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A novel tag-free probe for targeting molecules interacting with a flavonoid catabolite

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ABSTRACT

3,4-Dihydroxyphenylacetic acid (DOPAC) is one of the colonic microflora-produced catabolites of quercetin 4′-glucoside (Q4′G). Although the interaction of DOPAC with cellular proteins might be involved in its biological activity, the actual proteins have not yet been identified. In this study, we developed a novel tag-free DOPAC probe to label the targeted proteins by the copper(I)-catalyzed azide alkyne cycloaddition (CuAAC) and verified its efficacy. Various labeled proteins were detected by the DOPAC probe with the azide labeled biotin and a horseradish peroxidase (HRP)-streptavidin complex. Furthermore, a pulldown assay identified Keap1 and aryl hydrocarbon receptor (AhR) as the target proteins for the phase 2 enzyme up-regulation.

1. Introduction

Quercetin is one of the most widely distributed flavonoids in vegetables and fruits and has attracted much attention because of its beneficial biological effect, such as anti-oxidation [1,2] and anti-inflammation [3]. In general, quercetin exists in food stuffs as a glycoside form with a sugar moiety. Quercetin 4′-glucoside (Q4′G) is one of the major quercetin glycosides in onion and consumption of onions accounts for 29% of the total flavonol and flavone intake [4]. It is known that the fates of quercetin glycosides after intake are different depending on its sugar moiety. Mullen and his colleagues investigated the bioavailability of Q4′G in rats and proposed that Q4′G reaching the colon is subjected to hydrolysis by intestinal microbiota into 3,4-dihydroxyphenylacetic acid (DOPAC) [5]. DOPAC is subsequently converted into 3-hydroxyphenylacetic acid (OPAC) or 3,4-dihydroxybenzoic acid, which is also known as protocatechuic acid in the large intestine, and OPAC undergoes further conversion to hippuric acid [5]. Among the phenolic acid catabolites, DOPAC has been reported to have the significant ability to induce the phase 2 enzyme gene expression [6,7].

A previous study indicated that DOPAC is oxidized to form *o*-quinone, then covalently binds to sulfhydryls in GSH or protein due to its catechol structure [6]. Protein modification affects the protein function, consequently leading to effects on the cellular signaling pathway and gene expression. For example, covalent modification of Keap1 *via* cysteine residues subsequently causes activation of the Keap1/Nrf2/antioxidant response element (ARE) pathway [8]. The underlying mechanism in which DOPAC exerts an antioxidant effect *via* inducing the phase 2 enzyme is not fully understood. Therefore, developing a new probe of DOPAC provides an important clue to reveal the mechanism of its biological activity, including the phase 2 enzyme induction.

Click chemistry is a modular synthetic approach introduced by Sharpless and his co-workers in 2001 [9]. The reaction of this chemistry assembles molecules rapidly and efficiently under simple conditions and provides extremely high yields of the products, which can be easily isolated. The copper (I)-catalyzed alkyne azide 1,3-dipolar cycloaddition (CuAAC), which is known as the “cream of the crop” of click chemistry yields 1,4-disubstituted 1,2,3-triazols by conjugating the terminal alkyne and azide functional groups [10,11]. Over the last decade, it has been widely used as a major conjugating method in modern chemistry because of its high selectivity and tolerance to a wide range of reaction conditions.

In the present study, we designed a novel DOPAC probe that can be used for the CuAAC reaction. We introduced an alkyne moiety into DOPAC by esterification with 2-propyn-1-ol to afford the DOPAC propargyl ester (DPE, Fig. 1A). To confirm its efficacy as a protein thiol modifier, DPE was incubated with a model protein in the cell lysate, followed by CuAAC click reaction with an azide-

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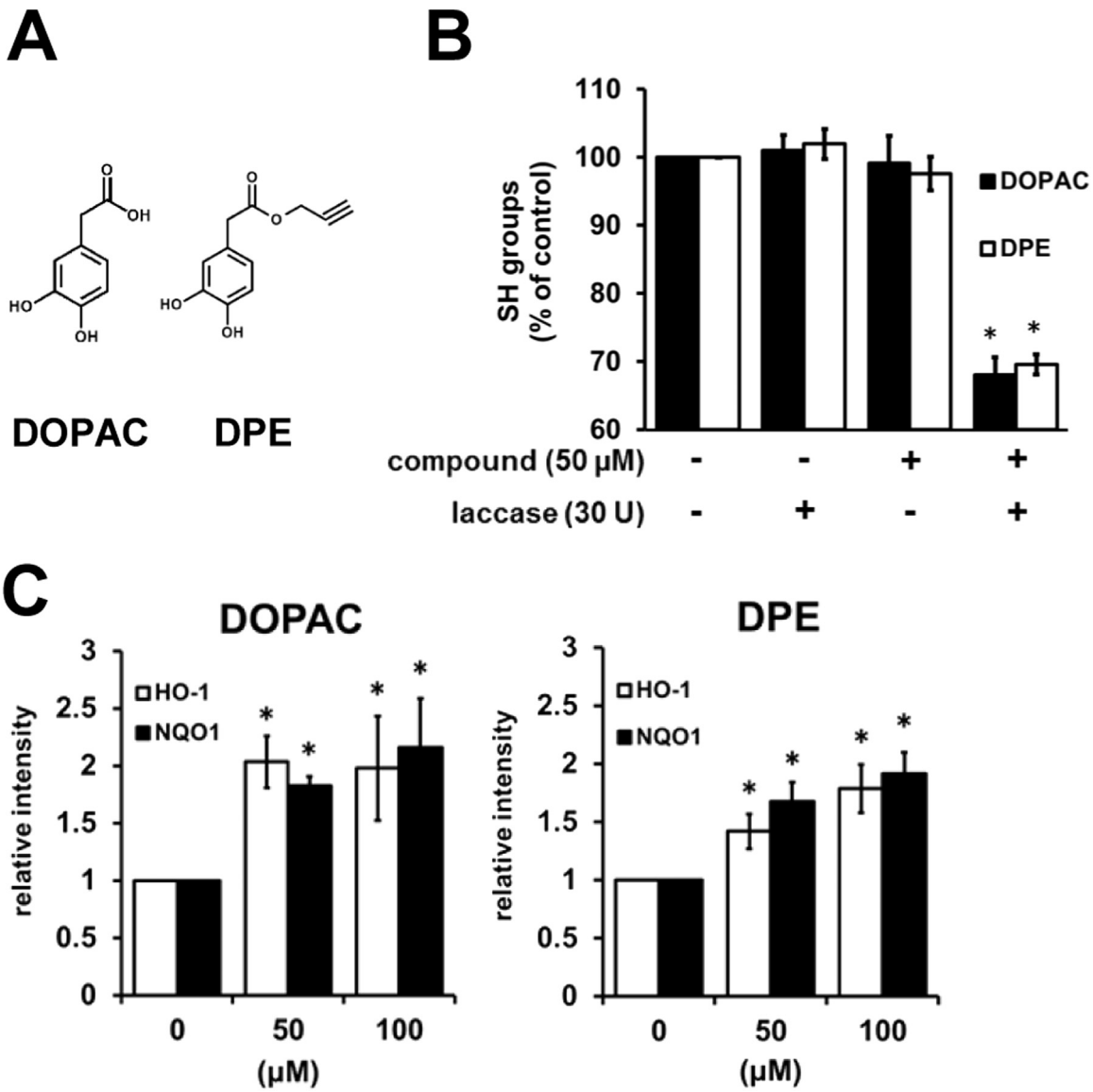

Fig. 1. DPE shows the similar chemical and biological properties to DOPAC. (A) Chemical Structures of DOPAC and DPE. (B) Modification of sulfhydryl groups in GAPDH by DOPAC and DPE. GAPDH (500 μg/ml) was incubated with DOPAC or DPE in 50 mM sodium phosphate buffer (pH 7.2) for 1 h at 37 °C in the presence or absence of laccase (30 units). The level of residual sulfhydryl groups in GAPDH was measured by the spectrophotometric method using DTNB. (C) Induction of the gene expression of HO-1 (black bars) and NQO1 (white bars) by DOPAC (left) or DPE (right). Hepa1c1c7 cells were treated with DOPAC or DPE for 24 h and total RNA was extracted. The values represent means ± S.D. of more than three separate experiments ($^*p < 0.05$ compared with control; Student's *t*-test.).

labeled biotin and finally detected using the horseradish peroxidase (HRP)-conjugated streptavidin. The use of DPE combined with the subsequent introduction with an azide-linked biotin by the CuAAC reaction is expected to enable the highly effective tagging of the DOPAC modified protein.

2. Materials and methods

2.1. Materials

DOPAC, laccase from *Rhus vernicifera*, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA), bis(4-nitrophenyl) phosphate (BNPP) and azide-PEG_3-biotin conjugate were obtained from Sigma Aldrich (St. Louis, USA). *p*-Toluensulfonic acid monohydrate (PTSA), *n*-butanol, toluene, copper (II) sulfate pentahydrate, protease inhibitor cocktail and Chemi-Lumi One Super were purchased from nacalai tasque (Kyoto, Japan). 2-Propyl-1-ol was obtained from Tokyo Chemical Industry (Tokyo, Japan). Streptavidin, HRP conjugate was purchased from Funakoshi (Tokyo, Japan). Anti-actin antibody, anti-aryl hydrocarbon receptor (AhR) antibody, anti-Keap1 antibody, horseradish peroxidase-linked anti-mouse IgG and horseradish peroxidase-linked anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Streptavidin Mag Sepharose was purchased from GE health care (Little Chalfont, UK). All other chemicals such as benzyl azide were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. General procedure

MS was recorded on ESI-mode by using Bruker MicrOTOF II and MicrOTOF Control 3.0. Data analysis was carried out using Data Analysis 4.0 SP2. ^{1}H NMR spectra were recorded on Varian Mercury 300. Chemical shift are described in parts per million (ppm) and coupling constants in Hz. Multiplicity and qualifier abbreviations are as follows: *s*=singlet, *d*=doublet, *t*=triplet, *quint*=quintet, *sext*=sextet, *m*=multiplet.