Pergamon

PII: S0031-9422(97)00754-1

Phytochemistry, Vol. 47, No. 7, pp. 1215–1218, 1998
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Printed in Great Britain
0031-9422/98 $19.00+0.00

INHIBITION *IN VITRO* LINOLEIC ACID PEROXIDATION AND HAEMOLYSIS BY CAFFEOYLTRYPTOPHAN

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(*Received in revised form* 28 *July* 1997)

Key Word Index—Caffeoyltryptophan; coffee; free radical; haemolysis; peroxidation.

Abstract—Antioxidant activities of caffeoyltryptophan were investigated by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging system, the superoxide anion generation system and the superoxide anion-mediated linoleic acid peroxidation system. At 10 μM, caffeoyltryptophan showed greater scavenging activity on DPPH than *dl*-α-tocopherol or ascorbic acid. DPPH radical scavenging activity of caffeoyltryptophan increased dose-dependently at concentrations ranging from 1 to 50 μM; 1 mol of caffeoyltryptophan reacted with *ca* 4 mol of radical.

Caffeoyltryptophan caused 80% inhibition of superoxide anion generation at 50 μM. The inhibitory activity of caffeoyltryptophan was as strong as that of 5-caffeoylquinic acid. Caffeoyltryptophan inhibited the formation of conjugated diene from linoleic acid. The inhibitory activity increased in the order caffeic acid < 5-caffeoylquinic acid < caffeoyltryptophan < *dl*-α-tocopherol.

Effects on the *in vitro* haemolysis and peroxidation of mouse erythrocytes induced by H_2O_2 were also examined. Caffeoyltryptophan exhibited strong inhibitory activities; Tryptophan was ineffective in these systems. These data suggest that caffeoyltryptophan may be a natural antioxidant in the human diet and, as such, may intervene in toxicological processes that are mediated by radical mechanisms. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Caffeoyltryptophan (Fig. 1) has been isolated and identified from unroasted robusta coffee beans [1]. It is contained in robusta green coffee beans at about 110 mg per 100 g [2]. However, little is known of the biological activity of caffeoyltryptophan. Since caffeoyltryptophan contains the dihydroxyphenol group, it is assumed that caffeoyltryptophan may exhibit some biological activities in oxido-reduction.

In the previous paper, we reported antioxidant activities of 5-caffeoylquinic acid which were hydroxycinnamic acid derivatives [3]. 3,5-Dicaffeoylquinic acid which contained two dihydroxy functions per molecule was the most antioxidant activity among the other catechols tested.

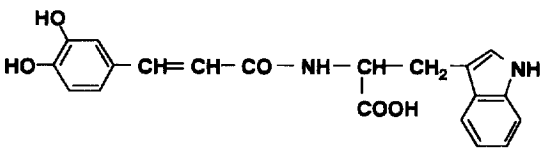

Fig. 1. Structure of caffeoyltryptophan.

In the present paper, we report the radical scavenging activity of caffeoyltryptophan, its inhibitory effect on superoxide anion generation, its inhibitory activity on linoleic acid peroxidation, and its effect on the haemolysis and peroxidation of mouse erythrocytes *in vitro* induced by H_2O_2. Therefore, the objective of this study was to investigate the biological property of caffeoyltryptophan.

RESULTS AND DISCUSSION

Scavenging activity on DPPH radicals

Caffeoyltryptophan exhibited scavenging activity on the DPPH radical. Its activity increased dose-dependently at concentrations ranging from 1 to 50 μM. Its activity was similar to those of caffeic acid and 5-caffeoylquinic acid (Fig. 2). Since 1 mol of caffeic acid or 5-caffeoylquinic acid reacted with *ca* 4 mol of radical in preliminary reports [3, 4], 1 mol of caffeoyltryptophan was trapped with *ca* 4 mol of radical (Table 1). It was stronger than that of *dl*-α-tocopherol or ascorbic acid (Fig. 2 and Table 1).

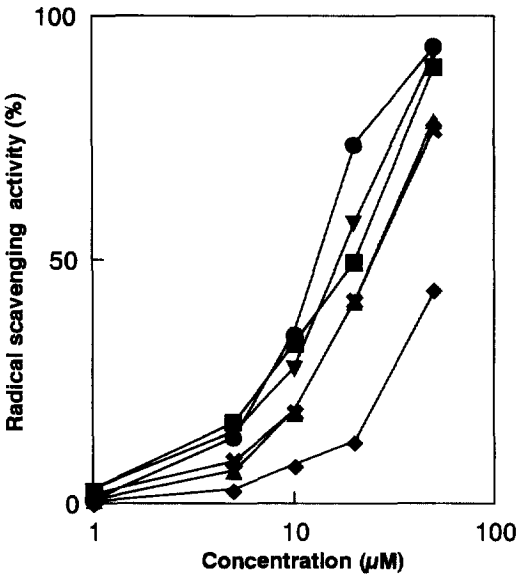

Fig. 2. Radical scavenging activities of caffeoyltryptophan and related compounds on DPPH. The scavenging activity was measured as the decrease in absorbance of the DPPH radical expressed as a percentage of the absorbance of a control DPPH radical solution without test substance. Symbols used in the panel were as follows: ●, caffeic acid; ■, caffeoyltryptophan; ▼, 5-caffeoylquinic acid; ▲, *dl*-α-tocopherol; ×, ascorbic acid; ◆, cysteine.

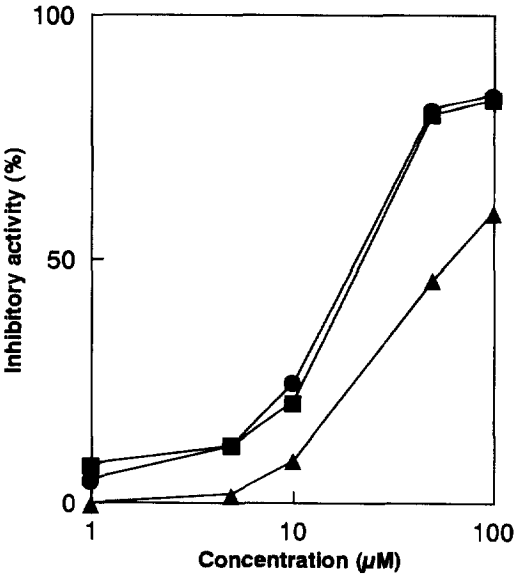

Fig. 3. Inhibitory activity of caffeoyltryptophan and related compounds on superoxide anion generation by xanthine-XOD system. Symbols used the in panel were as follows: ■, caffeoyltryptophan; ▼, 5-caffeoylquinic acid; ▲, ascorbic acid.

Table 1. Scavenging activities against DPPH radical relative to the activity of cysteine

Addition (10 μM)	Relative ratio
Cysteine	1.0
Caffeoyltryptophan	4.5
Caffeic acid	4.4
5-Caffeoylquinic acid	3.5
Ascorbic acid	2.4
dl-α-Tocopherol	2.4

The data of Fig. 2 is represented as a ratio of scavenging activity, compared with the activity of cysteine [22].

Tryptophan showed no reaction with the DPPH radical.

Inhibitory effect on superoxide anion generation

Caffeoyltryptophan inhibited the generation of superoxide anion from the xanthine-xanthine oxidase (XOD) system in a concentration dependent manner. At 50 μM, caffeoyltryptophan caused 80% inhibition of superoxide anion generation. Scavenging activity of caffeoyltryptophan was as effective as that of 5-caffeoylquinic acid, and stronger than that of ascorbic acid (Fig. 3). Tryptophan showed no inhibitory effect.

The inhibitory effect of caffeoyltryptophan on the generation of superoxide anion showed a good correlation with its scavenging activity on the DPPH radical. Thus, it may be concluded that the inhibition of the generation of superoxide anion by caffeoyltryptophan is due to its radical scavenging activity, and not due to its inhibitory activity upon the enzyme.

Antioxidant activity on linoleic acid peroxidation

Caffeoyltryptophan inhibited the formation of conjugated diene in the early stage of linoleic acid peroxidation. Its activity increased dose-dependently at concentrations ranging from 1 to 50 μM. Tryptophan showed no inhibitory activity. The inhibitory activity of caffeoyltryptophan was stronger than that of caffeic acid or 5-caffeoylquinic acid, and weaker than that of *dl*-α-tocopherol (Fig. 4). The greater inhibitory power of caffeoyltryptophan compared with caffeic acid or 5-caffeoylquinic acid is interesting.

It is known that caffeic acid and 5-caffeoylquinic acid exhibit higher stoichiometric numbers and reactivity with peroxyl radical. It is conceivable to be the cause of these activities that these compounds possess in their structure an *o*-dihydroxy group which is the putative radical target site [4–6]. Accordingly, since caffeoyltryptophan contains an *o*-dihydroxy group, it might strongly inhibit linoleic acid peroxidation by scavenging superoxide anion or peroxyl radicals.

Inhibition of haemolysis and lipid peroxidation of erythrocytes

Caffeoyltryptophan exhibited inhibitory activities on haemolysis and lipid peroxidation of mouse eryth-

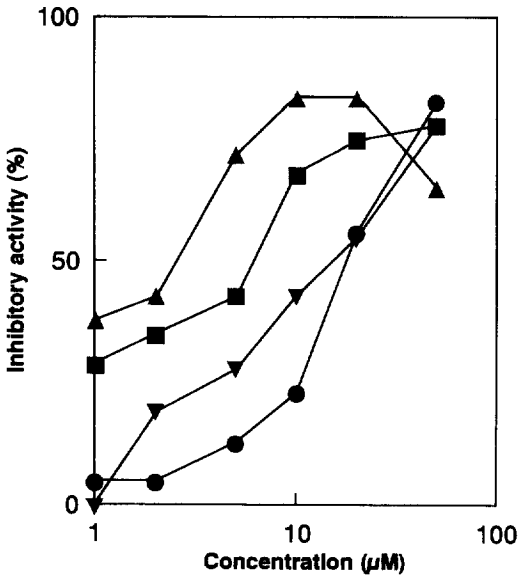

Fig. 4. Inhibitory activity of caffeoyltryptophan and related compounds on conjugated diene formation. Symbols used in the panel were as follows: ●, caffeic acid; ■, caffeoyltryptophan; ▼, 5-caffeoylquinic acid; ▲, *dl*-α-tocopherol.

rocytes *in vitro*. Caffeoyltryptophan, caffeic acid, 5-caffeoylquinic acid and *dl*-α-tocopherol (50 μM) were inhibited by 89, 99, 89 and 63% respectively, the haemolysis induced by H_2O_2 (Table 2).

Caffeoyltryptophan, caffeic acid, 5-caffeoylquinic acid and *dl*-α-tocopherol were inhibited by 79, 90, 92 and 65% respectively, of the lipid peroxidation induced by H_2O_2 (Table 2). Tryptophan showed no and a little inhibitory activities in this system.

It is known that caffeic acid or 5-caffeoylquinic acid inhibits the oxidation of metmyoglobin induced by H_2O_2. Its protection is attributed to oxoferryl reduction to ferric form [7, 8]. Thus, it may be concluded that the peroxyl radical scavenging activity of caffeoyltryptophan is a part of the mechanism of erythrocytes protection from the lipid peroxidation by H_2O_2.

5-Caffeoylquinic acid and caffeic acid have been shown to exhibit anticarcinogenic or antimutagenic properties *in vitro* and *in vivo* [9–11]. 5-Caffeoylquinic acid prevents lipid peroxidation mediated by carbon tetrachloride and ^{60}Co-irradiation [12, 13]. 5-Caffeoylquinic acid has been shown to protect against paraquat-induced oxidative stress and γ-radiation-induced damage *in vivo* [14, 15]. Thus, it is expected that caffeoyltryptophan, as well as 5-caffeoylquinic acid, indicates antioxidant effects *in vivo*.

The absorption, metabolism, and toxicity of caffeoyltryptophan remain to be clearly elucidated. However, the data suggest that caffeoyltryptophan may act as a natural antioxidant and partially protects against free radical mediated chemical toxicity.

Table 2. Inhibitory activities of caffeoyltryptophan and related compounds on haemolysis and lipid peroxidation by hydrogen peroxide

	Inhibitory activity (%)	
Addition (50 μM)	Haemolysis	Peroxidation
Caffeoyltryptophan	89	79
Caffeic acid	99	90
5-Caffeoylquinic acid	89	92
Tryptophan	0	18
dl-α-Tocopherol	63	65

EXPERIMENTAL

Materials. Linoleic acid (99.9%), XOD, 5-caffeoylquinic acid, caffeic acid, *dl*-α-tocopherol and bovine serum albumin were purchased from Sigma. 1,1-Diphenyl-2-picrylhyrazyl (DPPH) and nitro blue tetrazolium (NBT) were from Wako. All other chemicals used were commercial products of the highest grade.

Animals. Male ddY mice, 6-weeks-old, were obtained from the SLC (Japan).

Radical scavenging activity on DPPH radical. Radical scavenging activity was assayed in triplicate according to the method of ref. [16]. The reaction mixt. contained, in a total vol. of 3.5 ml, 3 ml of 0.1 mM DPPH (in 95% EtOH) and 0.5 ml of the test compound (in 95% EtOH). After allowing the mixt. to stand at room temp. for 20 min, A_{517} was determined. The scavenging activity was measured as the decrease in A_{517} of the DPPH radical expressed as a percentage of the control value.

Inhibitory effect on superoxide anion generation. Superoxide anion generation was assayed by the method of ref. [17]. The reaction mixt. contained, in a total vol. of 3 ml, 2.4 ml of 50 mM Na_2CO_3 buffer (pH 10.2), 0.1 ml of 3 mM xanthine, 0.1 ml of 3 mM EDTA, 0.1 ml of 1.5 mg ml^{-1} bovine serum albumin, 0.1 ml of 0.75 mM NBT, 0.1 ml of 0.3 U ml^{-1} XOD and 0.1 ml of the test compound. Reactions were initiated by adding XOD. After the mixt. was incubated for 20 min at 37°, 0.1 ml of 6 mM $CuCl_2$ was added to the mixt. to stop the reaction. A_{560} was determined, and percentage inhibition calculated relative to A_{560} for control.

Antioxidant activity of linoleic acid peroxidation. Linoleic acid peroxidation was assayed according to the method of ref. [18]. The reaction mixt. contained, in a total vol. of 5.02 ml, 4 ml of 1.25 mM linoleic acid micelles, 0.5 ml of 1 mM EDTA-Fe^{3+} (1.1 mM EDTA, 1 mM $FeCl_3$), 0.5 ml of 0.3 M acetaldehyde, 0.01 ml of 18.5 U ml^{-1} XOD and 0.01 ml of the test compound, in 30 mM NaCl (pH 7.0). Reactions were initiated by adding XOD; A_{234} was determined. The inhibitory activity was expressed by the decrease in conjugated diene formation after 10 min.

Inhibition of haemolysis and lipid peroxidation of

erythrocytes. Haemolysis and lipid peroxidation of mouse erythrocytes were carried out using H_2O_2 and a modification of the method of ref. [19]. The reaction mixt. contained, in a total vol. of 2 ml, 0.5 ml of 10% w/v erythrocytes of male mice (average body wt 25 g) in 0.02 M Na-Pi buffered saline (PBS, pH 7.4), 0.05 ml of the test compound, 1 ml of 0.6% H_2O_2 in 0.02 M PBS and 0.45 ml of 0.02 M PBS. The mixt. was incubated for 2 hr at 37° and centrifuged at 1500 g for 10 min. Haemolysis was determined on 0.2 ml of supernatant using the cyanomethaemoglobin method [20]. Lipid peroxidation was determined using 1.5 ml of supernatant and a quantitative assay of ref. [21].

Acknowledgments—We would like to thank Professor Tomihiko Higuchi, Tokushima University, for his advice.

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