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Polymerization of EGCG using polyphenol oxidase and the purification of EGCG dimer

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화학생물공학부
최진경

Polymerization of EGCG using polyphenol oxidase and the purification of EGCG dimer

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폴리페놀 산화효소를 이용한 에피갈로카테킨갈레이트의 중합반응과 이합체의 분리정제

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Abstract

Tea leaves from *Camellia sinensis* can be classified into four different types of tea: white, green, oolong and black tea according to degree of fermentation. Black tea is produced by a fermentation process from the green leaves and contains oxidized catechins. These catechins form dimers, oligomers, and polymers in a heterogeneous mixture known as thearubigins. Thearubigins constitute more than 70% of the dry mass of an average black tea aqueous infusion. However, the structures of the catechins in thearubigins are remained poorly characterized. The monomeric catechins are oxidized with polyphenol oxidases(PPO), peroxidases(POD) and catalases which are critical in fermentation process.

In this study, epigallocatechin-3-O-gallate(EGCG), the most abundant monomeric catechins, was oxidized to EGCG dimers through radical oxidative reaction using laccase, a member of the tea PPOs family. Electrospray ionization tandem LC-MS (ESI-LC/MS) was applied for the characterization of the dimers, theacitrin(TC), dehydrotheasinensin(DhTS), and theasinensin(TS), which were produced by the radical oxidative reaction. They were

also analyzed by HPLC and LC/MS/MS to identify their structures according to their fragmentation patterns. The dimers were dismutated with heat treatment after oxidation step in order to mimic the burning and drying in the last step of fermentation in the laboratory. The result of the heat treatment, theasinensin A(TSA) was the major dimer product and was separated through Sephadex LH-20 and C18 column. Finally, 27.6mg of purified TSA was obtained in 100ml scale, and it is the first time that TSA has been produced by enzymatic reaction and isolated.

Keyword : epigallocatechin-3-O-gallate(EGCG), laccase, theasinensin A(TSA), LC/MS, purification

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1. Introduction

Tea catechins are mainly derived from two kinds of tea trees, *Camellia sinensis var sinensis* and *Camellia sinensis var. assamica*.[1] In food industry, tea is classified into white, green, oolong, and black tea depending on the degree of fermentation. Green tea is prepared by dehydration of tea leaves which does not lead to the oxidation of polyphenols. However black tea, obtained by tea leaves fermentation, is oxidized and contains mainly multimeric polyphenols whose is one of the most popular beverages in the world, accounts for 78% of the global tea production.[2, 3] Black tea produced from young green shoots of the tea plant, which are converted to black tea by a fermentation. There are two major processes, the 'orthodox' and the 'cut-tear-curl'. During these processes, polyphenol oxidases(PPO) contact with phenolic compounds and oxidize monomeric catechins which have four major non-oxidized forms: (-)-epicatechin (EC), (-)-epicatechin-3-gallate(ECG), (-)-epigallocatechin(EGC), and(-)epigallocatechin-3-gallate(EGCG)[4]

Black tea 85% of the catechins are oxidized into complex phenolic compounds. Table 1 shows that theaflavins(TF) and thearubigins(TR) are the major components in polyphenols. [5] The

Compound	Type of tea	
	Green	Black
catechins	10-30%	3-10%
	(51.5-84.3mg/g)	**(5.6-47.5mg/g)**
Theaflavins(TF)	0%	2-6%
Thearubigins(TR)	0%	10-20%
Phenolic acids	2%	1%
Flavonols	2%	1%
Other polyphenols	3-6%	3-10%
Total amount of polyphenols	**87-106.2mg/g**	**80.5-134.9mg/g**
Caffeine, theobromine, theophylline	3-6%	3-6%
Amino acids	~10mg/g	~5mg/g
Theanine	2%	
Peptides, proteins	6%	6%
Organic acids	2%	2%
Mono-, disaccharides	11mg/g	11mg/g
Mineral substances	10-13%	10-13%

Table 1. The variation in composition of green and black teas

former are reddish-orange pigments of black tea by oxidative condensation between EC and EGC. The latter are the most abundant group of polymeric polyphenols that are formed during the enzymatic oxidation and condensation of two gallocatechins. The TFs and TRs in black tea infusion contribute to rusty color and richness taste but many components of TRs have been uncharacterized yet. A total amount of catechins is higher in green tea, composing 10-30% of the dry weight percent that is 50-80mg/g, but there are no TF and TR compounds. [6] The synthesis of TFs and TRs has been studied because they have anti-oxidant, anti-inflammatory and anti-obesity effects better than monomeric catechins.[7-9]. So, it is necessary to develop an enzymatic process in order to analyze structures of TF and TR and increase the product yield.

Oxidative enzymes in tea leaves are polyphenol oxidases(PPO) and peroxidases(POD).[10] The PPOs are copper containing enzymes that include laccase and tyrosinase. As shown in figure2 laccases need oxygen as oxidizing agent for catalysis and these are able to catalyse the oxidation of o-diphenols into o-quinones via a radical reaction.[11, 12] The mechanism is that four-electron substrate oxidaized and results in reductive cleavage of dioxygen bond. Cu metal atoms within the enzymes play key roles in the

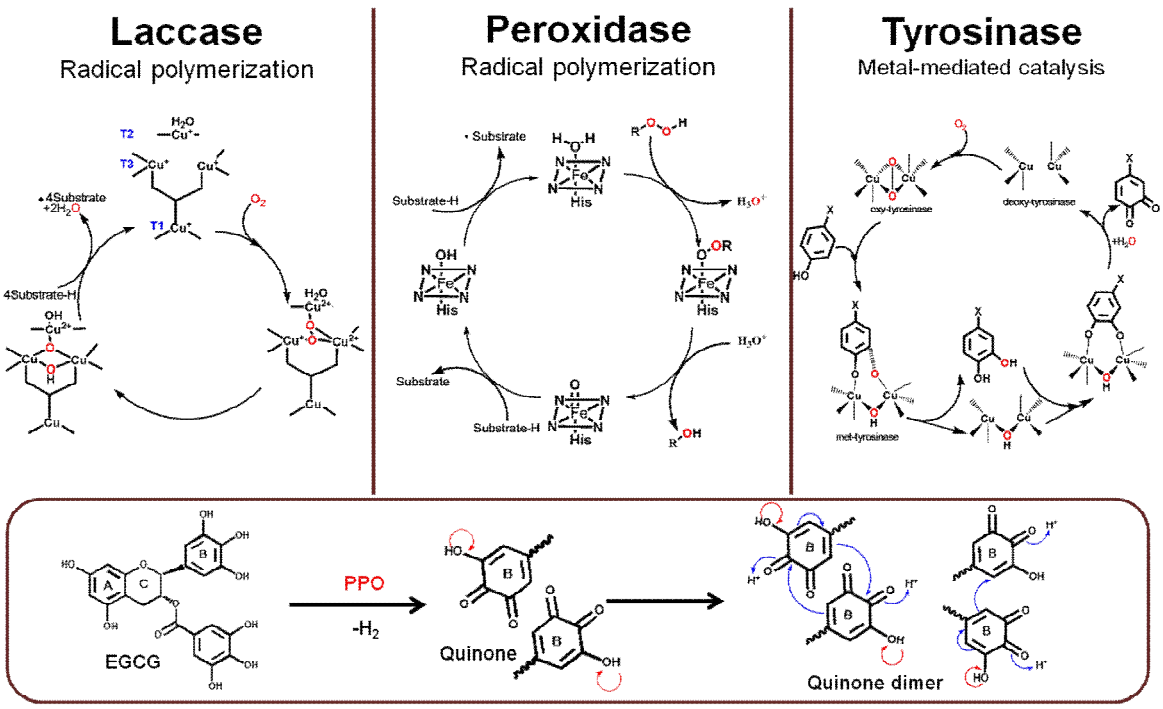


Figure 1. Comparison of PPOs and POD enzymatic mechanism

reduction of O2 to H2O.[13, 14] The latter, tyrosinase activity is hydroxylation of a mono-phenol into an o-diphenol that oxidized to an o-quinone. The PODs are heme-enzymes that catalyse the oxidation of o-diphenols into o-quinones via a radical reaction and require a peroxide as oxidizing agent to catalyse the oxidation reaction, such as H_2O_2. In tea leaves, there are uncharacterized polyphenols more than characterized because the PPOs and PODs will participate in the metabolic reactions for synthesis of TF and TR. TFs are formed from oxidative reaction with PPO between catechol and galloyl groups. During catalytic reaction, H_2O_2 is genetrated. Then, POD will participate in the oxidation for TRs and TRs. However, theasinensin(TS) and theacitrins(TC) are both formed from two galloyl groups and included in TR.[7, 15, 16] (Figure 2) The dimers are connected through B-rings of catechins and TR dimers are major polyphenols in black tea. Since , the gallo-catechin, EGCG is the most amount of total catechins as 45-65%, followed EGC, ECG, and EC. So, we focused EGCG and dimers drived from only EGCG as substrate by enzymatic oxidation. The possible structures of the dimers are theacitrin A(TCA), dehydrotheasinensin A(DhTSA), theasinensin A(TSA), EGCG dimer P2 and so on. Among them, TSA has two catechin B-rings are connected through C-C bond and more stable than other

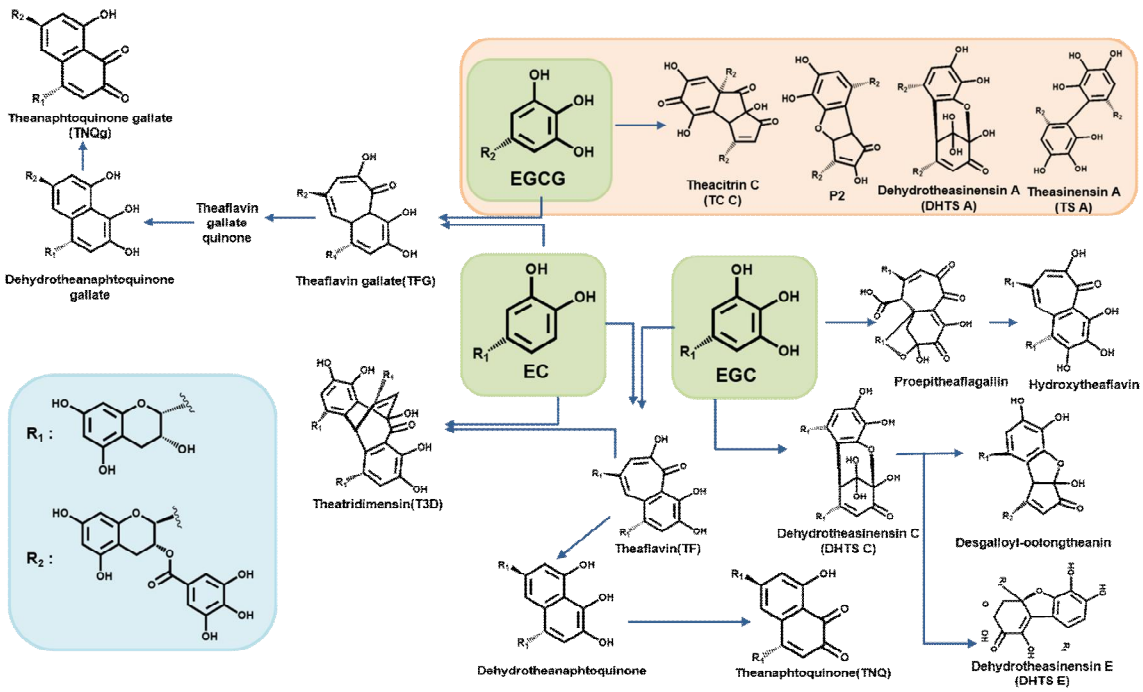


Figure 2. Possible catechin dimer structures

EGCG dimers. It is produced during heating and drying of tea leaves in the final stage of black tea production. DhTSA and other unstable dimers undergo redox dismutation to give reduction product, TSA and atropisomer theasinensin D(TSD).

TSA is a biologically active black tea polyphenol, The effects of TSA have been reported that has anti-oxidant, anti-cancer, anti-inflammation hypoglycemic effects and anti-obesity because potent inhibitor of squalene epoxidase, a rate-limiting enzyme in cholesterol synthesis.[17-21] Although, there are few studies about dimerization of catechins via enzymatic reaction and mass production to use in goods , such as, cosmetics and health food.

In this study, we have synthesized catechin dimers using PPO and mimicked the process of fermentation by heat treatment after the reaction to produce TSA.(Figure 3) The products analyzed by ESI-LC/MS for identification of structures of synthesized dimers. And then purified the TSA through two steps column chromatography.

EGCG was reacted with three representative enzymes of PPO, *Tramates versicolor*_laccase(TvLac),Horseradish peroxidase(HRP) and mushroom_tyrosinase(MuTyr).[3] The laccase reaction without hydrogen peroxide is more efficient than peroxidase, so the enzyme is used to oxidase EGCG further experiments.

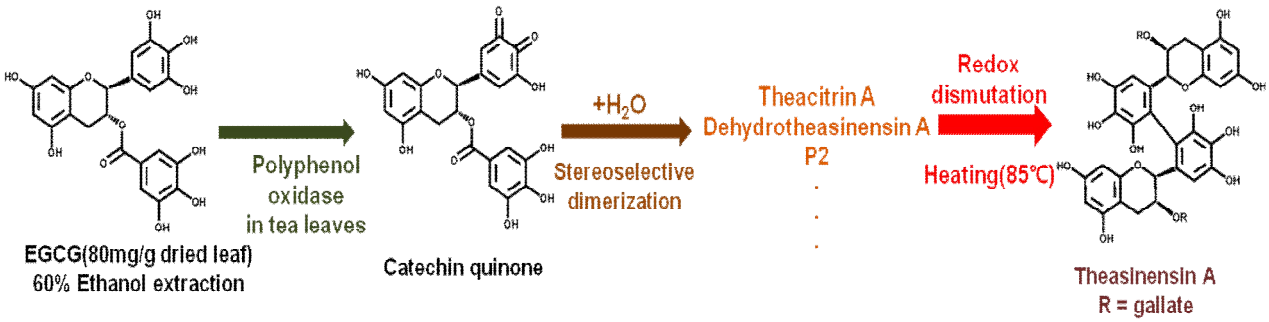


Figure 3 Formation of theasinensin A(TSA) from EGCG by polyphenol oxidases(PPOs)

The analysis of dimers using HPLC-ESI/MS/MS and reaction conditions were optimized. Also, we used deuterium oxide as reaction buffer to identify the structures of dimers have increased molecular weight successively other than TCA, TSA, and DhTSA. [15, 22, 23]

Additionally, as a proof of concept, cell fermentation was carried out using fungal strains, *aspergillus oryzae* and *aspergillus niger* for obtaining laccase or polyphenol oxidases expressed through fermentation. It has been reported that laccase(PPO) obtained from *aspergillus sp.*[26-28] In this study, we used $CuSO_4$ and xylidine as inducers to secrete laccase that oxidizes the EGCG.[26, 29] If this research will be optimized for production of catechin dimers, mass production and application of health food and cosmetics are possible.

2. Materials and methods

2.1 Chemicals and instruments

Laccase from *Trametes Versicolor,* Horseradish peroxidase, mushroom tyrosinase and citric acid were purchased from Sigma aldrich and used without any further purification. Potassium phosphate monobasic(Fisher Scientific Korea, Seoul, South Korea), potassium phosphate dibasic(Fisher Scientific Korea, Seoul, South Korea), acetone(Merck Korea, Seoul, South Korea), Sephadex LH-20(GE healthcare, Sweden), C18 bead(Alltech, USA), and seasand(Junsei, Japan) were purchased and used without any further purification. Epigallocatechin gallate(98%) was provided by Amorepacific company(Korea). Amicon ultracel 10k cellulose filter(Millipore, Ireland) was used for enzyme filtration after reaction. Matrix-assisted laser desorption/ionization-time of flight(MALDI-TOF) mass spectroscopy(Bruker Daltonics Microflex LRF, Bruker, Germany)and LC-MS(TSQ Quantum access max) were employed to investigate the mass spectra.

2.2 Dimerization of EGCG into theasinesin A

2.2.1 Dimerization in 1ml reaction volume

1 M of EGCG was dissolved in pure methanol to make a stock and enzyme mixture was prepared with 1 U/ml of laccase in 50 mM citrate potassium phosphate buffer(pH 5.0)in total volume 1ml. 5 μL of EGCG stock was mixed with the buffer containing enzyme solution in the 2 mL eppendorf tube. Reaction mixture was incubated in the shaking incubator at 100 rpm in 25 ℃ for 30 minutes. After reaction, the reaction solution was heated for 15 minutes at 85 ℃ for redox dismutation then stored in 4℃.

2.2.2 Dimerization in 100ml reaction scale

1 M of EGCG wwas dissolved in pure methanol to make a stock and prepare enzyme mixture was prepared with 1 U/ml of laccase in 50 mM citrate potassium phosphate buffer(pH 5.0) in total volume 100ml. 500 μL of EGCG stock was mixed with the buffer containing enzyme solution in the 500 mL flask. Rest of the process was the same as in 1ml scale.

2.2.3 Comparison of oxidative reaction products with laccase, peroxidase and tyrosinase

EGCG(5mM) was incubated with 1U of laccase frome *Tramates*

versicolor(TvLac), 1U of Horse radish peroxidase(HRP) 1U and 4U of tyrosinase from mushroom(Mutyr). Then, 5mM hydrogen peroxide was added in case of peroxidase reaction. Since the tyrosinase has lower activity than others, we used greater amount of enzyme to control the conversion similarly.[3] All reactions were performed in 50mM citrate potassium phosphate buffer(pH5.0) in total volume 1ml. The reaction conditions were the same as in 1ml scale.

2.3 Manufacturing column using size exclusive and hydrophobic resin

2.3.1 Column manufacturing with size exclusive resins

Sephadex LH-20 is usually used in gel filtration chromatography and the bead size is 25-100 ㎛ for molecular sizing of natural product. The bead should be swelled in methanol at 4 ml / g for 3 hours before filling the column. When swelled in methanol, the bead size grows in size to a maximum of 163 ㎛ and exhibits better resolution. In order to separate and purify reaction products of 100ml scale, 60g of beads were swelled in methanol and packed into a 5cm inner diameter size column and 26cm in height of column was made. (Figure 4A)

(A) (B)

Figure 4. Manufactured column from packing beads. (A) Sephadex LH-20 column, (B) C18 column

2.3.2 Column manufacturing with hydrophobic resins

According to HPLC data, C18 beads were used because various dimers or other degradation products and TSA can be separated by their hydrophobicity. So, 30g of beads was filled into a 2.5cm inner diameter size column up to the height of the column was 20cm. (Figure 4B) After the resin packing was completed, the washing process was performed with the same composition as the eluent used.

2.4 Separation and purification of TSA

The reaction solution of oxidized in 100ml is concentrated using vacuum evaporator and dissolved in 50% methanol 2.5ml. The solution is subjected to Sephadex LH-20 column chromatography(5cm inner diameter x 25cm) with eluents with gradually increasing water content of acetone 55%, 300ml and finally 60% acetone 200ml to collect 20 fractions. All eluent contains 1% acetic acid to maintain stable dimer compounds in acidic condition. The fractions were evaporated using vacuum evaporator at 60℃ and re-dissolved in 50% MeOH for analysis with LC/MS. Some fractions including TSA were further separated by C18 column chromatography(2.5cm inner diameter x 20cm, 50%

MeOH elution) to obtain purified TSA. When fractionating with C18 column, pressure was applied using air to maintain a constant flow rate. Otherwise, the sample will be diffused in the column because the flow rate is very slow. The fractions were analyzed by LC/MS same as above and fractions containing pure TSA were evaporated to remove MeOH and then lyophilized.

2.5 Electronspray Ionization-Mass Spectrometry (ESI-MS)

Mass spectrometry data was obtained from TSQ Quantum access max (Thermo Scientific) equipped with an ESI probe coupled to the HPLC system. Nitrogen was used as both sheath gas and auxiliary gas. Data were collected over the 400-1500 m/z ranges through full scan mode, and data dependent MS/MS analysis was performed with a normalized collision energy of 20%. The MS/MS fragmentation was performed on the most intense product ion in the MS spectrum and the system was tuned in positive ionization mode. The transfer tube temperature was 300℃ and the source voltage was 3.8 kV. Data acquisition and reprocessing was performed with Xcalibur 2.07 (Thermo Scientific). The mass spectrometer was run in three different modes: Full MS, MS/MS on selected ions with the intensity greater than e^5. The substrate, EGCG was quantified by the MS signal intensity area and the

calibration curve (ranging from 0.05-2mM) has correlation coefficient of 0.87.

2.6 Reverse phase-HPLC analysis

Samples were analyzed on an Accela HPLC system equipped with a pump, autosampler at 10℃ and a photodiode array(PDA) detector. Sample (10 ㎕) was injected on to Agilent Eclipse Plus C18 column (4.6x100mm, particle size of 3.5um). Water acidified with 0.1%(v/v) formic acid(eluent A) and Acetonitrile acidified with 0.1%(v/v) formic acid(eluent B) were used as eluents. The flow rate was 600㎕/min, and the column oven temperature was set to 30℃. The following elution profile was used: 0-5min, isocratic on 5% (v/v) B: 5-20min, linear gradient from 5 to 50% (v/v) B; 20-21min, linear gradient 50 to 95% (v/v) B; 21-23min, linear gradient 95 to 5% (v/v) B; and 23-25min, isocratic on 5% (v/v) B.

2.7 Aspergillus fermentation for secretion of laccase

2.7.1 Strains, media and culture conditions

Aspergillus is well known as a fermentation strain of tea leaves. Among them, two strains of *aspergillus oryzae* and *aspergillus niger* (ATCC 16888) were grown under minimal media conditions and

EGCG reaction was performed with secreted enzyme. Long-term stocks were maintained on potato dextrose agar(PDA) slants stored at 4℃. The strains were cultivated for oxidase production in following aspergillus minimal medium : 10gL^{-1} dextrose, 300gL^{-1} NaNO3, 0.5gL^{-1} KCl, 0.5gL^{-1} $MgSO_4$:7H_2O, 0.4mlL^{-1} Cove' s trace elements (x1000) in 12mM potassium phosphate pH 6.8.

2.7.2 EGCG reaction with expressed laccase from fugal strains without induction

Two loopfuls of strains were transferred from a PDA slant to 50ml of the minimal media in a 250ml Erlenmeyer flask. The flasks were incubated aerobically(27℃, 200rpm, 4days). The culture broth was centrifuged at 4000g for 10min at 4℃ and the supernatant was concentrated with a 10K ultra filtration filter. The cell pellet was dissolved in 5ml tris-HCl buffer(5mM Tris-HCl buffer pH 7.5) in order to disrupt the cell and centrifuged 16000 rpm for obtaining cytosolic expressed enzyme in soluble fraction.

The concentrated supernatant and soluble fraction reacted EGCG to confirm the laccase activity. Reaction conditions are same in 1ml reaction volume scale and the amount of enzyme is 25ul of concentrated supernatant and 200ul of soluble fraction.

2.7.3 EGCG reaction with expressed laccase from fugal strains without induction using $CuSO_4$ and xylidine

Two loopuls of the fungal strains grown on plates with PDA were transferred to 50ml of the minimal media in a 250ml Erlenmeyer flask. The flasks were incubated aerobically(27℃, 200rpm, 3 days) and induced by 1mM CuSO4 and 40uM 2,5-xylidine, respectively. After that, the flasks were incubated more 3 days as same culture conditions. The dry cell weight before induction was 3.6mg/ml. Cultures were centrifuged 4000g for 10min and the supernatant was filtrated with 10K ultra filtration filter to 0.5ml. EGCG reaction with concentrated enzymes was same as above.

3. Results and discussion

3.1 Comparison of catalytic activity and product structure profile of PPOs

TvLac and HRP were used 1U and MuTyr 4U, because tyrosinase has lower activity with EGCG than that of laccase and peroxidase. The conversion rate of the TvLac, HRP, and MuTyr was 64.4, 92.6, and 54 percent respectively though EGCG consumption by HRP depended on the concentration of H_2O_2. As shown in figure 5, the peak in 7.7−8.0 retention time is EGCG and broad peaks of both side are dimers. Enzyme activity is different, but the kinds of dimers produced are the same.[22, 30] The EGCG dimers are known to exist in four types which are theacitrin(TC) and dehydrotheasinensin A(DhTSA), and the other two are unknown, In mass profile of PPOs, TC is dominant products of TvLac and 948.3 m/z dimer is major of HRP and MuTyr. (Figure 6) However, MuTyr was used 4 units that four times more than others to make dimer products for comparison with others. Because tyrosinase typically oxidize monophenol compounds as substrate, gallyol groups are difficult to be oxidized by tyrosinase. Although TC, TS, and DhTSA have identified structures, three types molecular weights are unknown as 948.4, 964.3, 980.4 m/z. The analysis of reaction products was performed with LC/MS.

As a result, it was concluded that the oxidation by laccase or

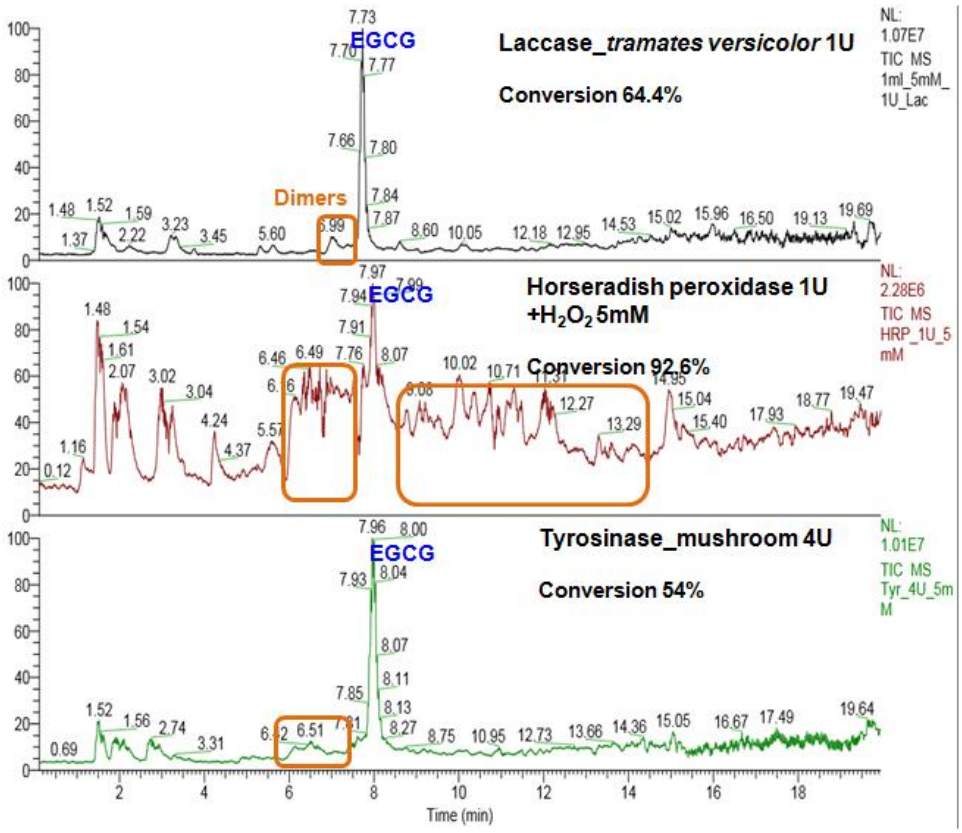


Figure 5. TIC of PPO and POD

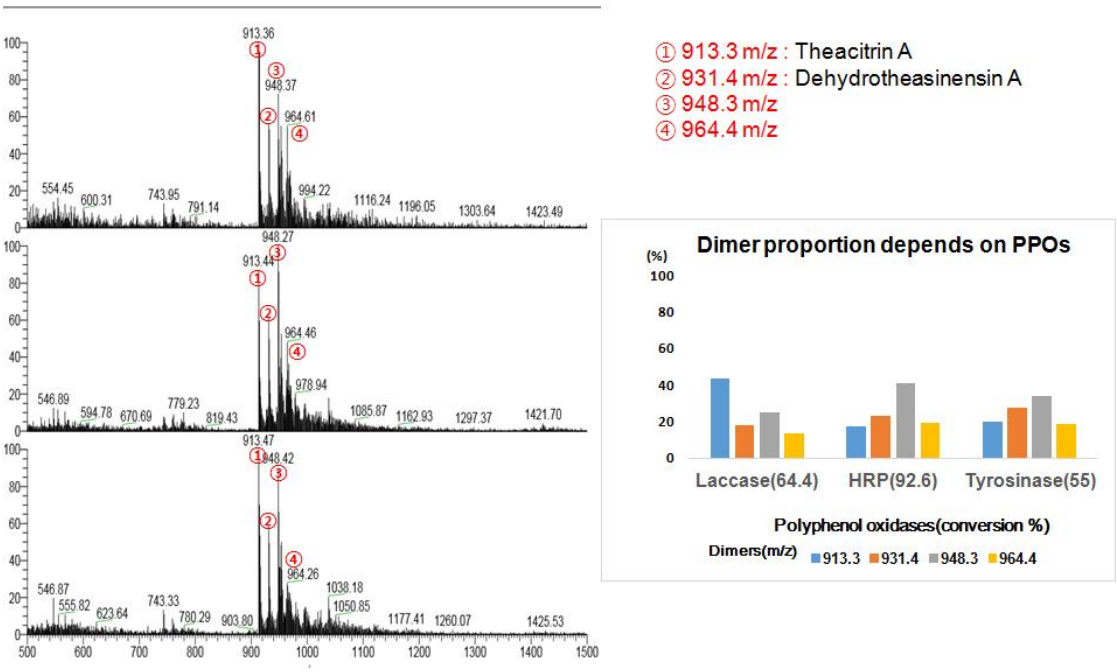


Figure 6. Mass profile of TvLac, HRP and MuTyr

peroxidase was dominant and the difference between the enzymes was not enough large active site to accept substrate which has galloyl group like EGCG. In addition, O-quinone is formed in the b-ring of EGCG, and the dimer is formed by radical bonding. Hydroxylation by the addition of water occurs during the reaction and dimers of complex structure with high molecular weight were produced.[31-33]

3.2 EGCG dimerization using laccase and analysis by LC-MS

Laccase was used to oxidize EGCG to dimer and we optimized the reaction conditions on a 100ml scale. If the reaction volume increased, oxygen transfer rate and amount of dissolved oxygen are changed then, the conversion of laccase will also increase. Because the reaction of laccase is highly influenced by oxygen. The reaction was performed under varying enzyme concentration and reaction time, without changing other conditions. Table 2 shows the conversion rate of TvLac according to varying conditions of enzyme units and reaction time. Condition 3 in which we used 1 unit of enzyme for 50 minutes showed the highest conversion rate. The result indicates that conversion rate is more time dependent on

reaction time than an amount of enzyme used, and we could find a condition with the greatest conversion rate(89%) for EGCG

	1	2	3
Units(U)	1	2	1
Reaction time(min)	30	30	50
Conversion(%)	61	69	89

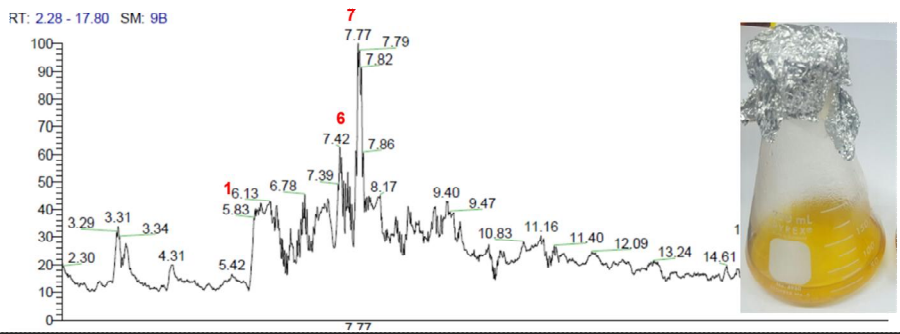
Laccase_*Tramates versicolor
50mM Citrate sodium phospate buffer pH5.0, 25℃, 100rpm
EGCG10mM, Total volume 100ml in 500ml flask

Table 2. Conversion(%) of the TvLac dependent on different reaction condition

dimerization. The oxidized products of EGCG were analyzed by TSQ. The data showed lots of broad peaks and was difficult to separate indivisual dimers. The detected dimers were identified through the LC-MS/MS fragmented ions and the intensity of unreacted EGCG was calculated to yield conversion rate by the laccase. As shown in figure 7A, before the heat treatment process, there were six different structure of dimers and two of theme were identified. Figrue 8A shows the mass fragmentation and analyzed structures of Theacitrin C(TCC)(913.3m/z). The ether in hetero cyclic ring C and ester bond to galloyl group can easily be broken, so the ions provide information to identify the synthesized dimer structure. There were m/z values of 787, 743, 635, 617, 573, 423, 164, 152 and 139. The four important fragment ions were 743, 635, 573 and 423, which their structures were elucidated in figure 8A. The 617 m/z ion was derived from a neutral loss of water molecule from 635 m/z ion. This data indicates that the TCC is synthesized by merging ring B of EGCG and has two five membered rings. Figure 8B shows fragmented ions and their structure of the DhTSA(931.4 m/z) that include m/z 787, 761, 591, 587, 435, 304 and 291 and 587, 555, 435 and 304 which were derived from loss

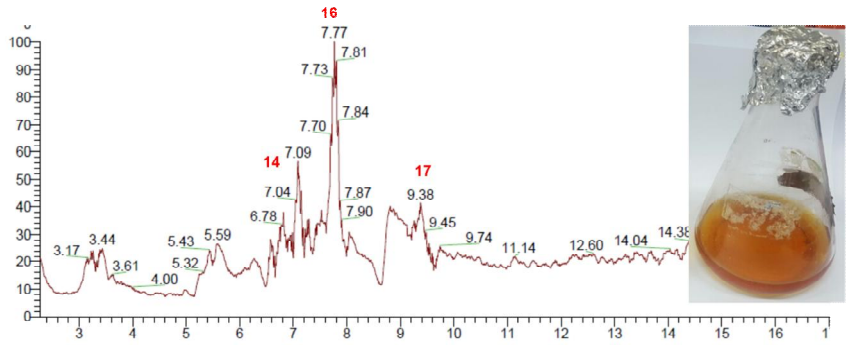
of two water molecules frome TCC. DhTSA was synthesized through EGCG quinone and addition of water, causing hydroxylation

(A)



Peak no.	RT(min)	LC/MS [M+H] (m/z)	fragment ion LC-MS2 (m/z)	Trivial name
1	5.88	510.3	474.7, 304.7, 287.3, 260.8, 152.6, 139.1	
2	6.21	948.5	806.1, 787.9, 757.2, 744.4, 634.5, 572.8, 439.1, 330.9, 315.2	unknown
3	6.33	949.2	932.2, 914.1, 896, 636.5, 574.5, 404.4, 382.4	unknown
4	6.44	948.3	946.4, 912.6, 743.4, 717.4, 605.4, 562.9, 555.4	unknown
5	6.79	913.3	787.2, 616.8, 591, 573.4, 563.4, 453.2, 423.2, 368.4, 152.3, 139.1	Theacitrin C
6	7.66	931.1	590.9, 416.6, 333.1, 302.9, 153, 139.2	Dehydrotheasinensin A
7	7.76	459.3	288.9, 205, 152.7, 151, 139	Epigallocatechin gallate
8	8.56	966.2	948.9, 913, 894.9, 743.3, 724.8, 573.4, 555.4, 538.2, 288.9	unknown
9	9.18	948.4	913.5, 778.2, 760.5, 725, 573.1, 286.8, 154.2	unknown
10	10.35	980.4	908.6, 895, 756.8, 559, 393.4, 379	unknown
11	11.29	930.4	894.9, 725.1, 573.2, 537.3	

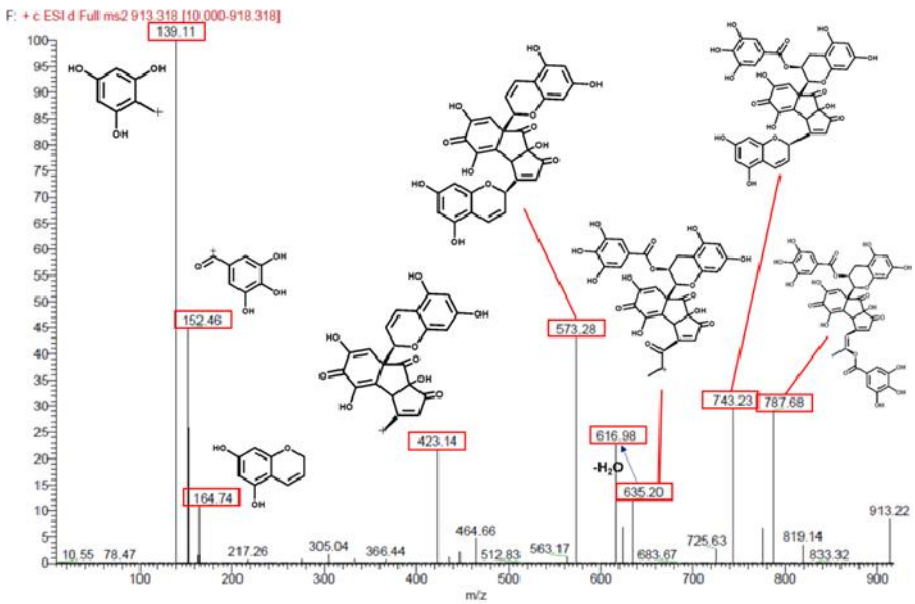
(B)



Peak no.	RT(min)	LC/MS [M+H] (m/z)	fragment ion LC-MS2 (m/z)	Trivial name
12	5.43	713.3	713.1, 694.9	Unknown
13	6.57	964.4	777.1, 758.8, 715.2, 563.3, 490.9, 471.5, 461	Unknown
14	6.7-7.2	915.3	789.1, 745.1, 619.1, 575.3, 557.2, 466.8, 449.2, 423.1, 332.9, 314.5, 276.6, 152.9	Theasinensin A
15	7.24	745	789.1, 745.1, 619.1, 575.3, 557.2, 466.8, 449.2, 423.1, 332.9, 314.5, 276.6, 152.9	Unknown
16	7.67	459.2	289.1, 228.8, 151.1, 139	EGCG
17	9.5	885.3	758.5, 715.2, 560.7, 545.4, 293, 288.5	EGCG dimer P2

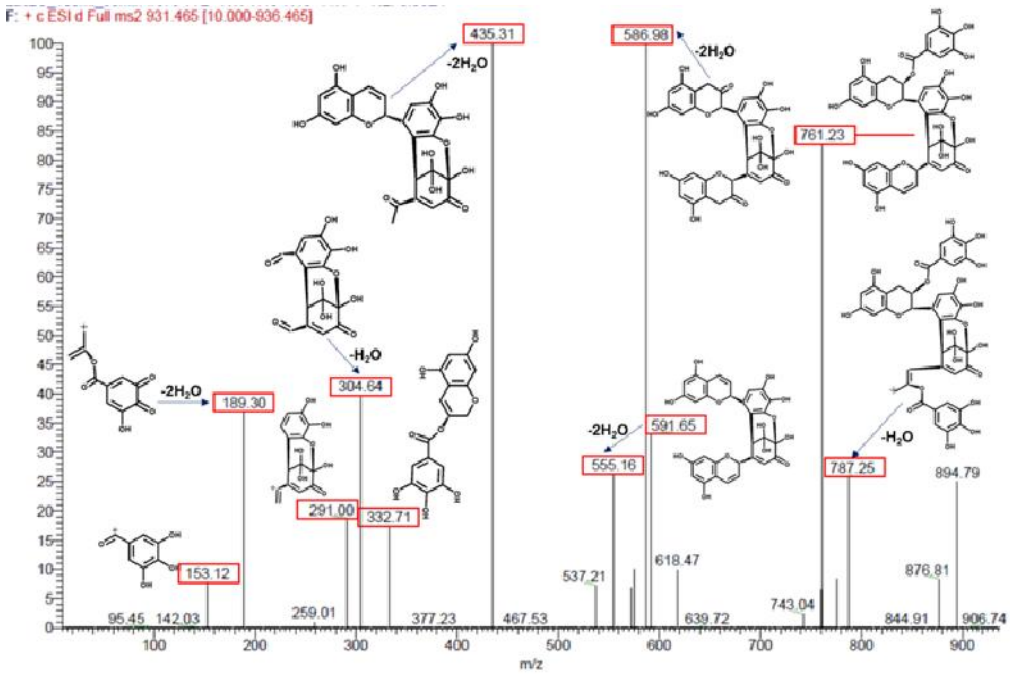
Figure 7. The total ion chromatography(TIC) of laccase reaction products and the identified peaks and their MS2 fragment ions. (A)reaction products before the heat treatment, (B)after heat treatment process.

(A)



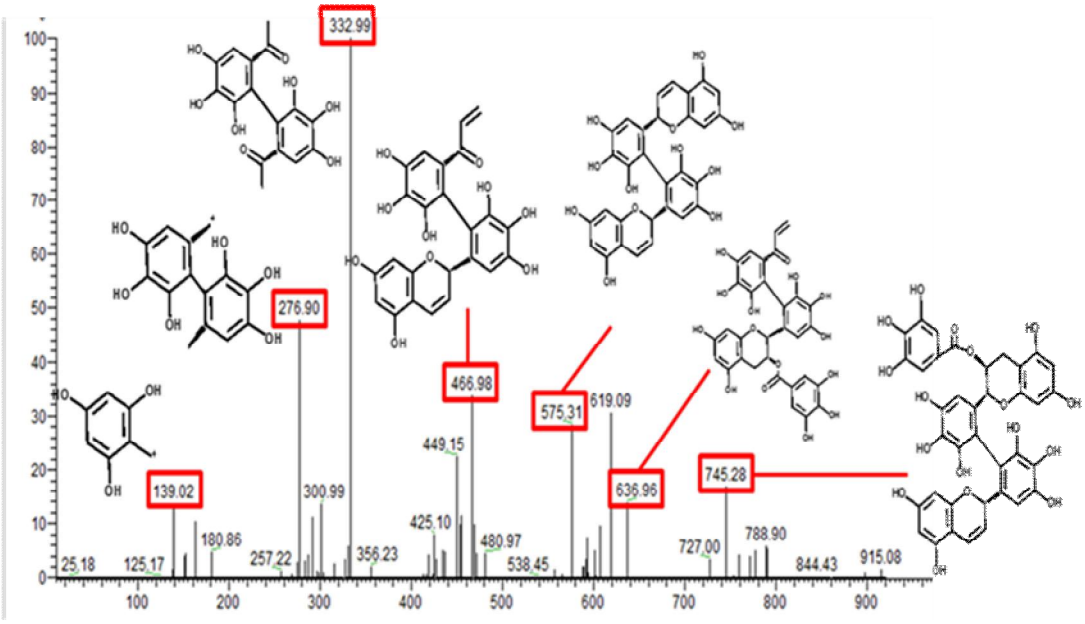
Theacitirn C (913.3m/z)

(B)



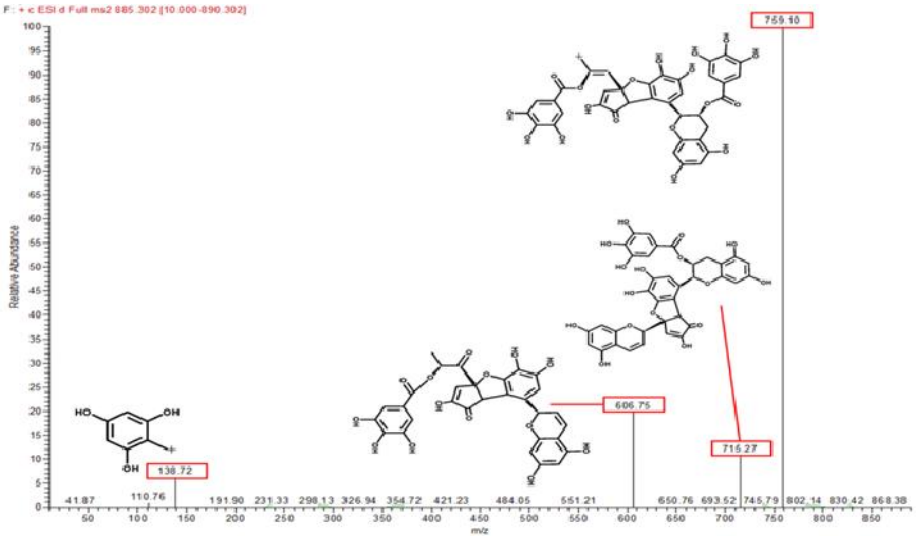
Dehydrotheasinensin A (931.4m/z)

(C)



Theasinensin A (915.3m/z)

(D)



P2 (885.3 m/z)

Figure 8. Analysis of synthesized dimers from mass fragmentation

at ring B during The structure is unstable because of the weak ether bond linkage that breaks after heat treatment.

Figure 7B shows TIC of three kinds of dimers and unreacted EGCG after the heat treatment process of the products. The dimer structures were modified through redox-dismutation. As the result of the heat treatment, TSA(915.3 m/z) and P2(885.3 m/z) were formed which unobserved compounds before the heat treatment. The two dimers were stable at 85℃ and TSA was more stable than other dimers because of TSA consists of only stable C-C bond linkage rather than ether bonds or more hydroxy groups. Figure 8C and 8D show the structure analysis of TSA and P2, respectively. An exact mechanism of the redox-dismution to produce TSA has not been revealed, but it is reported that the heat drying process results in modification on oligomer structures and increased amount of TSA.[34]

3.3 Redox dismutation of EGCG dimers for theasinensin A

The laccase oxidizes EGCG to ortho-quinone and subsequent dimerization of the quinone affords an unstable dimers such as DhTSA. Redox dismutation occurs during the heat treatment of the dimers at 85 ° C for 15 minutes after the oxidation of EGCG.[24, 25, 35] Dimers with 913.3, 931.4, 948.3, and 964.4 m / z values produced after the reaction are converted to theasinensinA at 915.4 m / z.(Figure 9) The dehydrotheasinensin A(931.3 m/z) and others undergo redox dismutation to give reduction products TSA produced by oxidative cleavage of aromatic rings with R-biphenyl bond. After the reaction, the products and the products after heat treatment were compared by LC / MS data and it was confirmed that TSA was formed as a major dimer.

3.4 Effects of differents heat treatment temperature on dimers

Heat treatment step is a key role in production of the TSA as described above, so we optimized the heat treatment temperature. The product structures vary according to the heat treatment temperature and our study revealed that TSA can be obtained at a certain temperature or higher. After 30 minutes of reaction at 25 ° C, 15 minutes of heat treatment at 40, 60, 80, and 100 ° C was performed. The study showed an increase in amount of TSA above 80 ° C, and 67% of TSA in total dimers at 100 ° C.(Figure 11)

As shown in figure 10, the amount of EGCG remained and the amount of total dimer was almost same as all temperature. This result indicates that EGCG and synthesized dimer were unstable and decomposed at this temperature. On the other hand, above 80 ℃,

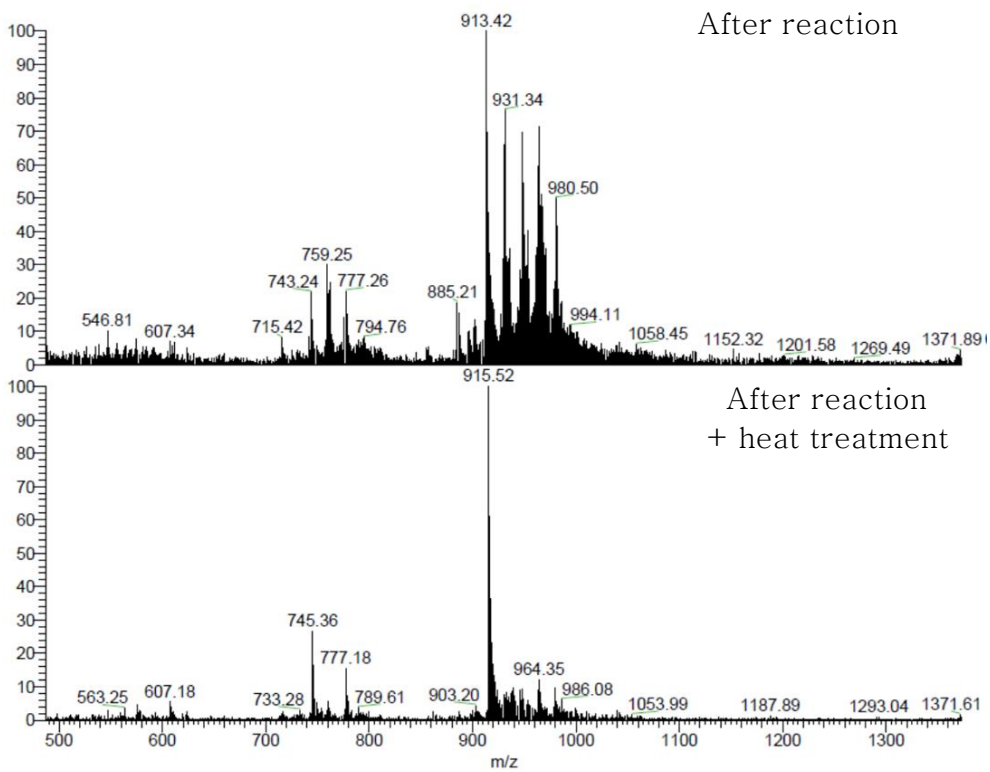


Figure 9. Comparison of mass profile from effects of heat treatment on products after reaction

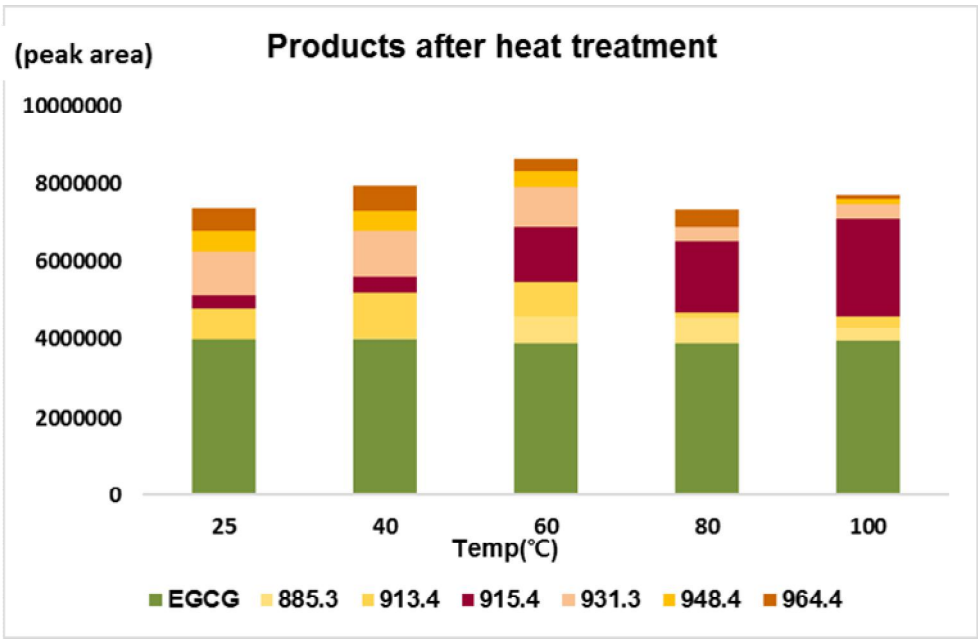


Figure 10. Comparison of reaction products depend of heat treatment temperature

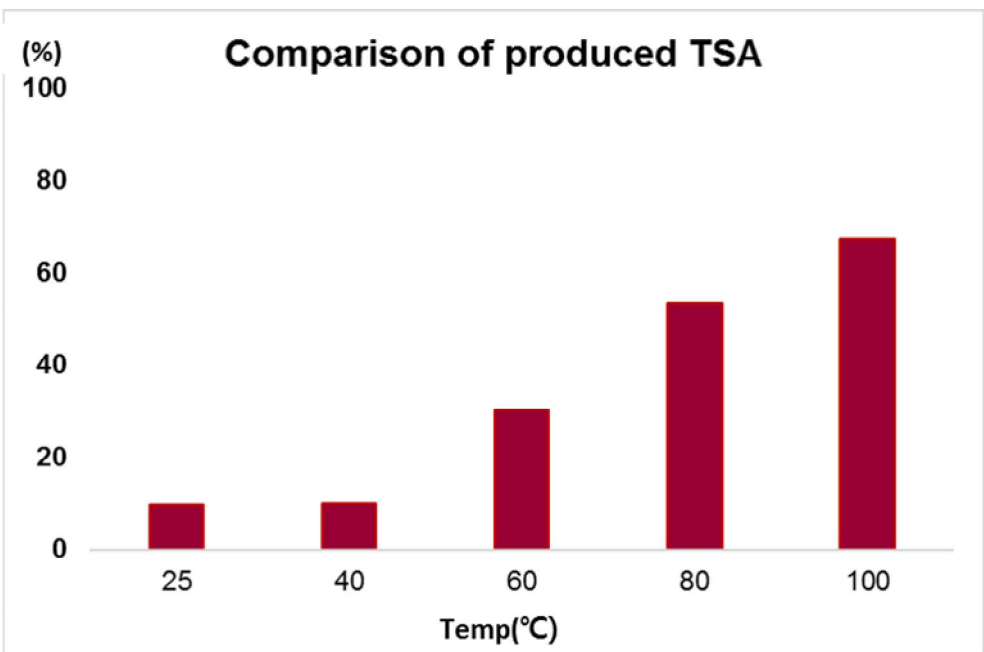


Figure 11. Percentage of TSA in dimers according to heat temperature

the ratio of TSA increased while maintaining the total dimer content. However, we cannot confirm exact quantitative analysis of the ratio because we did not obtain the authentic sample of EGCG dimers. As a result, the oxidized EGCG dimer structures above 80 ℃ inform us that dismutation occurs on TSA and the resulting structure is a stable one at high temperature.

3.5 Separation and purification using Sephadex LH-20 and C18 column

Figure12 shows column work to separate dimer from EGCG and other degradation products. Since the Sephadex LH-20 column is size exclusive, small amount of trimers, major products of dimers, and small molecules of monomers were fractionated sequentially according to the molecular size. In consequence of the Sephadex LH-20 column chromatography, EGCG and various dimer fractions could be separated and five of them included TSA. Figure 14 shows that Friaction 1 and 3 contained several kinds of dimers and degradation products of dimers. On the other hand, fraction 7-9

were EGCG contained elution. Pure TSA could be obtained from fraction 11; however, the remaining four fractions were needed to be further purified through the Sephadex LH-20 column since they

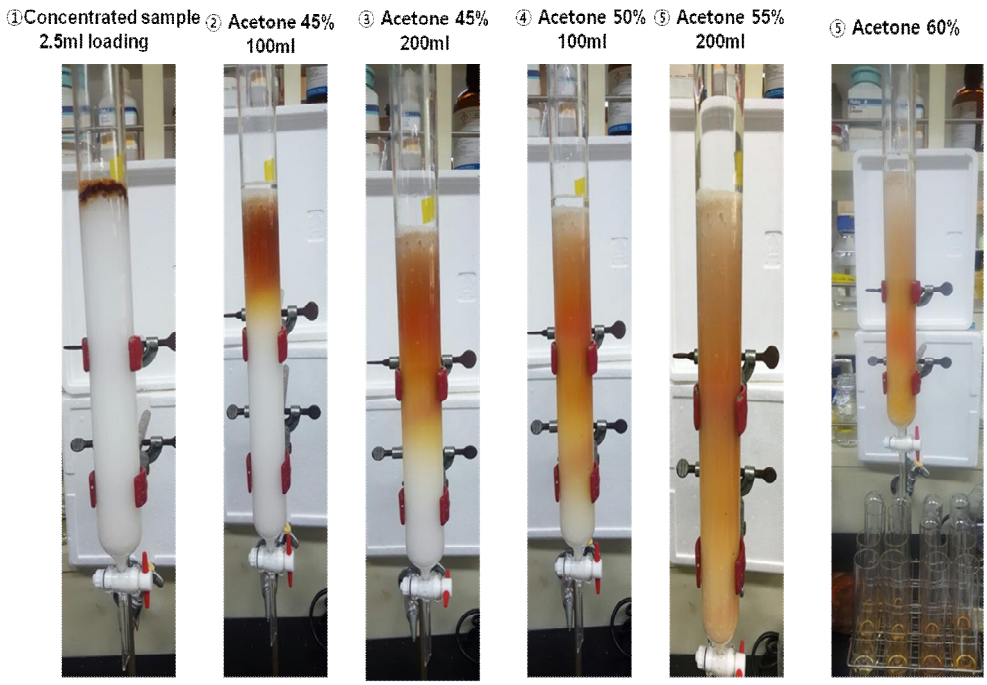


Figure 12. Sephadex LH-20 column

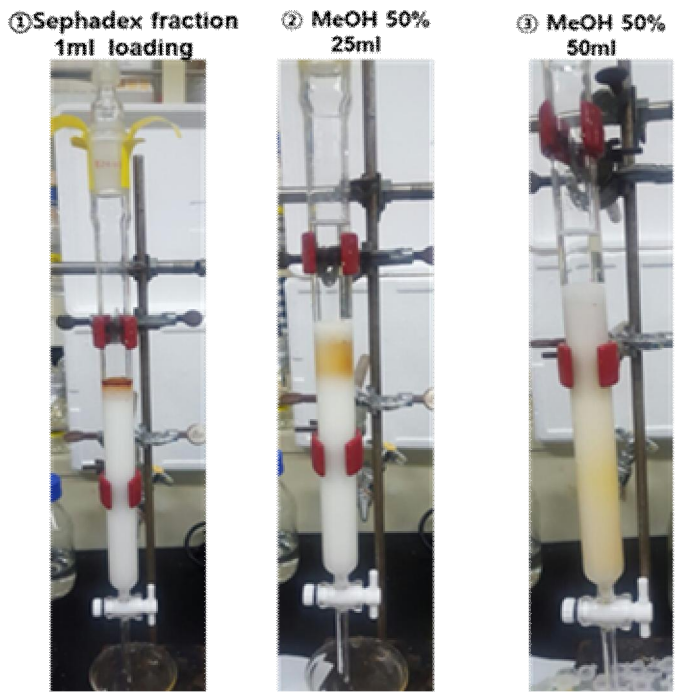


Figure 13. C18 column

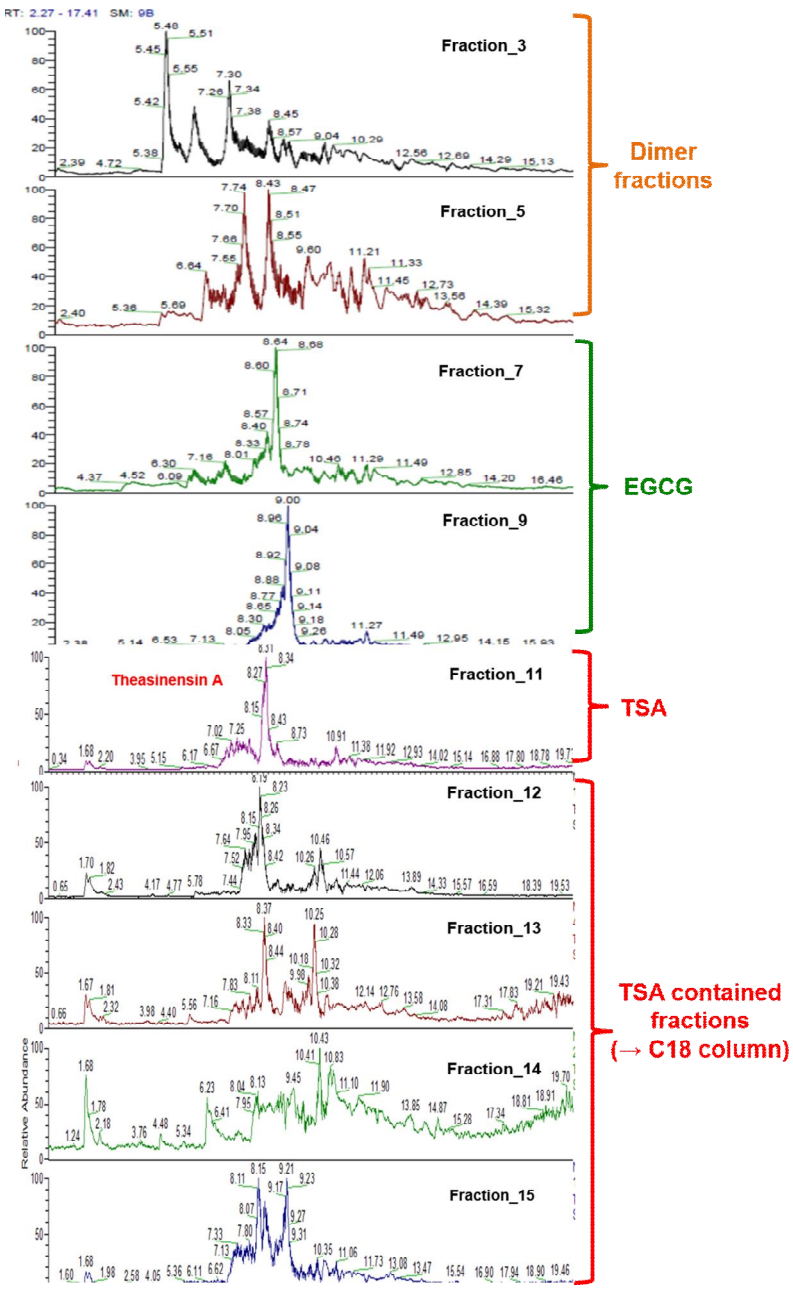


Figure 14. TIC of fractions from Sephadex LH−20

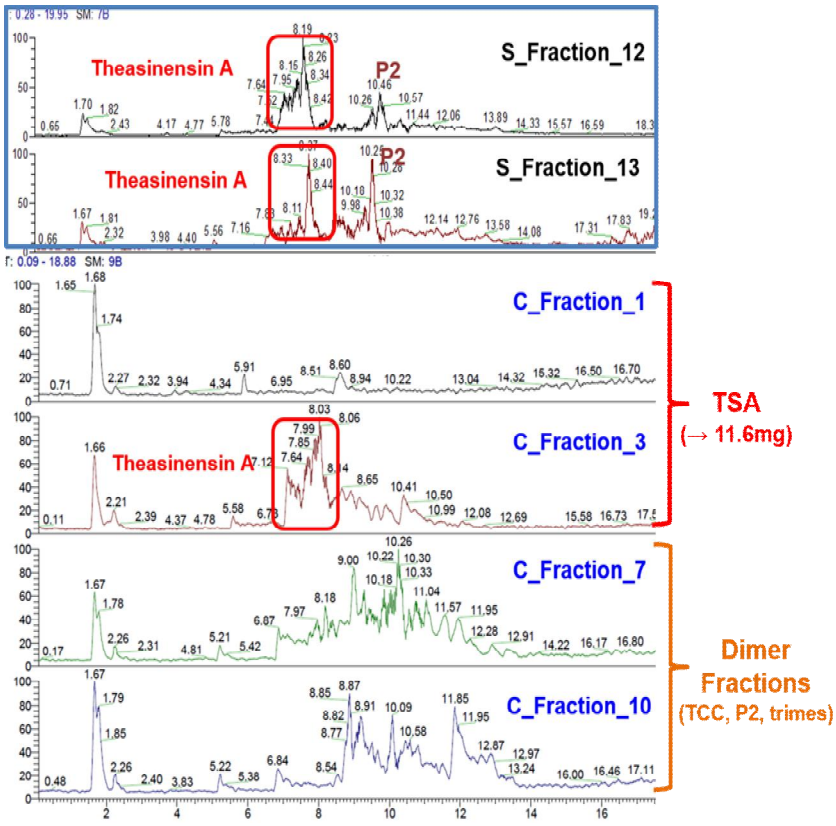


Figure 15. TIC of fractions from C18 column

are not pure enough.

As shown in figure 13, fraction 12,13, and 14 from sephadex LH-20 were concentrated to 1ml to load into C18 column. The fraction 13 and 14 were performed same as above. The TSA is more hydrophilic than other dimers, so it is eluted earlier than other compounds are. Fractions of 1-3 contained 11.6mg of purified TSA.(Figure 15) At the end of the all purification process, we could obtain 27.6mg of TSA and the yield is about 7% when 89% conversion of EGCG. The separation of TSA using column chromatography can vary depending on the condition of the loading sample, eluent composition, and size of the column. In addition, if the amount of TSA is sufficiently produced through a proper enzymatic reaction then heat treatment at temperature above 80℃ helps to obtain TSA in higher yield.

3.6 EGCG oxidation with expressed oxidase from aspergillus strains

In no induction system, the largest amount of dimers was produced from the supernatant of *A.oryzae* and the patterns were similar with the ones with laccase. The difference comes from the dimers proportion of soluble fraction of *A.oryzae* has different pattern that TSA is the major dimer in products.(Figure 16) It could be

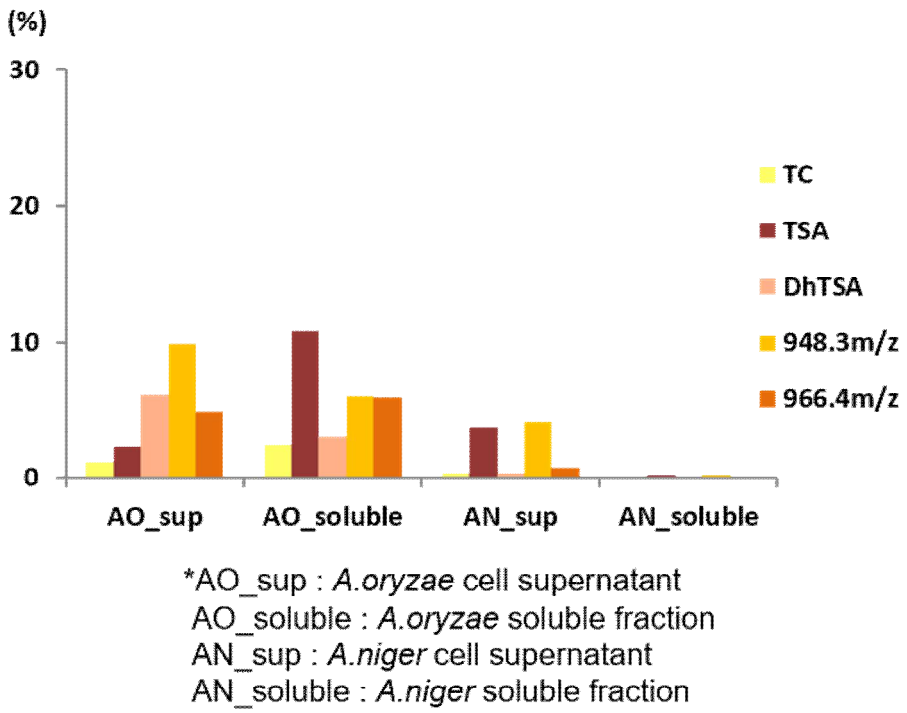


Figure 16. Produced dimers from aspergillus culture without induction

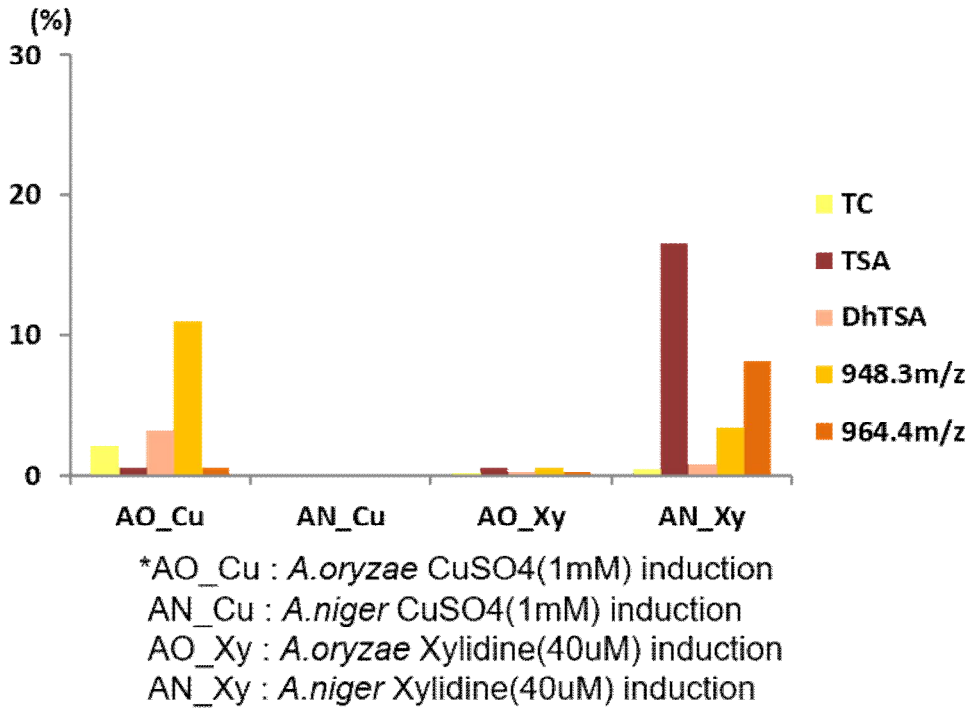


Figure 17. Produced dimers from aspergillus culture with induction

predicted that PPO other than laccase oxidized EGCG, thus increased amount of TSA may be an effect of complex enzymes. In the case of *A.niger*, only handful of EGCG dimers were produced.

However, result was opposite in the induction system. When 2,5-xylidine was used as inducer in *A.niger*, the more dimers were produced than in other conditions.(Figure 17) Surprisingly, significant amount of TSA was formed with fungal strain even without the heat treatment while almost no TSA formed in in vitro reaction without heat treatment. Enzymes expressed from *A.oryzae* were better induced by the inducer, $CuSO_4$.

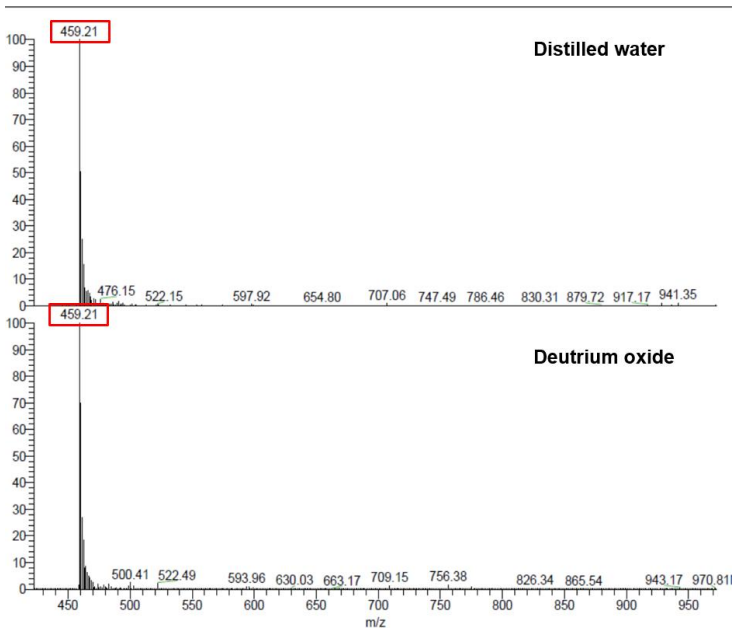
This experiment was performed as proof of concept that different oxidized EGCG products are produced depending on induction and culture conditions.

3.7 Disccusion

Although different forms of catechins from black tea extracts have been studied and reported, there are still many unknown dimers and oligomers of catechin. Since synthesis may begin with many kinds of catechins and other chemicals in tea extract, also they can be oxidized easily by PPO. So, it is difficult to understand the exact mechanism. Therefore, we oxidized the EGCG as the only

substrate using enzyme *in vitro* and predicted structures of unknown dimers by LC/MS/MS. The dimers of 948.3, 964.3 and 980.4 m/z were increased by 16 Da, which indicates that there is a successive hydroxylation with water molecule during enzymatic oxidation. We could experimentally prove that the adduct is a hydroxyl group by using deuterium oxide instead of the buffer solution. When D_2O was used as the reaction solution under the same reaction conditions, all m / z value of the dimer were increased by 1. As a result, it was found that the dimer of 948.4, 964.3, 980.4 m / z was affected by water.(Figure 18) When the fragment ion were analyzed by LC / MS / MS, the overall patterns were hard to distinguish each other and the precise position of hydroxylation could not be determined. When EGCG was used as the only substrate, a small amount of trimer was generated, but no other oligomer was formed. Most of the dimer structure consists of a B ring bond and the elongation of the radical site is not achieved in the D ring gallate. The oligomers or catechin polymers present in black tea are composed of catechin complex and are made up of various PPO enzymes. Based on our study, it can be predicted that oligomers may be synthesized if EC or EGC are used as co-substrate and also by a natural product such as gallic acid as a mediator of a radical oxidative reaction.

(A)


459.21
Distilled water
476.15
522.15
597.92
654.80
707.06
747.49
786.46
830.31
879.72
917.17
941.35
459.21
Deutrium oxide
500.41
522.49
593.96
630.03
663.17
709.15
756.38
826.34
865.54
943.17
970.81
m/z

(B)

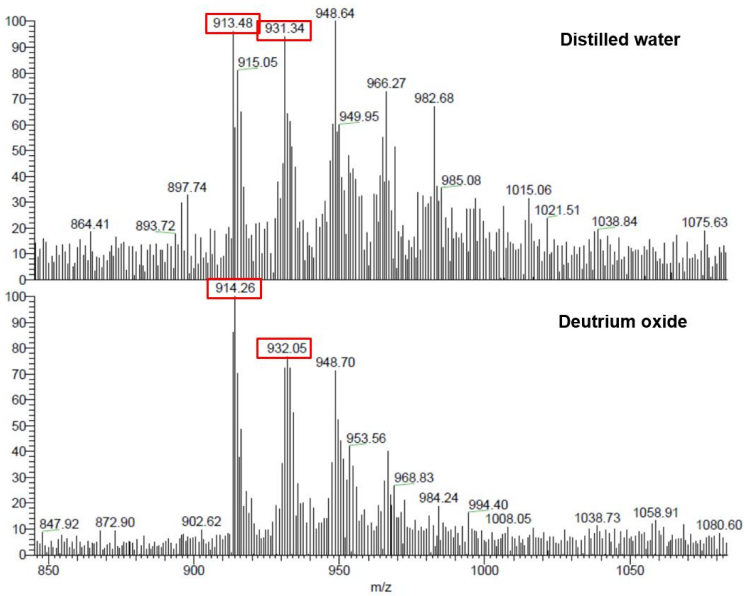


Figure 18. Comparison of products mass for water effects during oxidation (A) EGCG, (B) reaction products

4. Conclusion and further suggestion

Our purpose in this study was to produce the major catechin dimers in black tea through enzymatic reaction and characterize the structures of synthesized dimers by mass spectrometry. EGCG, the most abundant in tea catechin, was used as only substrate. In order to synthesize black tea dimers, it was necessary to understand the role of PPO in tea fermentation process. Three representative PPOs were laccase, peroxidase, and tyrosinase, and model enzymes for each PPO, TvLac, HRP, and MuTyr respectively, were selected to compare their enzymatic activity and structural profile of products. Among them, laccase was chosen as a optimal enzyme since laccase showed the fastest catalytic activity and greater proportions of dimer structure profile. With the optimal enzyme, we could produce a major dimer, TSA, by post-processing method that imitates the drying step of fermentation *in vitro*. Immediately after the enzymatic reaction, unstable and complex dimers were formed in large quantities, making us difficult to maintain certain amount and separate them. Therefore, no research was done on identifying these complex dimers. Thus, it is meaningful to study and analyze these structures. We used LC / MS / MS

fragmentation to analyze the formed structures. We confirmed that some of the dimer structure were identical to the ones obtained from fermentation process. It is the first time to mimic the process of fermentation *in vitro* and check EGCG dimer formation.

An important last step in this experiment is the separation of the products. EGCG and dimers have many hydroxyl groups so there is little difference in polarity between dimers. Since each dimer was present in a small amount immediately after the reaction, the dimers were converted to TSA by heat treatment and thus amout of TSA increased. Two columns, one with Sephadex LH-20 resin and the other with C18 resin were used to separate TSA from other dimers. Initially, the fraction containing TSA was separated via Sephadex LH-20 and the pass-thru was separated through C18. We could obtain 27mg of purified TSA and about 6% yield in 100ml scale laccase reaction as 89% conversion.

It is the first time that purified TSA has been produced through enzymatic reaction, and higher yield is expected if we optimize column work and reaction conditions. Additionally, if strains that can express PPOs can be screened for dimer production, mass production will be possible *in vivo*.

Furthermore, it can be used in food and cosmetic industries as well.

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국문초록

락카아제를 이용한 카테킨 단량체로부터 카테킨 이합체 합성과 분리정제

Camellia sinensis로부터 유래되는 차 잎은 발효 정도에 따라 백차, 녹차, 우롱차, 홍차로 분류될 수 있다. 홍차는 녹차로부터 발효과정을 통해 생산되며 산화된 카테킨 물질을 포함한다. 산화된 카테킨은 이합체, 삼합체, 올리고머 및 고분자를 형성하고 이 물질은 thearubigins으로 복합적 혼합물로 존재한다. Thearubigins은 홍차 추출물의 70% 이상을 구성하고 있다. 하지만 thearubigins의 구조는 아직 다 밝혀지지 않았다. 카테킨 단량체들은 발효 과정을 거치면서 폴리페놀 산화효소, 과산화효소 및 카탈라아제 효소에 의해 산화되고 이 효소들은 발효 과정에서 중요한 역할을 한다.

이 연구에서는 카테킨 단량체에서 가장 많이 존재한는 에피갈로카테킨 갈레이트를 폴리페놀 산화효소 중 하나인 락카아제를 사용하여 라디칼 산화 반응으로 에피갈로카테킨 갈레이트 이합체를 합성하였다. 라디칼 산화반응으로 합성된 이합체 theacitrin, dehydrotheasinensin 그리고 theasinensin를 분석하기 위해 액체 크로마토그래피 질량분석기를 사용하였다. 또한 구조분석을 위해 LC/MS/MS를 사용하여 분해된 이온 조

각의 패턴을 통해 분석하였다. 이합체들은 산화 반응 후에 열처리를 통하여 구조의 변환이 일어났고 이 과정은 발효과정의 마지막 순서인 태우고 건조하는 방법을 모사한 것이다. 열처리의 결과로 theasinensin A(TSA)가 주요 이합체로 만들어졌고 이 물질은 Sephadex LH-20 과 C18 컬럼을 이용하여 분리정제를 진행하였다. 결론적으로 효소반응을 이용하여 분리정제를 통해 TSA를 얻어낸 연구는 처음으로 진행된 것이고 100ml 반응을 통해 27.6mg의 TSA를 얻어낼 수 있었다.

주요어 : 에피갈로카테킨 갈레이트, 락카아제, 분리정제, 진량분석기, 데아시넨신

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