Phenylpropanoid 2,3-dioxygenase involved in the cleavage of the ferulic acid side chain to form vanillin and glyoxylic acid in *Vanilla planifolia*

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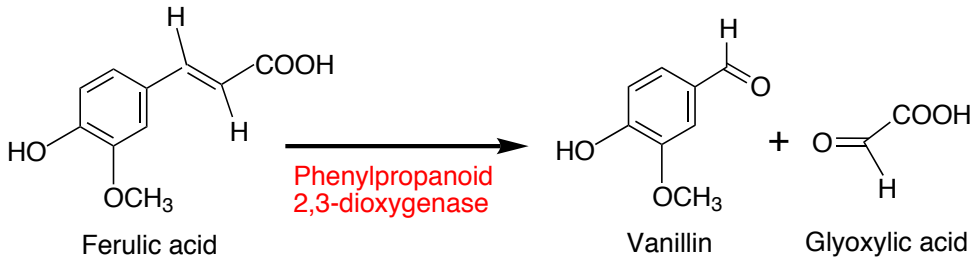
Abbreviations: DTT, dithiothreitol; GSH, glutathione; GA, glyoxylic acid; qNMR, quantitative NMR; UDP-glucose, uridine 5'-diphosphoglucose.

Abstract

Enzyme catalyzing the cleavage of the phenylpropanoid side chain was partially purified by ion exchange and gel filtration column chromatography after $(NH_4)_2SO_4$ precipitation. Enzyme activities were dependent on the concentration of dithiothreitol (DTT) or glutathione (GSH) and activated by addition of 0.5 mM Fe^{2+}. Enzyme activity for ferulic acid was as high as for 4-coumaric acid in the presence of GSH, suggesting that GSH acts as an endogenous reductant in vanillin biosynthesis. Analyses of the enzymatic reaction products with quantitative NMR (qNMR) indicated that an amount of glyoxylic acid (GA) proportional to vanillin was released from ferulic acid by the enzymatic reaction. These results suggest that phenylpropanoid 2,3-dioxygenase is involved in the cleavage of the ferulic acid side chain to form vanillin and GA in *Vanilla planifolia* (Scheme 1).



Scheme 1. Phenylpropanoid 2,3-dioxygenase is involved in the cleavage of the ferulic acid side chain to form vanillin and GA in *Vanilla planifolia*

Key words: *Vanilla planifolia;* biosynthesis of vanillin; glyoxylic acid; ferulic acid; phenylpropanoid 2,3-dioxygenase

Introduction

Vanillin is accumulated as a glucoside in the green vanilla pods (*Vanilla planifolia*) and the sweet aroma is released only by the "curing" treatment. For the formation of vanillin in the green pods, researchers proposed several kinds of biosynthetic pathways, however, the complete biosynthetic pathway for vanillin from phenylpropanoids has not been clarified for a long time.[1)] Previously, we demonstrated that the biosynthetic pathway for vanillin is 4-coumaric acid → caffeic acid → ferulic acid → vanillin → vanillin glucoside in *Vanilla planifolia* by pulse-chase experiments with ^{14}C-labeled compounds,[1)] and carried out partial purification and characterization of the enzyme involved in the conversion of ferulic acid to vanillin in vanilla pods.[2)] Enzyme activities were dependent on the presence of DTT and activated by the addition of 0.5 mM Fe^{2+}. Based on our preliminary work dealing with a partially purified enzyme from vanilla pods, we proposed that the enzyme forming vanillin and 4-hydroxybenzaldehyde from ferulic acid and 4-coumaric acid, respectively, is 2,3-dioxygenase, and also proposed a reaction mechanism for 2,3-dioxygenase in green vanilla pods after consulting other studies.[3–5)] In brief, the double bond of the phenylpropanoid side chain is twice attacked by oxygen atoms of the enzyme–ferrous oxygen complex *via* the epoxide structure to form two aldehydes. We detected GA in an enzyme reaction mixture with a high-performance anion exchange chromatography-pulsed amperometric detection system after concentration of the product with a SAX cartridge.[6)] On the one hand, Podstolski et al.[7)] reported an enzyme involved in the reaction that converts 4-coumaric acid to 4-hydroxybenzaldehyde in tissue cultures of vanilla. They provided a biochemical characterization of one type of plant chain-shortening enzyme system, and confirmed a non-β-oxidative mechanism most likely involving hydrolyase activity that promotes hydration of the side chain 2,3 double bond of 4-coumaric acid with subsequent cleavage of the side chain to yield acetate and 4-hydroxybenzaldehyde. On the other hand, Gallage et al.[8)] also carried out feeding experiments with putative ^{14}C-labeled precursors to tissue slices of vanilla pods and obtained results similar to our findings.[1)] Their results indicated that 4-hydroxybenzaldehyde was not converted to vanillin glucoside and they proposed a biosynthetic pathway in which vanillin is glucosylated after the conversion of ferulic acid to vanillin. Moreover, they demonstrated the substrate specificity of vanillin synthase obtained from a coupled transcription/translation of the PCR-generated DNA for VpVAN encoding a two-carbon chain-shortening enzyme.[8)] This enzyme catalyzed the reactions from ferulic acid and ferulic acid glucoside to vanillin and vanillin glucoside, respectively, whereas no activity was found using 4-coumaric acid, caffeic acid and those glucosides as substrates. They proposed a reaction

mechanism catalyzed by a hydratase/lyase-type enzyme that produces acetate and vanillin (or vanillin glucoside) as well as the mechanism suggested by Podstolski et al.[7] The two kinds of enzyme activities mentioned above are necessary for vanilla pods to metabolize both 4-coumaric acid and ferulic acid to 4-hydroxybenzaldehyde and vanillin, respectively, because large amounts of both 4-hydroxybenzaldehyde glucoside and vanillin glucoside accumulate in the mature pods.[1] However, the release of acetate other than vanillin and 4-hydroxybenzaldehyde by the enzymatic reactions has not been determined in vanilla pods[7,8] and other organisms,[9,10] even though acetyl-CoA produced from feruloyl CoA by 4-hydroxycinnamoyl-CoA hydratase/lyase in *Pseudomonas fluoresens* has been detected.[11]

On the other hand, in addition to these non-β-oxidative enzyme reactions, heme and non-heme iron dioxygenase reactions are indicated to catalyze the C–C double bond cleavage. Dioxygenase enzymes that cleave aliphatic C–C double bonds of substrates such as β-diketone dioxygenase,[12] quercetin 2,3-dioxygenase,[13] and phenylpyruvate dioxygenase[14] have been identified. In carotenoid cleavage oxyganases,[4,5] β-ionone and retinal are directly produced with cleavage of β-carotene at the 9,10 (or 9',10') and 15,15' positions, respectively. These are non-heme iron-containing oxygenases, while the O_2-dependent oxidation of L-tryptophan to *N*-formylkynurenine is a reaction catalyzed by a heme dioxygenase.[3] Moreover, fatty acid hydroperoxide lyase is a cytochrome P450 enzyme that cleaves fatty acid hydroperoxides to form short-chain aldehydes and oxo-acids and has been isolated from tea leaves, bell pepper fruits, *Arabidopsis* leaves, and tomato fruits. All have high substrate specificity for the 13-hydroperoxide of α-linolenic acid.[15]

In a non-β-oxidative enzyme reaction, an initial hydration addition reaction is likely followed by a retro-aldol elimination reaction that results in the formation of an aldehyde and an acid,[7–10] whereas in a dioxygenase reaction the C–C double bond is cleaved to form the corresponding aldehydes or ketones using dioxygen as the reactant and ferrous iron as a cofactor through either an epoxide or a dioxetane intermediate or through Criegee rearrangement.[3–5] One of the products in these reactions is an aldehyde compound, so the identification and determination of the other reaction product may demonstrate a part of the enzymatic reaction mechanism and characterize the enzyme involved in the C–C double bond cleavage. In order to clarify the reaction mechanism for the enzyme catalyzing the cleavage of the ferulic acid side chain in *Vanilla planifolia*, we analyzed the enzymatic reaction products more accurately with qNMR in this study. qNMR is one of the most suitable techniques for quantitative measurement of multicomponents in a complex mixture (for example: cell and tissue

extract, natural product isolates, and drug formulations) and has been widely applied to metabolomics, pharmaceuticals and natural products.[16–18] This method is believed to provide absolute and relative quantification of several products in a chemical reaction or an enzymatic reaction mixture without separation of individual components similar to NMR-based metabolomics in metabolic studies.[16] As a result, we detected aldehyde compounds with proton signals that are likely to separate from other signals by ^{1}H-NMR in the enzymatic reaction mixtures. This report focuses mainly on determination of the enzymatic reaction products using qNMR.

Materials and methods

Vanilla pods. Green vanilla pods (*Vanilla planifolia*), cultivated for 6 months after pollination in 2007 by a farmer in Central Java, Indonesia, were transported by air to Japan and stored at −40°C.

Chemicals. DTT, GSH, UDP-glucose, ATP, ferulic acid, vanillin, 4-hydroxybenzaldehyde, GA monohydrate, D_2O, DCl, 3-(trimethylsilyl)propanonic acid sodium salt were purchased from Wako Pure Chemical Industry, Osaka, Japan. 4-Coumaric acid, caffeic acid, 3,4-dihydroxybenzaldehayde were obtained from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. Vanillin glucoside was synthesized and supplied by Takasago International Corporation, Tokyo, Japan.

Preparation and purification of enzyme. Frozen green vanilla pods (500 g) were cut into small pieces and homogenized with 2 L of cold acetone (−20°C) in a Waring blender, and further with Polytron (Kinematica AG, Switzerland). The homogenate was passed through a filter paper under suction, and the residue washed three times with 1 L of cold 80% acetone–water solution (v/v). After removing the acetone from the residue, the enzymes were extracted with 2 L of 0.1 M K_2HPO_4 solution (pH 7.0) containing 0.6% ascorbic acid, 1 mM DTT, and 0.5% Triton X-100. After $(NH_4)_2SO_4$ precipitation by 80% saturation and dialysis for 18 h with 0.02 M acetate buffer (pH 5.5) containing 1 mM DTT, the enzymes were partially purified with a DEAE-Toyopearl 650M column (Tosoh Corporation, Japan, 2.5 × 25 cm) and an SP-Toyopearl 650S column (Tosoh Corporation, Japan, 2.5 × 30 cm) equilibrated with 0.02 M acetate buffer (pH 5.5) containing 1 mM DTT and Toyopearl HW-55S column (Tosoh Corporation, Japan, 1.5 × 96 cm) equilibrated with 0.02 M MES–KOH buffer (pH 6.5). The extraction and purification procedures were carried out at 4°C.

Assays for enzymes. Assays for enzymes were based on the separation of products from substrates by HPLC. Reaction mixtures for standard assays contained 0.1 M MES–KOH buffer (pH 6.5), 2 mM substrate, 0.5 mM $FeSO_4$, 10 mM DTT, 10 mM ATP for 2,3-dioxygenase; 0.25 M Bicine–KOH buffer (pH 8.0), 0.1 mM vanillin, 1 mM $MnCl_2$, 10 mM UDP-glucose for glucosyltransferase; and 0.2 M acetate buffer (pH 5.0), 1 mM vanillin glucoside for glucosidase with enzyme solution added to total volumes of 25 μL. Reactions were carried out at 30°C for 2 h and stopped by addition of 125 μL of 0.04 N HCl. A 50-μL reaction mixture was analyzed with a Shimadzu LC-10A HPLC System, Cadenza CD-C18 column (3 μm, 250 × 4.6 mm ID, Imtakt Corporation, Japan) and 40% MeOH (1% AcOH) for 20 min at 40°C and a flow rate of 0.5 mL/min. The eluates were monitored by absorbance at 280 nm. One katal (kat) was defined as the amount of enzyme that produced 1 mol of vanillin, 4-hydroxybenzaldehyde, or vanillin glucoside per sec under standard assay conditions.

Anion exchange HPLC for GA. A 100-fold scaled enzymatic reaction as the standard assay for dioxygenase was carried out[6] and the reaction mixture was treated with a SAX cartridge.[19] GA in the solution eluted with 0.1 N HCl from the SAX cartridge was analyzed with a Dionex DX-500 high-performance anion exchange chromatography-pulsed amperometric detection (HPAE–PAD) system (Dionex Corporation, Sunnyvale, CA) equipped with a Dionex CarboPac PA1 column (250 mm × 4 mm ID) and a PA1 guard column (50 mm × 4 mm ID). Elution was performed with 500 mM NaOH solution for 15 min at 25°C and a flow rate of 1.0 mL/min. GA was eluted at 6.2 min.

NMR analysis of enzymatic reaction products. Reagents for enzymatic reactions were all prepared with D_2O and water (H_2O) in the enzyme solution was exchanged with D_2O by lyophilization of the enzyme solution. Twenty-fold scaled enzymatic reactions (500 μL) as a standard assay for 2,3-dioxygenase were carried out at 40°C and the reaction was stopped by addition of 20 μL of 3 N DCl. After standing for 1 h to hydrate aldehyde groups of the reaction products in the existence of DTT and DCl,[6,20] 50 μL of 3-(trimethylsilyl)propanonic acid sodium salt solution (1.2 mg/mL D_2O) was added as the internal standard for NMR. The ^{1}H-NMR spectra of the enzymatic reaction mixtures (pH 1.4) were quantitatively measured with the following parameters: acquisition time of 6.56 s, relaxation delay of 5.00 s, 90° pulse width of 5.40 μs, 64 k data points, and 256 scans for approximately 50 min at 30°C with a JNM-A400 spectrometer (399.65 MHz, JEOL, Japan). The proton signal areas of reaction products were integrated and their ratios to the signal area of the internal standard substance (0 ppm) calculated. Integrations of the signal areas were repeated five times and the values averaged. The

amounts of the reaction products were obtained by comparing the calibration curves with the corresponding authentic compounds.

Results and discussion

Purification of enzymes

The enzyme solution after $(NH_4)_2SO_4$ precipitation and dialysis was charged to the DEAE-Toyopearl 650M column. The pass-through fraction from the DEAE-Toyopearl 650M column that contained enzyme activity catalyzing the formation of vanillin and 4-hydroxybenzaldehyde from ferulic acid and 4-coumaric acid, respectively, was charged to an SP-Toyopearl 650S column connected to a DEAE-Toyopearl 650M column and both columns were washed thoroughly with 0.02 M acetate buffer (pH 5.5) containing 1 mM DTT. After removal of the DEAE-Toyopearl column, the enzymes adsorbed on the SP-Toyopearl column were separated into four fractions by a NaCl gradient (Fig. 1-A). Further purification of these fractions (No. 9–15, 17–22, 25–33 and 34–56) was each done by gel-filtration column chromatography on a Toyopearl HW-55S. The main pooled fraction (No. 17–22) in Fig. 1-A was further separated into three enzyme fractions (Fig. 1-B). The main peak with high activity was detected in Fig. 1-B, No. 46–53. These fractions were pooled, concentrated, and used for further enzyme reactions. The enzyme solution appeared to contain enzymes that catalyzed the cleavage of ferulic acid and 4-coumaric acid. On the other hand, enzymes adsorbed on the DEAE-Toyopearl 650M column were eluted with 0.4 M NaCl without separation and chromatographed with a Toyopearl HW-55S column (Fig. 1-C). Previous studies[21–23] reported that glucosyltransferase and glucosidase activities were adsorbed on DEAE-cellulose. In this study, however, the existence of glucosyltransferase activity (conversion of vanillin to vanillin glucoside) was not clearly demonstrated by column chromatography,[6] although a little activity was detected in the concentrated fraction eluted from the DEAE-Toyopearl 650M column. By contrast, strong activity of glucosidase activity (Fig. 1-C, hydrolysis of vanillin glucoside) was reported in a previous study.[23]

In our preliminary study,[2] we reported some properties of 2,3-dioxygenase. The optimum pH and the molecular weight of the enzyme in one fraction were approximately 7 and 17,000, respectively, but activities were very weak. DTT was necessary for the appearance of activity, and the enzyme activity toward ferulic acid in comparison with 4-coumaric acid in the same fraction was approximately 16%, which is higher than that in tissue cultures of vanilla.[7]

Effects of ferrous ion, DTT, and GSH on 2,3-dioxygenase

A previous report indicated the effect of Fe^{2+} on enzyme activities for ferulic acid and 4-coumaric acid.[2] In the presence of Fe^{2+} and DTT, a non-enzymatic formation of vanillin and 4-hydroxybenzaldehyde occurred.[7] At 0.5 mM Fe^{2+} and 5 mM DTT, however, the enzyme activities toward ferulic acid and 4-coumaric acid were approximately 3 and 7 times, respectively, as high as the activities toward these substrates at 10 mM Mg^{2+} and 10 mM DTT in the 4-h reaction (Supplemental Fig. S1). Furthermore, 5 mM GSH was effective for enzyme activities during the early time of incubation. In this study, the enzyme activities degrading ferulic acid, 4-coumaric acid, and caffeic acid to yield their corresponding aldehydes were measured with different concentrations of DTT or GSH at 30°C for 30 min and products were analyzed by HPLC (Fig. 2). Enzyme activities depended on the concentrations of the SH reagents. At 0.5 mM Fe^{2+} and more than 20 mM DTT, non-enzymatic reactions to 4-coumaric acid were larger than the enzymatic reaction. DTT was more effective than GSH on enzyme activities to degrade the three substrates, but GSH was effective on enzyme activities to degrade ferulic acid and 4-coumaric acid. The results suggest that GSH may act as an endogenous reductant in vanilla pods, because GSH is the most abundant non-protein thiol compound widely distributed in living organisms.[24] In plants, the content of GSH is for example, 0.5 μmol/g FW in barley leaf (*Hordeum vulgare*),[25] 6.0 μmol/g FW in winter rape (*Brassica napus*)[26] and 0.65 μmol/g FW in cucumber leaf (*Cucumis sativus*)[27] and other vegetables.[28] β-Carotene 15,15'-dioxygenase activity also depends on SH-reagents such as DTT and GSH.[29] On the other hand, Podstolski et al.[7] reported that GSH was much less effective for enzymatic chain shortening and the enzyme activity was specific for 4-coumaric acid. In our study, however, the enzyme activity was clearly activated by the addition of Fe^{2+} and our results indicate that 4-coumaric acid and ferulic acid are equally good substrates for enzyme catalyzed cleavage of the side chains in the presence of GSH (Fig. 2).

NMR Analyses of enzymatic reactions and reaction mechanisms

Our previous report indicated that GA in addition to vanillin was formed from ferulic acid by the enzymatic reaction (Supplemental Fig. S2).[6] Vanillin was determined easily by HPLC with an ODS column and UV detector (Fig. 3-A), while GA was analyzed with an HPAE–PAD system after the treatment of the reaction mixture with a SAX cartridge (Fig. 3-B). GA was detected, but large amounts of the enzymatic reaction mixture was necessary to concentrate and desalt with a SAX cartridge[19] for

analysis with HPLC and another method without loss of the products for determination of GA is required. In this study, to obtain a more accurate measurement of GA, we analyzed the reaction mixture using qNMR. ^{1}H-NMR spectra of reaction products with 256 scans for approximately 50 min were measured. In the ^{1}H-NMR analyses, the singlet ^{1}H signals of aldehyde groups in the chemical structures of both authentic vanillin and GA are usually observed at chemical shifts of about 9.71 ppm (-C<u>H</u>O). However, a water solution of GA immediately led to a hydrate compound[30] and ^{1}H signal of the hydrated aldehyde group was observed at 5.175 ppm (-C<u>H</u>$(OH)_2$) (Fig. 4-A). Furthermore, we found that the chemical shifts of aldehyde groups in vanillin and GA changed to 5.377 and 5.451 ppm, respectively, in the presence of both DTT and DCl (Fig. 4-B). These results indicate that the hydration of aldehyde compounds can be performed for about 1 h under the presence of (10–40 mM) DTT in addition to 0.115 N DCl.[6] Vanillin and GA in the enzymatic reaction mixture (Fig. 4-C: DCl is lacking for the hydration) were identified after hydration by further addition of DCl solution to the mixture (Fig. 4-D, 5-A+) and compared with hydrated authentic compounds (Fig. 4-B, Supplemental Fig. S3). In the enzymatic reactions with 4-coumaric acid (Fig. 5-B+) and caffeic acid (Fig. 5-C+), the corresponding degraded products, 4-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, and GA after hydration were detected, identified, and quantified by comparison with hydrated authentic compounds (Supplemental Fig. S3). All signals of enzymatic reaction products for the three substrates (Fig. 5-A+, B+, C+) were larger than the non-enzymatic reaction products (Fig. 5-A-, B-, C-), suggesting that the enzyme catalyzed these reactions.

Amounts of the products in the enzymatic reaction carried out at 40°C for 1 h and measured by qNMR are shown in Table 1. Concentrations of DTT were 40 mM in ferulic acid and caffeic acid, and 10 mM in 4-coumaric acid. Amounts of vanillin and GA were calculated from the calibration curves in Fig. 6 and amounts of 4-hydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde from the qNMR analysis results of 100 nmol of the authentic compounds, because the signal areas of the aldehydes were changed after the treatment with DCl (Figs. 4 and 6). From ferulic acid and 4-coumaric acid, similar amounts of the corresponding benzaldehydes were detected in the enzyme reaction for 1 h (Table 1). Enzyme activity toward caffeic acid was much lower. Furthermore, the reactions from ferulic acid to vanillin and GA were more precisely investigated during the intervals. The results in Fig. 7 show that vanillin and GA similarly increased for 3 h. Some of the GA may have been oxidized and decomposed to formic acid over time.[19,31] In the ^{1}H-NMR spectra, signals of formic acid (<u>H</u>COOH) were detected at 8.452 ppm and 8.229 ppm as seen in Fig. 4-C and D (Supplemental Fig. S3),

respectively. Furthermore, $C\underline{H_3}$ signals (2.085 ppm) of acetic acid (AcOH) were also observed in spectra C and D (Supplemental Fig. S3). However, the acetic acid likely originated from the enzyme solution, because no signal of acetic acid was detected in the reaction without enzyme solution and the amount of acetic acid (29.2 nmol/tube) obtained by qNMR did not change during the enzymatic reaction (Fig. 7).

In this study, the enzyme degraded phenylpropanoids to yield the corresponding aldehydes and GA. These reactions appear to involve dioxygenase in the presence of Fe^{2+} and DTT, even though it is difficult to demonstrate a mechanism passing through either an epoxide[3)] or a deoxetane intermediate,[3–5)] or through Criegee rearrangement.[3,5)] On the other hand, Podstolski et al.[7)] and Gallage et al.[8)] explained a non-β-oxidative mechanism involving hydrolyase activity proceeds hydration of the side chain 2,3 double bond of 4-coumaric acid and ferulic acid with subsequent cleavage of the side chains to yield acetate and 4-hydroxybenzaldehyde or vanillin. In both mechanisms by dioxygenase and hydrolyase, the side chain 2,3 double bond of phenylpropanoids appears to be oxidized first. Then the C–C double bond of the side chain seems to be cleaved by a lyase that belongs to an oxygenase such as fatty acid hydroperoxide lyase. Moreover, two steps of the reactions are catalyzed by one enzyme. Therefore, we hypothesize that this series of reactions are catalyzed by one type of dioxygenase. The enzyme catalyzed the cleavage of the ferulic acid side chain to form vanillin and GA appears to be ferulic acid 2,3-dioxygenase or phenylpropanoid 2,3-dioxygenase. Co-factors of Fe^{2+}, GSH and ATP are necessary for enzyme activity. The enzyme activity seems to become stable and higher[7)] by the addition of ATP (data not shown). In the new work, it is proposed that millimolar concentrations of ATP may act to keep proteins soluble.[32)] The activity of the partially purified enzyme is low, while vanillin synthesized from ferulic acid appears to be immediately glucosylated to form vanillin glucoside and accumulate in the vanilla pods.[1,33)] The existence of enzyme activity catalyzing the glucosylation of vanillin was not demonstrated in our study. A revised biosynthetic pathway for vanillin is indicated in Fig. 8. In this study, we demonstrated that the reactions from (a) to (b) and (d) to (e) are catalyzed by phenylpropanoid 2,3-dioxygenase(s). Further investigations on ferulic acid 2,3-dioxygenase (or phenylpropanoid 2,3-dioxygenase) and vanillin 4-*O*-glucosyltransferase (or hydroxybenzaldehyde 4-*O*-glucosyltransferase) are in progress.

Conclusions

Previously, we demonstrated that the biosynthetic pathway for vanillin is 4-coumaric acid → caffeic acid → ferulic acid → vanillin → vanillin glucoside in *Vanilla planifolia* by pulse-chase experiments with ^{14}C-labeled compounds,[1)] and carried out partial purification and characterization of the enzyme involved in the conversion of ferulic acid to vanillin in vanilla pods to confirm the biosynthetic pathway. Enzyme activities were dependent on the concentration of DTT or GSH and activated by addition of 0.5 mM Fe^{2+}. Enzyme activity for ferulic acid was as high as for 4-coumaric acid in the presence of GSH, suggesting that GSH acts as an endogenous reductant in vanillin biosynthesis. Analyses of the enzymatic reaction products with qNMR indicated that an amount of GA proportional to vanillin was released from ferulic acid by the enzymatic reaction. These results suggest that phenylpropanoid 2,3-dioxygenase is involved in the cleavage of the ferulic acid side chain to form vanillin and GA in *Vanilla planifolia*.

Author contributions

O.N. and Y.N. designed the study. O.N. performed the experiments and wrote the manuscript. The authors reviewed and approved the final version of the manuscript.

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Table 1. Enzymatic reaction products from the three substrates

	Amount of reaction product (nmol/tube)	
Substrate	Corresponding benzaldehyde (%)	GA (%)
Ferulic acid	23.8 (100)	19.8 (100)
4-Coumaric acid	22.0 (92.4)	15.5 (78.3)
Caffeic acid	11.8 (49.6)	9.3 (47.0)

Notes: Enzymatic reactions were carried out at 40°C for 1 h and the amounts of products measured by qNMR. Concentrations of DTT were 40 mM in ferulic acid and caffeic acid, and 10 mM in 4-coumaric acid. (%) indicates the ratio of each reaction product to that from ferulic acid.

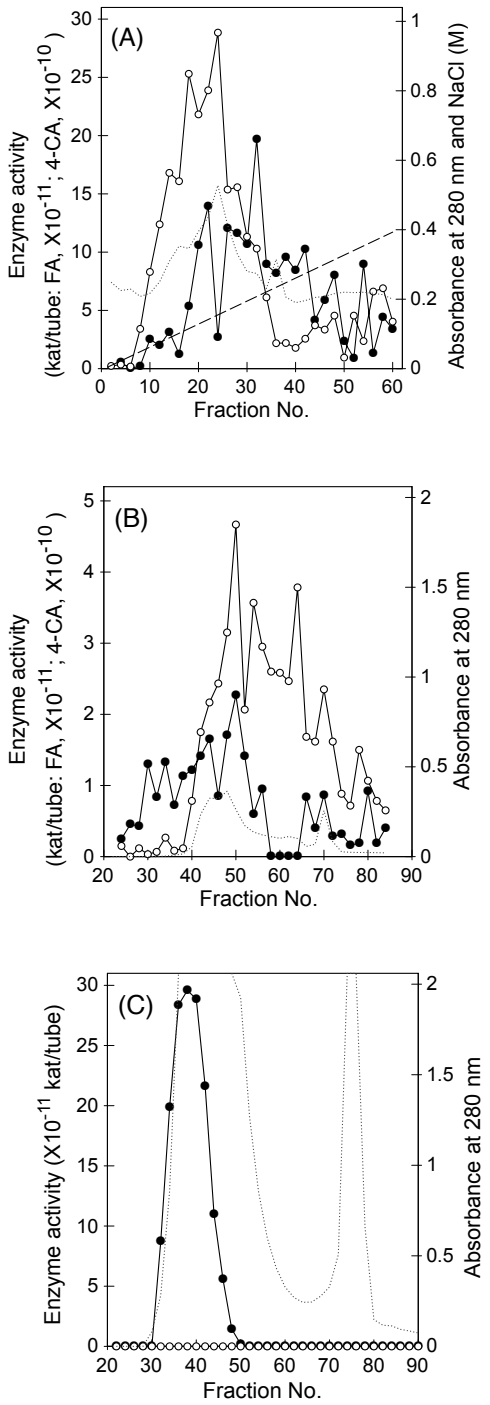


Fig. 1. Column chromatography on SP-Toyopearl 650S (A) and Toyopearl HW-55S (B and C). One dioxygenase fraction (Tube Nos. 17–22) in (A) was purified by Toyopearl HW-55S column (B). Chromatogram (C) is a purification of the glucosidase fraction eluted from the DEAE-Toyopearl column. ●, dioxygenase activity with ferulic acid (FA) and ○, dioxygenase activity with 4-coumaric acid (4-CA) (A and B); ●, glucosidase activity and ○, glucosyltransferase activity (C); --------, A_{280}; – – –, NaCl; fraction volume, 10 mL/tube (A) and 2 mL/tube (B and C).

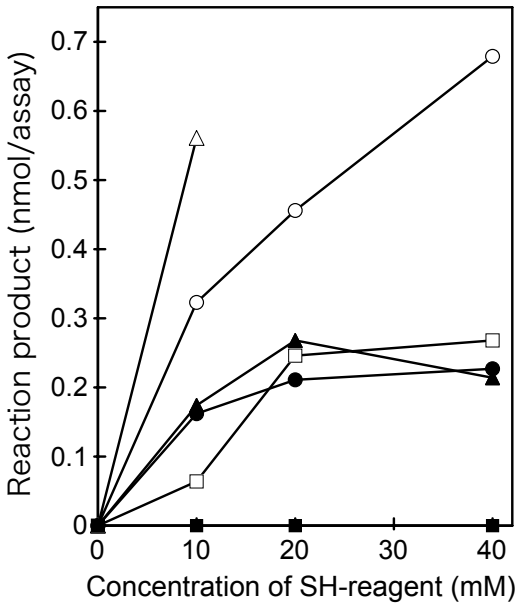


Fig. 2. Enzyme activities for three phenylpropanoids at several concentrations of SH reagents. The reactions were carried out at 30°C for 30 min with ferulic acid (○, ●), 4-coumaric acid (△, ▲) and caffeic acid (□, ■) in the presence of SH reagents (DTT, GSH, respectively), and their products analyzed by HPLC. At 0.5 mM Fe^{2+} and more than 20 mM DTT, non-enzymatic reactions to 4-coumaric acid were larger than the enzymatic reaction.

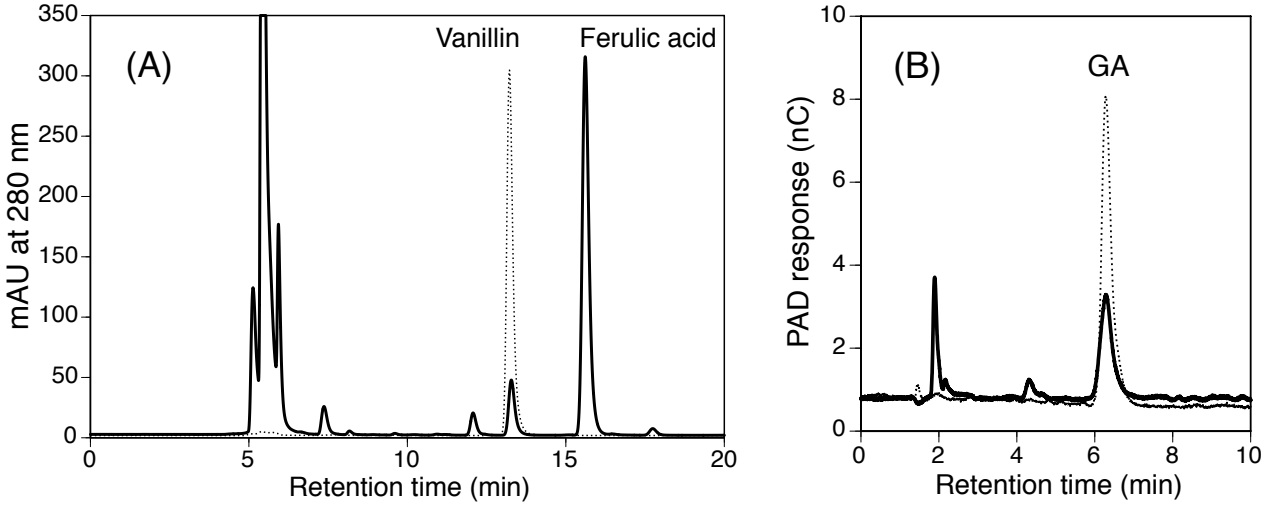


Fig. 3. Separation of enzymatic reaction products by HPLC with an ODS column (A) and an anion exchange column (B). Solid lines indicate the analyses of reaction mixtures and dotted lines those of authentic vanillin and GA.

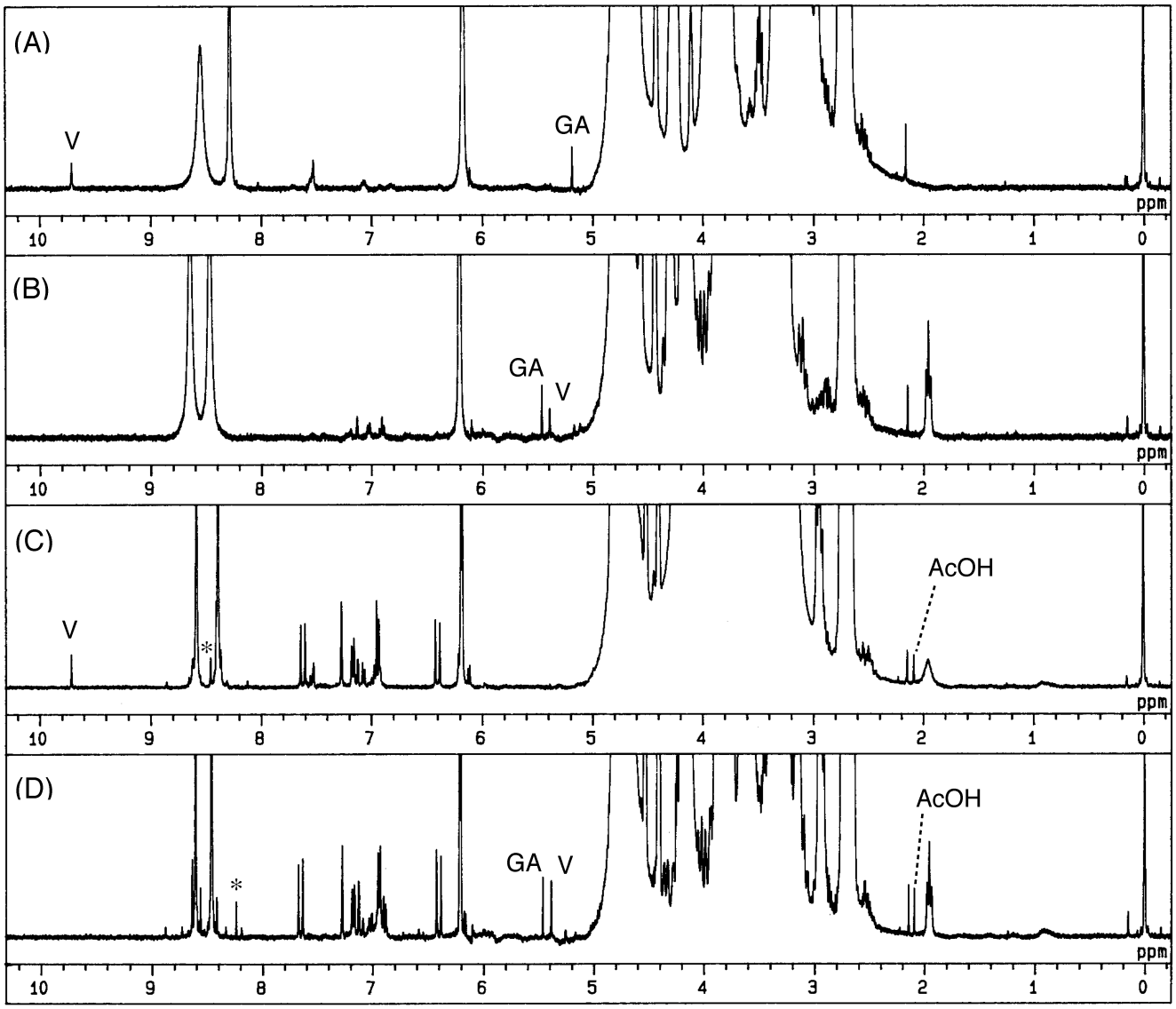


Fig. 4. ^{1}H-NMR spectra of reaction mixtures contained glyoxylic acid (GA) and vanillin (V). Signals of authentic GA and vanillin were detected in the solutions under the standard assay conditions, before (spectrum A) and after (spectrum B) the addition of 20 μL of 3N DCl. Enzyme reactions with ferulic acid were carried out for 4 h and stopped by the additions of 10 μL of 3N DCl (spectrum C) or 20 μL of 3N DCl (spectrum D). In addition, $C\underline{H_3}$ signals of acetic acid (AcOH) and $\underline{H}COOH$ signals (∗) were observed in spectra C and D.

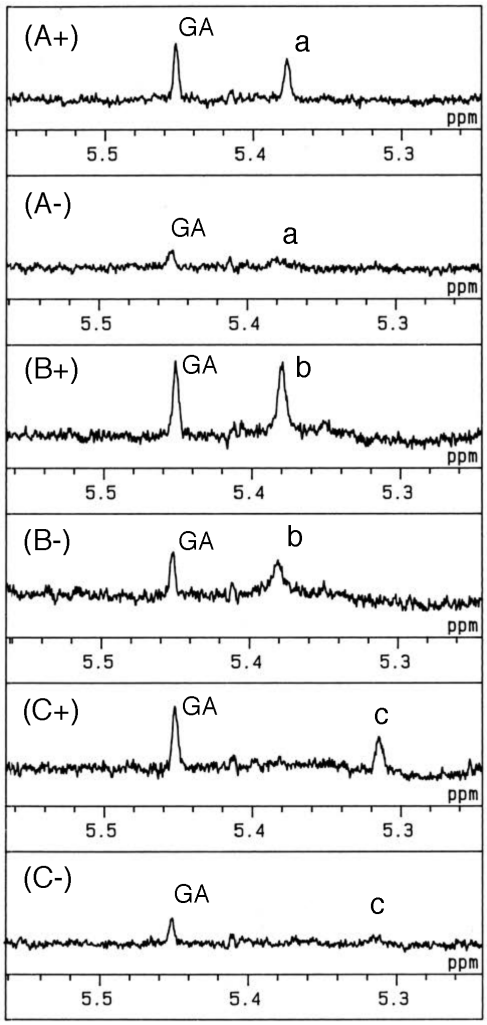


Fig. 5. Expanded ^{1}H-NMR signals of aldehyde groups in glyoxylic acid (GA), vanillin (a), 4-hydroxybenzaldehyde (b) and 3,4-dihydroxybenzaldehyde (c) formed in the enzymatic reactions at 40°C for 1 h with substrates of ferulic acid (A+), 4-coumaric acid (B+) and caffeic acid (C+) and the respective non-enzymatic reactions, (A-), (B-) and (C-) without the addition of enzyme solution.

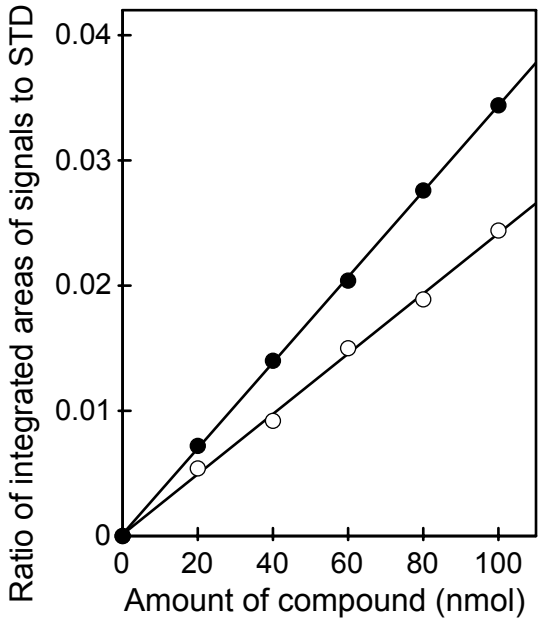


Fig. 6. Calibration curves of vanillin (○) and GA (●) in qNMR.

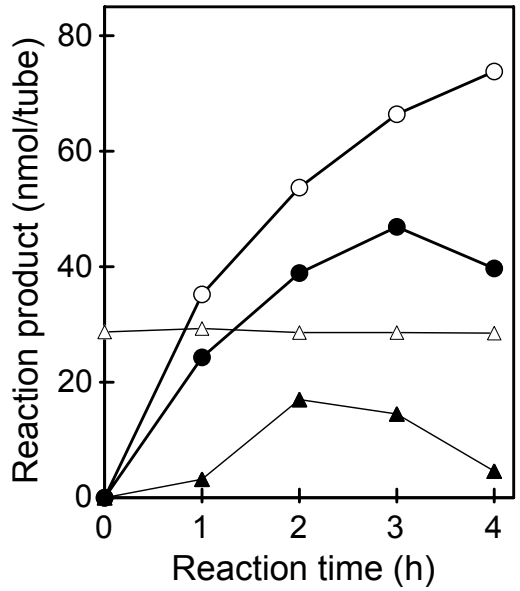


Fig. 7. Formation of vanillin, GA and two acids in the enzyme reaction with ferulic acid. The amounts of vanillin (○), GA (●), formic acid (▲) and acetic acid (△) were determined by qNMR.

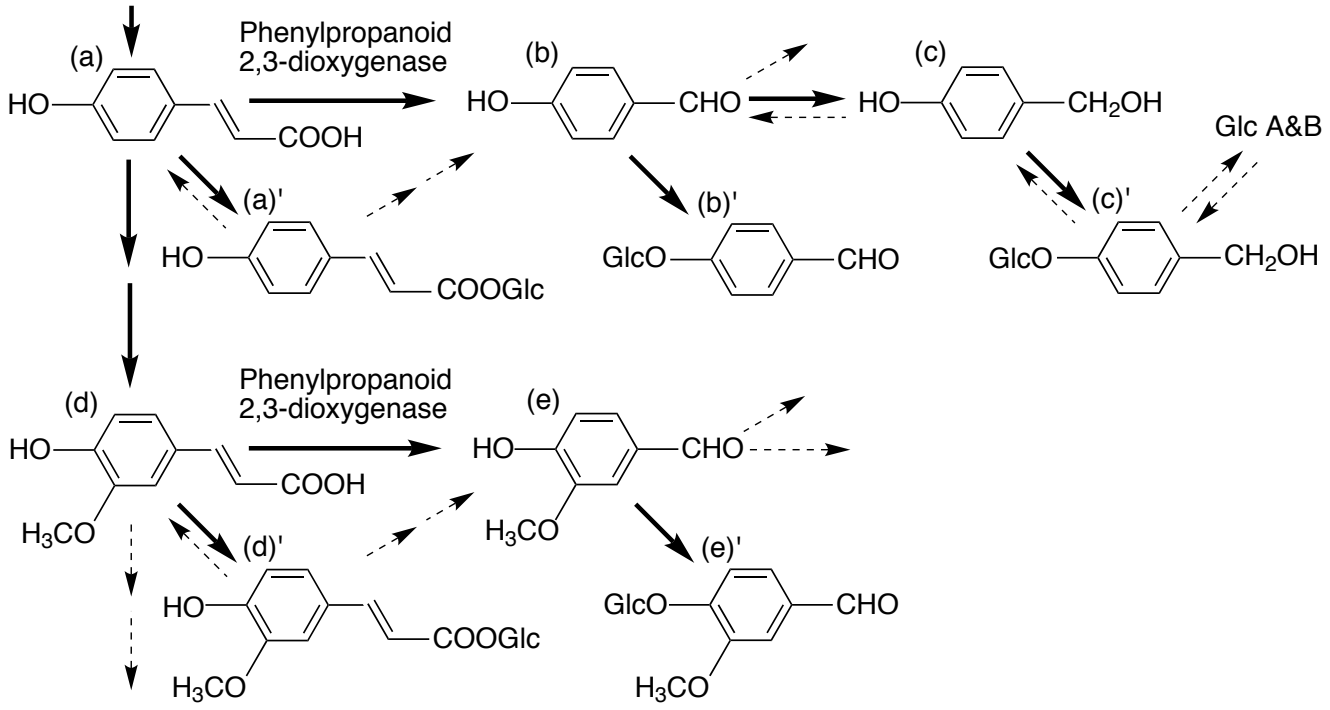


Fig. 8. Proposed biosynthetic pathway for vanillin and related compounds from phenylpropanoids, and formation of their glucosides and glucose esters in *Vanilla planifolia*. (a) 4-coumaric acid; (b) 4-hydroxybenzaldehyde; (c) 4-hydroxybenzyl alcohol; (d) ferulic acid; (e) vanillin; and (a)' (b)' (c)' (d)' (e)' show the respective glucose esters or glucosides. Glc A and B are esters of tartaric acid derivatives and 2 molecules of (c)' (bis[(β-D-glucopyranosyloxy)benzyl]-2-isopropyltartrate and bis[(β-D-glucopyranosyloxy)benzyl]-2-(2-butyl)tartrate, respectively). Bold and dotted arrows indicate identified and unidentified routes, respectively. In this study, we demonstrated that the reactions from (a) to (b) and (d) to (e) are catalyzed by phenylpropanoid 2,3-dioxygenase(s).

Supplemental material

Effects of ferrous ion, DTT and GSH on 2,3-dioxygenase

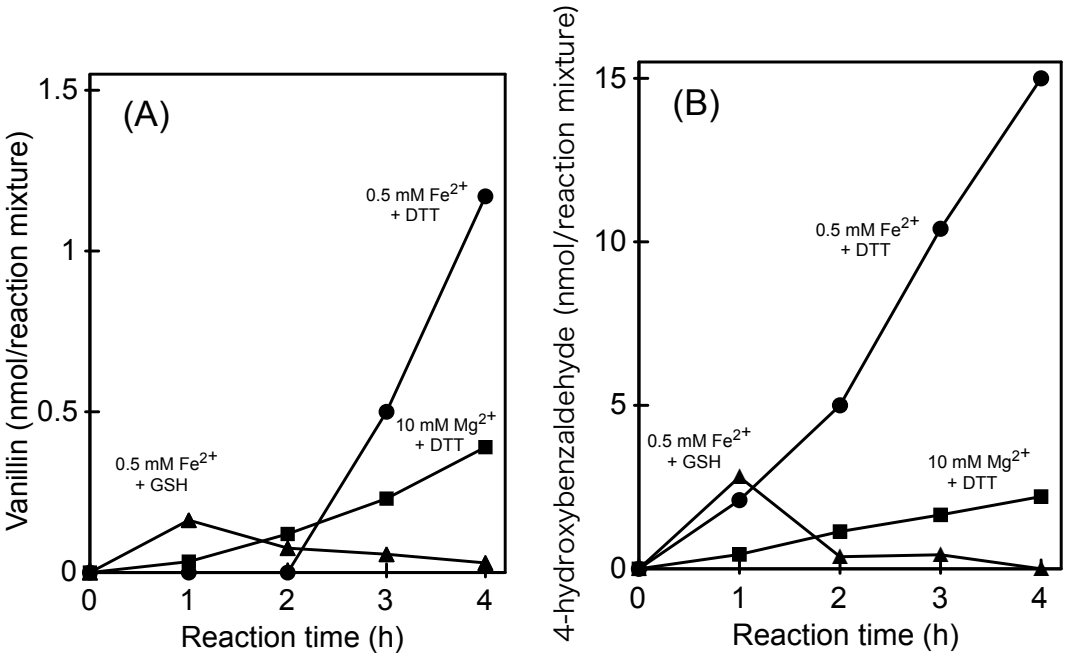


Fig. S1. Time-course for enzymatic reactions with ferulic acid (A) and 4-coumaric acid (B). ●, 0.5 mM Fe^{2+} + 5 mM DTT; ■, 10 mM Mg^{2+} + 10 mM DTT; ▲, 0.5 mM Fe^{2+} + 5 mM glutathione.

Determination of enzymatic reaction products by HPLC

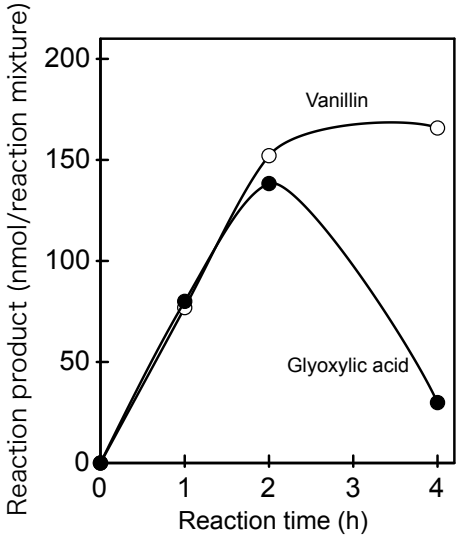


Fig. S2. Products in the enzymatic reaction with ferulic acid.

[1]H-NMR spectra of the authentic compounds

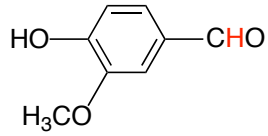
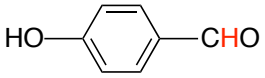
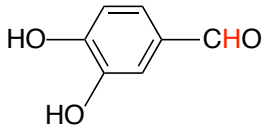
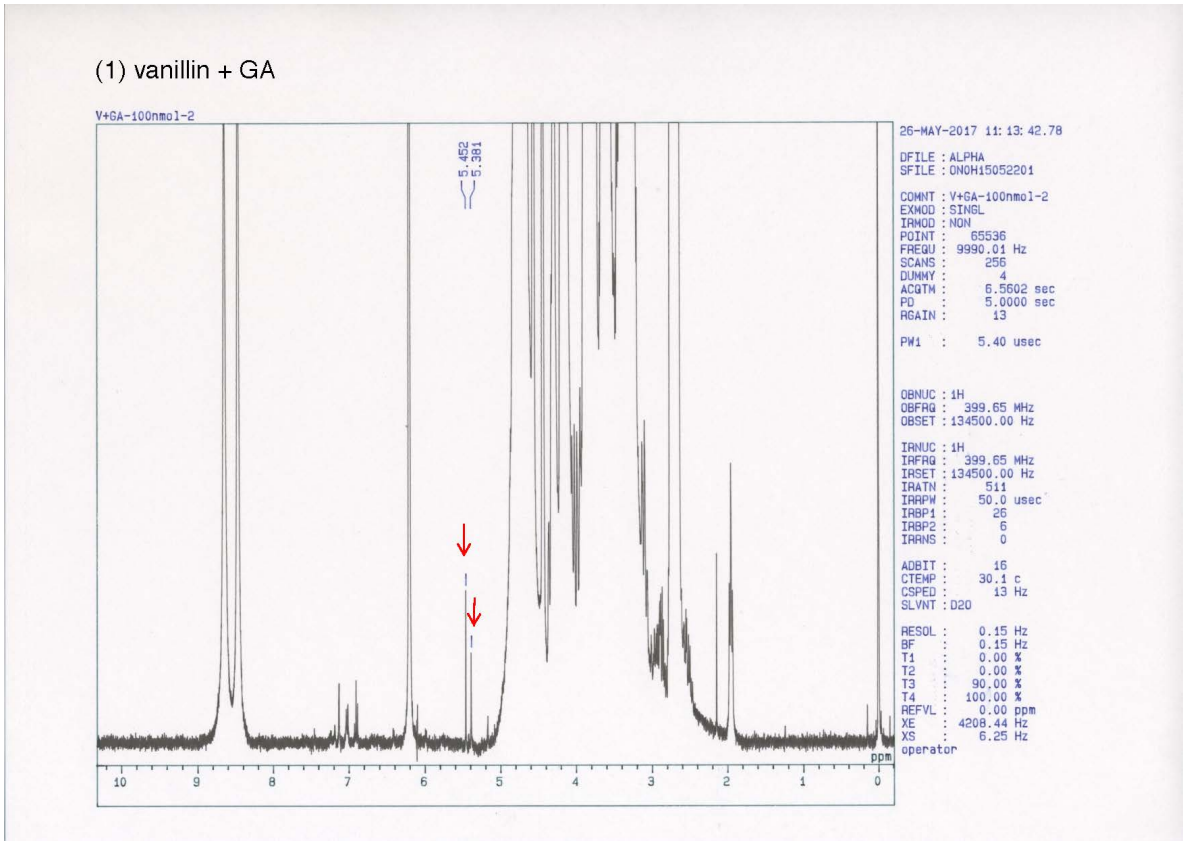
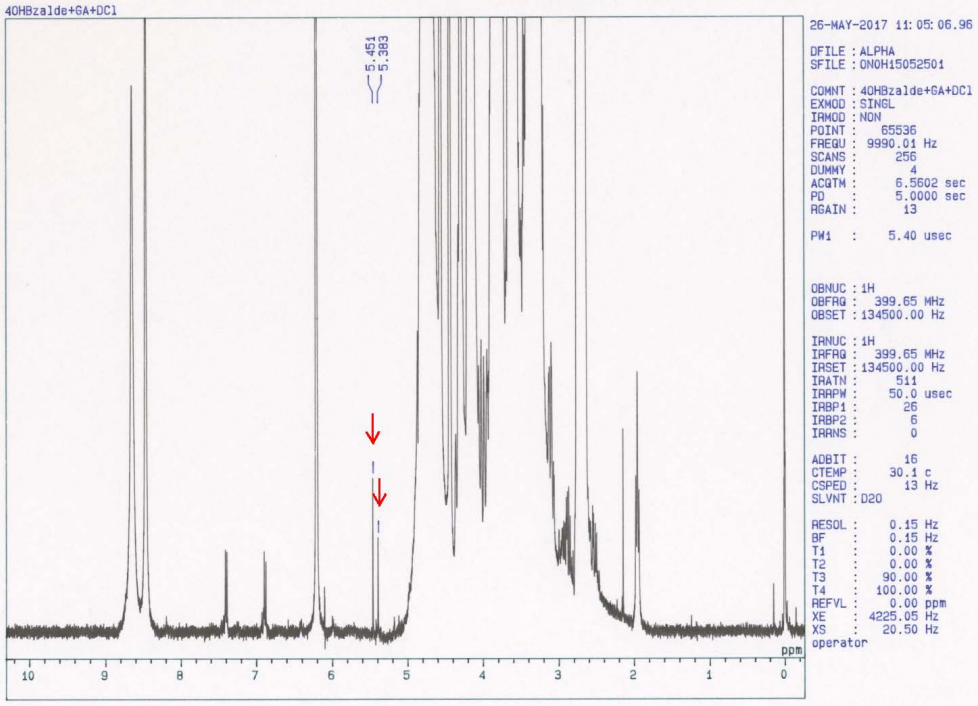
vanillin	**4-hydroxybenzaldehyde**	**3,4-dihydroxybenzaldehyde**
 (δ 5.381)	 (δ 5.383)	 (δ 5.316)
glyoxylic acid (GA)	**acetic acid**	**formic acid**
 (δ 5.451)	 (δ 2.085)	 (δ 8.228)

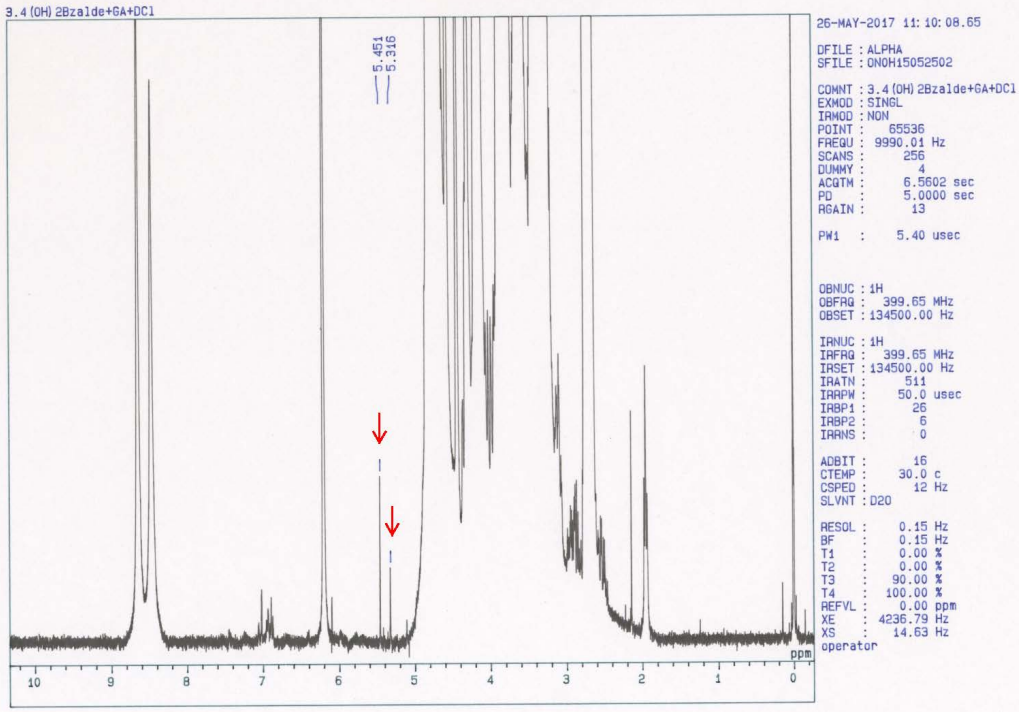
Fig. S3. [1]H-NMR spectra of the authentic compounds in the standard assay mixtures containing DCl were measured. The arrowed signals in the spectra were assigned to the protons in red in the above structural formulas of six compounds and their chemical shifts (δ) also indicated. The spectra of (1) vanillin + GA, (2) 4-hydroxybenzaldehyde + GA, (3) 3.4-dihydroxybenzaldehyde + GA, (4) acetic acid, and (5) formic acid are as follows.



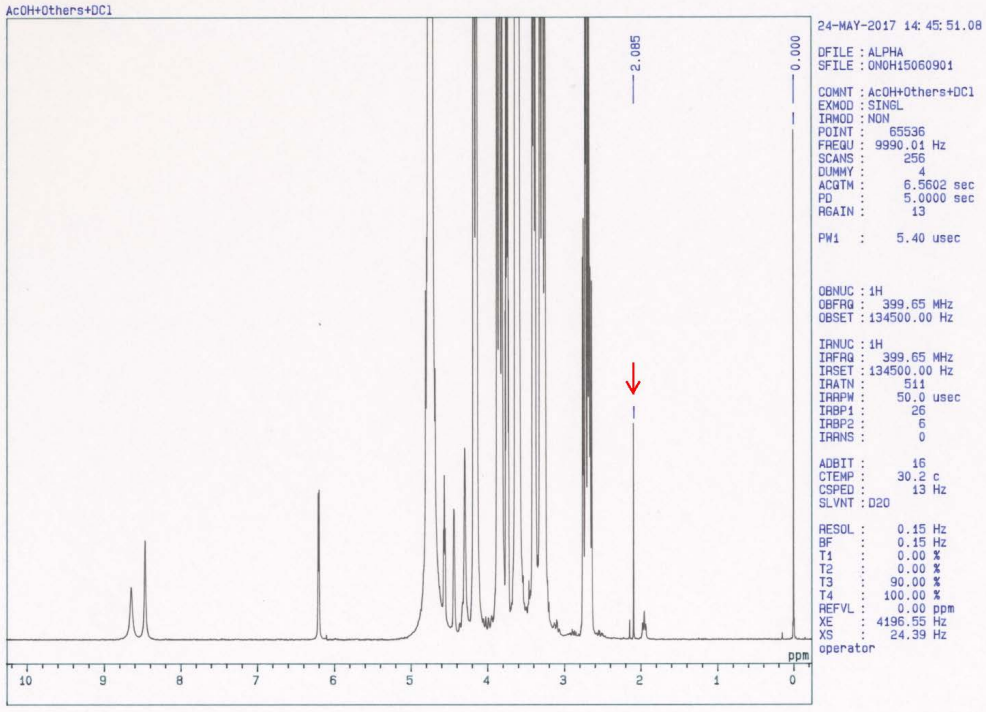
(2) 4-hydroxybenzaldehyde + GA



(3) 3,4-dihydroxybenzaldehyde + GA



(4) acetic acid



(5) formic acid