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Analysis of stereochemistry and biosynthesis of epicatechin in tea plants by chiral phase high performance liquid chromatography

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ABSTRACT

Tea (*Camellia sinensis*) is rich in flavan-3-ols (catechins), especially epicatechin (EC), which is the predominant extension unit of polymeric proanthocyanidins (PAs). However, studies assessing EC's stereochemistry are scarce. Here, a high performance liquid chromatography column using amylose tris-(3, 5-dimethylphenylcarbamate) immobilized on silica-gel as chiral stationary phases (CSPs) was applied to explore its stereochemistry and biosynthetic pathway in tea plants. The results revealed (−)-epicatechin [(−)-EC] was the predominant di-hyroxy-non-galloylated-catechins, while (+)-epicatechin [(+)-EC] was not detected. Interestingly, (−)-EC was the only product obtained from cyanidin using the partially purified native *C. sinensis* anthocyanidin reductase (CsANR) in the presence of reduction nicotinamide adenine dinucleotide phosphate (NADPH); meanwhile, (+)-EC was the main product using recombinant CsANR in the same conditions. In addition, (−)-EC could be obtained from (+)-catechin [(+)-C] using recombinant CsANR, which displayed C_3-epimerase activity in the presence of oxidation nicotinamide adenine dinucleotide phosphate ($NADP^+$). But the partially purified native CsANR did not possess this function. Finally, (−)-EC could result from the de-gallate acid reaction of epicatechin gallate (ECG) catalyzed by a novel partially purified native galloylated catechins hydrolase (GCH) from tea leaves. In summary, (−)-EC is likely the product of native protein from the tea plants, and (+)-EC is only produced in a reaction catalyzed by recombinant CsANR *in vitro*.

1. Introduction

Tea [*Camellia sinensis* (L.) O. Kuntze], originated in China, is one of the most widely cultivated and consumed beverages throughout the world. Flavan-3-ols (catechins) are the major class of secondary metabolites in tea plants; they are responsible for tea quality and beneficial to human health [1–4].

Owing to two asymmetric atoms at the C_2 and C_3 positions, four heterogeneous flavan-3-ol types are known, including (2R, 3R)-*cis*-flavan-3-ols (*i.e.*, (−)-epi-form), (2S, 3S)-*cis*-flavan-3-ols (*i.e.*, (+)-epi-form), (2S, 3R)-*trans*-flavan-3-ols (*i.e.*, (−)-form), and (2R, 3S)-*trans*-flavan-3-ols (*i.e.*, (+)-form) (Fig. 1A).

Previous studies have found that 2R-flavan-3-ols are mainly of two types (*i.e.*, epi-form and non epi-form) that consist of (−)-epigallocatechin gallate [(−)-EGCG], (−)-epigallocatechin [(−)-EGC], (−)-epicatechin gallate [(−)-ECG], (−)-EC, (+)-C and (+)-gallocatechin [(+)-GC] in tea plants, using the two-way paper chromatography technology [5]. The content of epicatechins (ECs), the collective term for (−)-EC, (−)-EGCG, (−)-EGC, and (−)-ECG, could be affected by different cultivation conditions; for instance, the content of ECs decreased by shading [6]. However, ECs constitute the main components of total catechins in tea leaves [7] (Fig. 1B). 2S-flavan-3-ols, being non-native flavan-3-ols, such as (−)-gallocatechin gallate [(−)-GCG], (−)-gallocatechin [(−)-GC], (−)-catechin [(−)-C], and (−)-catechin gallate [(−)-CG], were only detected in manufactured tea. For example, Chen et al. reported that (−)-GCG levels could reach as much as 50% of total catechins in some tea drinks, using HPLC [8]; in addition, Gotti et al. reported that (−)-GC and (−)-C were detected in tea infusion at 85 °C for

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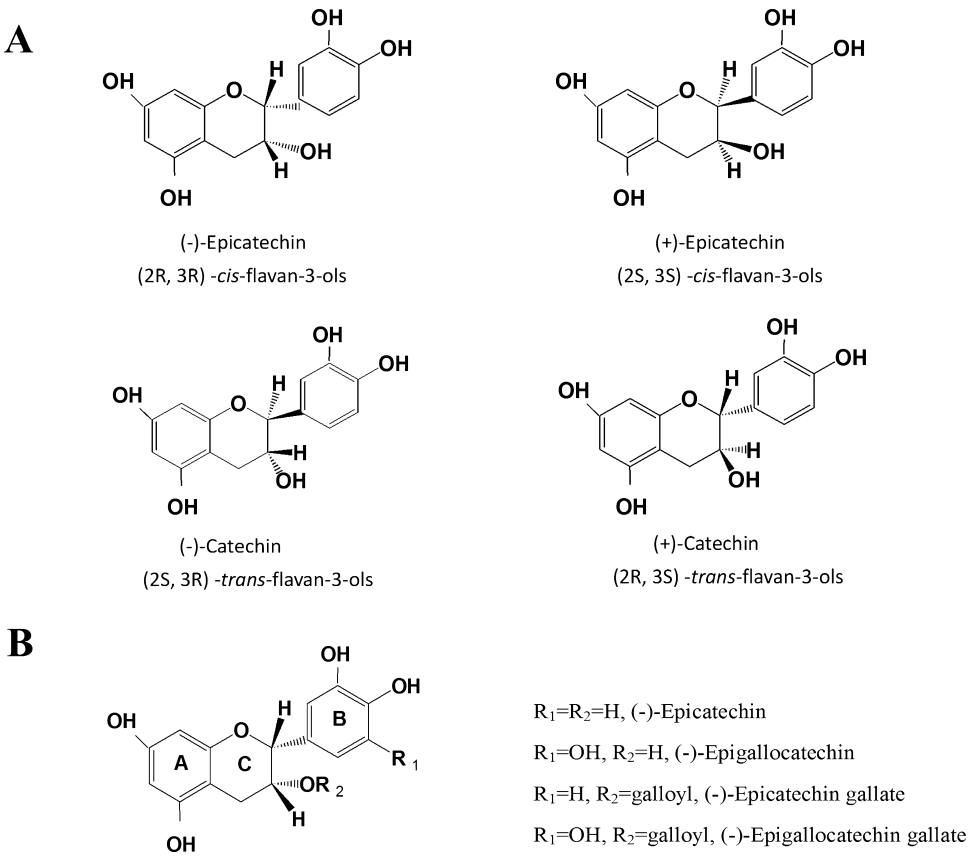

Fig. 1. Stereochemistry of flavan-3-ols. (A) Four types of flavan-3-ols; (B) typical epicatechins in tea plants.

5 min *via* a chiral cyclodextrin-micellar electrokinetic chromatography (CD-MEKC) method [9].

High performance liquid chromatography well known for direct enantioseparation based on different CSPs (*i.e.*, chiral HPLC) has received increasing attention due to its advantages, and the polysaccharide-based CSPs are most powerful, thanks to their high loading capacity and wide applicability [10,11]. To our knowledge, studies using chiral HPLC with amylose tris-(3,5-dimethylphenylcarbamate) immobilized on silica-gel to identify the stereochemistry of flavan-3-ols in fresh tea leaves are scare.

For (−)-EC biosynthesis, there may be three biosynthetic pathways in tea plants. The main one is *de novo* synthesis (Fig. 2). Xie et al. described anthocyanidins as the most effective substrates, and anthocyanidin reductase (ANR) as key enzymes encoded by important genes; for instance, *BANYULS (BAN)* from *Arabidopsis thaliana* and *Medicago truncatul* could convert cyanidin into (−)-EC in the presence of NADPH [12]. Further study revealed (−)-C as a minor by-product in the above enzyme reaction system, straight from (−)-EC after a non-enzymatic C_2-epimerization [13].

Recently, new developments have been proposed for the function of ANR. Gargouri et al. reported that (+)-EC and (−)-C are produced from cyanidin by using recombinant *Vitis vinifera* anthocyanidin reductase (VvANR) in the presence of NADPH [14]. Pang also reported that recombinant CsANR could convert cyanidin to the same products as above *in vitro* [15]. However, (+)-EC and (−)-C are not detected in the tea plant; specifically, (+)-EC is seldom found in the plant kingdom [16]. So far, previous studies have reported the stereochemistry of EC obtained from cyanidin by using recombinant ANR *in vitro*, but did not report that by native ANR *in vivo*.

The second biosynthetic pathway is assumed that (−)-EC results from (+)-C by epimerization. Extensive studies have shown that epimerization of flavan-3-ols can be classified into two types, namely non-enzymatic and enzymatic epimerization. Non-enzymatic epimerization often occurs at the C_2 position under extreme external environment conditions such as high temperature or pH higher than 6.0 [17–20]. Under moderate physical conditions, non-enzymatic epimerization between (−)-EC and (−)-C could be observed only after incubation of (−)-EC in 100 mM Tris–HCl buffer with pH 7 at temperatures above 30 °C for 30 min, but not in MES buffer [13]. Meanwhile, enzymatic epimerization occurs at the C_3 position. Interestingly, recombinant VvANR could act as a pure C_3-epimerase of 2R-flavan-3-ols in reverse direction, but not 2S-flavan-3-ols in the presence of excess $NADP^+$; in other words, (−)-EC was obtained from (+)-C by enzymatic epimerization*in vitro* [14].

In the third biosynthesis pathway, (−)-EC can result from ECG *via* de-gallate acid reaction. Our previous study indicated that a novel native protein, named galloylated catechins hydrolase (GCH), could catalyze ECG into EC [21]. This paper, it was identified by using chiral HPLC that (−)-EC could be obtained from de-gallate acid reaction.

According to previous reports, several questions arise as to (−)-EC biosynthesis in tea plants. For example, what is EC's stereochemistry in tea plants? Do the above mentioned three biosynthetic pathways of (−)-EC really exist in tea plants? Here, we used chiral HPLC to determine the stereochemistry of EC, and found (−)-EC, not (+)-EC, in tea plants. Subsequently, we demonstrated that (−)-EC was the only product of the partially purified native CsANR from fresh tea leaves using cyanidin as substrates, and the atypical flavan-3-ol (+)-EC was the main product of recombinant CsANR in the same conditions. In addition, (−)-EC could be obtained from (+)-C *via* C_3-epimerization catalyzed by recombinant CsANR*in vitro*.