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Production of caloxanthin 3′-β-D-glucoside, zeaxanthin 3,3′-β-D-diglucoside, and nostoxanthin in a recombinant *Escherichia coli* expressing system harboring seven carotenoid biosynthesis genes, including *crtX* and *crtG*

Ayako Osawa [a], Hisashi Harada [b], Seon-Kang Choi [c], Norihiko Misawa [b,1], Kazutoshi Shindo [a,*]

[a] Department of Food and Nutrition, Japan Women's University, Mejirodai, Bunkyo-ku, Tokyo 112-8681, Japan
[b] Central Laboratories for Frontier Technology, Kirin Holdings Co. Ltd., i-BIRD, Suematsu, Nonoichi-machi, Ishikawa 921-8836, Japan
[c] Marine Bio Industry Support Center, Gangnueung Science Industry Park, Daejeon-dong, Gangneung-city 210-340, South Korea

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ABSTRACT

The aim of this study was to produce rare β-carotene-modified carotenoids possessing 2-O (-H or -glu) and/or 3-O (-H or -glu) functionalities in their β-ionone ring(s) using a recombinant *Escherichia coli* approach. This involved expressing seven carotenoid biosynthesis genes (*crtE*, *crtB*, *crtI*, *crtY*, *crtZ*, *crtX* and *crtG*). From the cells of the recombinant *E. coli*, caloxanthin (β,β-carotene-2,3,2′,3′-tetrol)-3′-β-D-glucose, zeaxanthin (β,β-carotene-3,3′-diol) 3,3′-β-D-diglucoside, and nostoxanthin (β,β-carotene-2,3,3′-triol) (rare carotenoids) were isolated and identified. Caloxanthin 3′-β-D-glucose displayed potent $^1O_2$ quenching activity (IC_{50} 19 μM).

1. Introduction

More than 750 carotenoids with different molecular structures have been isolated from natural sources (Britton et al., 2004); however, a few carotenoid species can be obtained in sufficient amounts, including dicyclic carotenoids, such as β-carotene, α-carotene, β-cryptoxanthin, zeaxanthin, lutein, canthaxanthin, astaxanthin and fucoxanthin, and an acyclic carotenoid, lycopene. Among these carotenoids, β-carotene, α-carotene, β-cryptoxanthin, zeaxanthin, lutein, fucoxanthin and lycopene are present in edible plant sources, and their beneficial effects on human health such as cancer prevention have been extensively examined (Hosokawa et al., 2004; Nishino et al., 2000; Talegawkar et al., 2008). Astaxanthin, which was isolated from green alga *Haematococcus pluvialis*, was shown to inhibit low-density lipoprotein oxidation in human and prevent diabetic nephropathy for diabetic db/db mice (Iwamoto et al., 2000; Naito et al., 2004). With the exception of such carotenoids, it is difficult to find natural sources that can supply sufficient amounts of carotenoids (referred to as "rare carotenoids"), which hinder researchers from examining their biological activities.

Engineering biosynthetic pathways in heterologous organisms (pathway engineering) is one of the most powerful methods of generating plenty of natural compounds of interest. With this approach, a variety of rare carotenoids, including structurally novel ones, have been produced in either *Escherichia coli* or higher plants, using various combinations of carotenoid biosynthesis genes that were isolated from carotenogenic (carotenoids-producing) bacteria (Lee et al., 2003; Misawa, 2010; Shindo et al., 2008). These carotenogenic bacteria belonged to the class *Gamma-* or *Alpha-proteobacteria*, which can synthesize derivatives of a plant-type cyclic carotenoid β-carotene (Misawa, 2010; Nishida et al., 2005). A gene cluster needed for the biosynthesis of a carotenoid glycoside was first isolated from the soil bacterium *Pantoea ananatis* (re-classified from *Erwinia uredovora*) belonging to *Gammaproteobacteria*, and was structurally and functionally analyzed (Misawa et al., 1990). In the carotenoid gene cluster of *Pantoea* species, zeaxanthin (β,β-carotene-3,3′-diol) glucosyltransferase, named CrtX, was found to glucosylate zeaxanthin with UDP-glucose to yield zeaxanthin 3,3′-β-D-diglucoside (**2**) (structure shown in Fig. 1) (Misawa et al., 1990; Nakagawa and Misawa, 1991; Hundle et al., 1992). When genes encoding enzymatic steps from farnesyl

* Corresponding author. Address: Department of Food and Nutrition, Japan Women's University, 2-8-1 Mejirodai, Bunkyo-ku, Tokyo 112-8681, Japan. Tel./fax: +81 3 5981 3433.
E-mail address: kshindo@fc.jwu.ac.jp (K. Shindo).
[1] Present address: Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, Suematsu, Nonoichi-machi, Ishikawa 921-8836, Japan.

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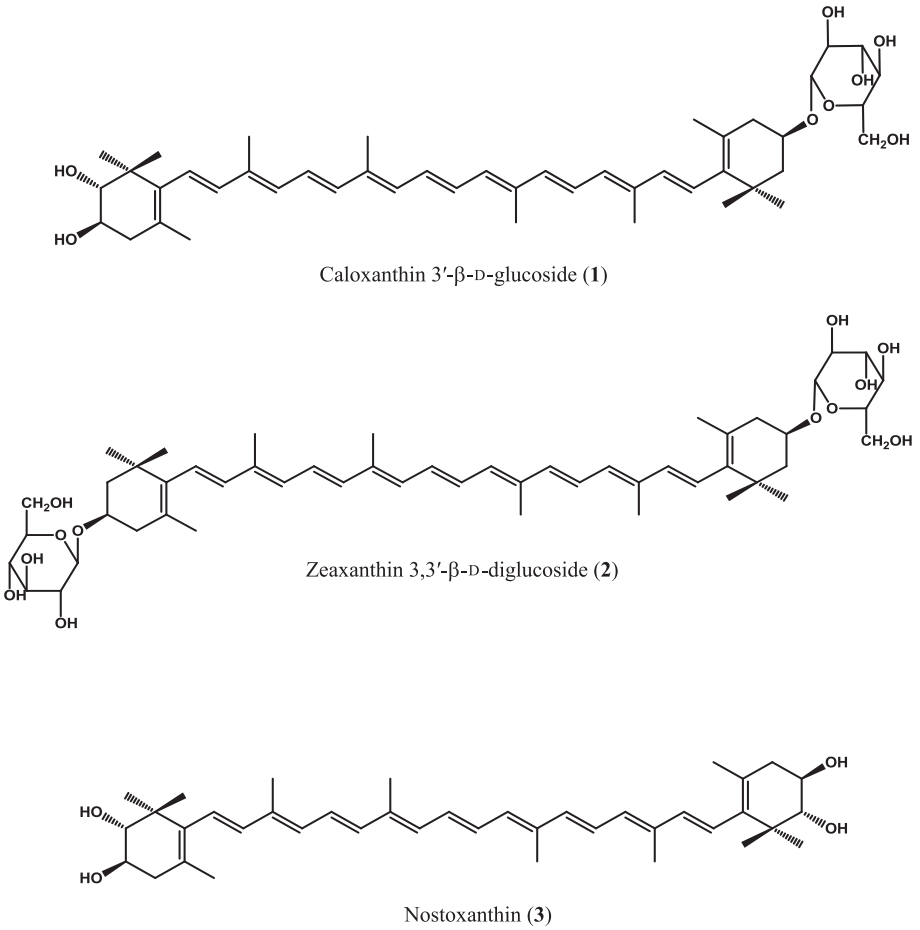

Fig. 1. Structures of caloxanthin 3′-β-D-glucoside (**1**), zeaxanthin 3,3′-β-D-diglucoside (**2**), and nostoxanthin (**3**).

diphosphate (FPP) to zeaxanthin (*crtE*, *crtB*, *crtI*, *crtY*, and *crtZ*), which were present in the *Pantoea* gene cluster, in addition to the *crtX* gene, were introduced into *E. coli* and expressed there, the resultant bacterial cells synthesized zeaxanthin 3,3′-β-D-diglucoside (**2**) as its predominant carotenoid product. This was also the main pigment present in the *Pantoea* species (Nakagawa and Misawa, 1991; Hundle et al., 1991). *E. coli* expressing the *P. ananatis crtE*, *crtB*, *crtY*, *crtZ* and *crtX* genes in addition to the *crtI14* gene (*in vitro* evolved gene of *crtI*) was shown to synthesize torulene-monoglucoside (Lee et al., 2003). The *crtX* gene was also found in a marine bacterium *Paracoccus* sp. strain N81106 (formerly called *Agrobacterium aurantiacum*) that produces astaxanthin 3-β-glucoside (Maruyama et al., 2007; Yokoyama et al., 1995). On the other hand, a marine bacterium, *Brevundimonas* sp. strain SD212, was reported to produce trihydroxy-keto-carotenoids, such as 2-hydroxyastaxanthin (Yokoyama et al., 1996). A carotenoid 2,2′-hydroxylase gene, named *crtG*, was first isolated from this bacterium, and *E. coli* that expressed the *crtG* gene in addition to the *crt* genes needed for the biosynthesis of zeaxanthin from FPP was shown to generate nostoxanthin (**3**) (β,β-carotene-2,3,2′,3′-tetrol) by way of caloxanthin (β,β-carotene-2,3,3′-triol) (Nishida et al., 2005). The *crtG* genes were also identified in a soil bacterium *Brevundimonas vesicularis* strain DC263 (Tao et al., 2006) and a cyanobacterium *Thermosynechococcus elongatus* strain BP-1 (Iwai et al., 2008).

The present study shows that co-expression of the *P. ananatis* carotenogenic genes (*crtE*, *crtB*, *crtI*, *crtY*, *crtZ*, and *crtX*) and the *Brevundimonas* SD212 *crtG* gene in *E. coli* results in the production of caloxanthin 3′-β-D-glucoside (**1**) (a novel carotenoid), zeaxanthin 3,3′-β-D-diglucoside (**2**), and nostoxanthin (**3**) (rare carotenoids) as the main pigments. The antioxidative ($^1O_2$ quenching) activity of caloxanthin 3′-β-D-glucoside (**1**) was also examined.

2. Results

2.1. Isolation and identification of carotenoids produced by recombinant proteins

Recombinant *E. coli* cells harboring two plasmids pUCBreG-CAR1 (Fig. 2) and pAC-MeV (Harada et al., 2009) were cultured in 12 L medium, and collected by centrifugation. Carotenoids were extracted by adding CH_2Cl_2:MeOH (1:1) solution after sonication of the cells. The solution was then centrifuged, and the yellow supernatant was concentrated to a small volume *in vacuo*, and partitioned between *n*-BuOH/H_2O without adjusting the pH. The *n*-BuOH layer was evaporated to dryness and analyzed by thin-layer chromatography (TLC) on silica gel (E. Merck 60 F-254 0.25 mm silica gel plates) using CH_2Cl_2–MeOH (10:1). By TLC analysis, three yellow spots were observed at Rf 0.4, 0.3, and 0.2, with compounds at Rf 0.4 and 0.2 identified as nostoxanthin (**3**) and zeaxanthin 3,3′-β-D-diglucoside (**2**) (Fig. 1), respectively, by HPLC analyses using the method of standard addition. The compound at Rf 0.3 (**1**) was not identified. The ratio of each peak area (at 450 nm) was nostoxanthin (**3**):unidentified carotenoid (**1**):zeaxanthin 3,3′-β-D-diglucoside (**2**) = 2:1:2, approximately.

To determine the structure of **1**, the *n*-BuOH extract was subjected to silica gel chromatography, and the fractions