

From the results obtained above, it can be concluded that EGCg among catechins and flavonols is the most effective in inhibiting kinin release in blood by Df-protease in mite. EGCg is contained in green tea plant; levels of 8.6 and 12.1 g/100 g of dry fresh leaves are

found in *Camellia cinensis* var. *sinensis* and var. *assamics*, respectively (Yamamoto et al., 1997). The physiological function of EGCg was reported in relation to inhibition in adherence of various bacteria onto oral epithelia cells and also to inhibition in propagation of rotavirus cultured in monkey kidney (Yamamoto et al., 1997).

ABBREVIATIONS USED

Df-protease, a serine protease from house dust mite (*Dermatophagoides farinae*); EC, epicatechin; EGC, epigallocatechin; ECg, epicatechin gallate; EGCg, epigallocatechin gallate; EGCgG-1, epigallocatechin gallate monoglucopyranoside; EGCgG-2, epigallocatechin gallate diglucopyranoside; FO-349, *N*-allyl-*N*-[4-(4-amidinophenoxycarbonyl)-α-methylcinnamoyl]glycine mesylate; PBS, phosphate-buffered saline.

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