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Study on the Synthesis of Theaflavin-3,3′-Digallate Catalyzed by *Escherichia coli* Expressing Tea Tree Polyphenol Oxidase Isozymes and Its Enzymatic Solution

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Abstract: *Polyphenol oxidase* and its isoenzymes are crucial enzymes in the tea tree that catalyze the synthesis of theaflavins. In this study, tea tree polyphenol oxidase was used as the research object, and various protein sequence treatments, such as *TrxA* fusion tag + N-terminal truncation, were tested for prokaryotic expression through the *Escherichia coli* expression system. Comparative analyses were conducted on the activities of the different recombinant enzyme proteins on the substrates of tea polyphenol fractions. Additionally, the enzyme with the highest catalytic efficiency on the TFDG substrate was immobilized using polyethylene glycol to investigate the yield of its synthesis of TFDG. Our results demonstrated that after N-terminal truncation and *TrxA* fusion expression, CsPPO1, CsPPO2, CsPPO3, and CsPPO4 were mostly expressed in the form of inclusion bodies in the cell and exhibited varying degrees of enhancement in substrate activity. Specifically, CsPPO1 exhibited significantly increased activity in EC and ECG, CsPPO2 showed enhanced activity towards ECG and EGCG, and CsPPO2 displayed the highest activity toward TFDG substrates. Homology modeling structural analysis of the polyphenol oxidase isozymes revealed that the active centers of CsPPO1, CsPPO2, and CsPPO3 consisted of double copper ion center structures, while the conserved histidine residues surrounding the active centers formed different catalytic activity centers in different structures. Furthermore, polyethylene glycol immobilization significantly increased the activity recovery of the CsPPO2 enzyme to 74.41%. In summary, our study elucidated that tea tree polyphenol oxidase is expressed as inclusion bodies in prokaryotic expression, and the activity of the recombinant enzyme towards substrates could be enhanced through N-terminal truncation and *TrxA* fusion expression. Moreover, immobilization treatment of the CsPPO2 enzyme greatly improved enzyme efficiency. These findings offer an important enzymatic basis and theoretical support for the synthesis of theaflavins.

Keywords: tea tree; polyphenol oxidase; theaflavin; *Escherichia coli* expression; immobilized enzyme

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1. Introduction

Theaflavins (TFs) are a class of compounds with a benzophenone structure that are formed through the oxidative polymerization of catechins and their derivatives. This process is catalyzed by tea polyphenol oxidase (PPO) during the processing of black tea [1,2]. The existence of this class of compounds was first documented in the studies conducted by Roberts E.A.H [3]. To date, more than twenty different structures of TFs have been identified and characterized. Among these, the main ones commonly found in black tea include theaflavin-3,3′-digallate (TFDG), theaflavin-3′-gallate (TF-3′-G), theaflavin-3-gallate (TF-3-G), and theaflavin (TF).

TFs are not only components that contribute to the quality of black tea but also possess various significant biological functions, such as antioxidant effects [4,5], the regulation of glycolipid metabolism [6,7], anti-inflammatory effects [8,9], antitumor effects [10,11], the protection of muscle health [12,13], the preservation of bone health [14,15], as well as inhibitory effects on bacteria and viruses [16,17]. Therefore, TFs hold substantial potential for application in multiple fields. These health effects provide TFs with considerable nutritional value and wide-ranging possibilities for development and use. Consequently, TFs have emerged as a prominent functional ingredient in the realm of tea science, garnering significant attention alongside tea polyphenols and theanine. In the market, several internationally renowned brands have launched TF products, including Life Extension's TFs capsule, which offers antioxidant properties, regulates blood lipids, and protects the heart. Given the abundance of tea resources in our country, exploiting these resources to develop TF products with independent intellectual property rights inevitably presents extensive market prospects.

The TF content in black tea is relatively low, accounting for only 0.5–2% of the dry weight [18]. Consequently, directly isolating TF monomers from black tea is challenging, inefficient, and costly. Additionally, the structure of tea polyphenolic chemicals closely resembles TFs, further complicating the isolation of high-purity TFs or theaflavin monomers from black tea, as it requires extensive steps and the use of numerous organic reagents. This method results in low yields and potentially leaves behind organic reagent residues that do not comply with the raw material standards in the food and pharmaceutical industries. However, the oxidative synthesis principle of TFs by externally added bio-enzymes catalyzed by catechin is similar to the natural formation process during black tea processing. Utilizing bio-enzyme-catalyzed TFs is considered an efficient approach to achieve industrial-scale TFs preparation due to its mild reaction conditions and exclusive use of food-grade substrates. Moreover, the resulting product complies with the requirements of the food and pharmaceutical fields. The selection of an appropriate enzyme source plays a crucial role in this technology. The chosen enzyme source should possess high catalytic activity, selectively catalyze the oxidation reaction of catechins to produce TFs, and avoid the formation of by-products. Additionally, the stability and sustainable supply of the enzyme source must be taken into consideration [1]. Identifying a suitable enzyme source will establish a viable pathway for the production of TFs and facilitate the development of the TFs industry. Tea tree contains various PPO isozymes, including CsPPO1, CsPPO2, CsPPO3, CsPPO4, CsPPO5, and CsPPO6, and is abundant in tea polyphenols, comprising 20–30% of the tea's dry weight. This presents a potential advantage for exploring TF study through tea-tree-derived PPOs. By expressing the genes that regulate PPO enzyme expression in tea tree within microorganisms and leveraging their prolific reproduction ability and diverse metabolites, the microorganisms were capable of producing enzyme preparations and preparing them as immobilized enzymes. This approach effectively enhances the catalytic efficiency of the bio-enzymes and increases their recyclability. Simultaneously, by leveraging the advantages offered by different PPO isoenzyme components, a strategy involving multi-strain, multi-species, and multi-enzyme sources, combined with mixed fermentation, biosensors, and modern isolation and purification techniques, holds the potential to achieve large-scale modern factory preparation of TFs. This would provide an opportunity to investigate the biological activities of TFs and their monomers with high contents and encourage further development in the TF industry. It is important to note that utilizing tea tree PPO for TF preparation is still in the exploratory stage, and the involved techniques and methods require further in-depth research and optimization. Nevertheless, this research direction demonstrates significant potential and is expected to yield new breakthroughs and advancements in the production and application of TFs. Currently, studies have been conducted on the heterologous expression and analysis of polyphenol oxidases (PPOs) from tea tree and pear sources. Wang et al. conducted research into the eukaryotic expression of tea tree PPO in Pichia pastoris, successfully obtaining the active target protein [19]. Liu et al. performed research into the prokaryotic expression

of the transit peptide of tea tree PPO, resulting in a three-fold increase in specific activity compared to the whole protein [20]. Gan et al. expressed excised truncated versions of the tea-tree-derived PPO protein, specifically the first 43 amino acids and the first 70 amino acids at the N-terminus [21]. They found that the former exhibited significantly higher activity than the latter, suggesting potential industrial application. Chen et al. cloned the PPO gene from Fung Shui pear and inserted the precursor and mature PPO genes into prokaryotic and eukaryotic expression vectors [22]. However, the results showed that most of the prokaryotic expression resulted in inclusion bodies and the eukaryotic expression activity was low. Nevertheless, these studies primarily focused on the cloning and expression of individual PPO genes, lacking a comparison and analysis of other isozymes. Furthermore, the activities of different PPOs on various tea polyphenol substrates within the same tea cultivar were not addressed. Therefore, in this study, we selected the published PPO isozymes (CsPPO1, CsPPO2, CsPPO3, and CsPPO4) from *Camellia sinensis var. assamica* for investigation. *E. coli* was used as the expression system to study the full-length, N-terminal truncated, and *TrxA* fusion-tagged + N-terminal truncated sequences of each protein. The aim was to promote the soluble expression of the target genes and enhance their viability. Additionally, we analyzed different recombinant crude enzyme proteins for their activities using tea polyphenol fractions as substrates. The enzyme with the best affinity for the TFDG substrate was immobilized to synthesize TFDG, providing a reference and ideas for subsequent enzyme development and screening of TF-synthesizing enzymes.

2. Materials and Methods

2.1. Main Reagents

Epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG), each with a purity exceeding 98%, were provided by Hunan Sanfu Biotechnology Co. (Changsha, China) The theaflavin mixture with TF labeling and samples (40–60%) were also provided by Hunan Sanfu Biotechnology Co. Tryptone and yeast extract were purchased from Oxoid (Shanghai, China). Agar powder, kanamycin sulfate, and sodium chloride were purchased from Beijing Tiangen Biotechnology Co. (Beijing, China).

Molecular biology reagents, including KOD-plus-Neo Polymerase, were purchased from TOYOBO (Shanghai, China). The expression vector pET-30a, thioredoxin fusion-tagged vector pUC19-TrxA, and host cell *E. coli* BL21(DE3) were provided by Hunan Fuleige Biological Co (Changsha, China). All primers, gene synthesis services, and sequencing services were performed at Beijing Tsingke Biotechnology Co. (Beijing, China).

2.2. Instruments and Equipment

The following equipment was used: PCR equipment (ABI EN61326, Foster City, CA, USA), a shaking incubator (Shanghai Zhicheng ZWY-2102C, Shanghai, China), a centrifuge (Beckman 368827, Krefeld, Germany), an enzyme-labeling instrument (Thermo Fisher MultwkanFC, Waltham, MA, USA), and high-performance liquid chromatography equipment (Agilent 1100, Santa Clara, CA, USA).

2.3. Synthesis of the Gene

According to the sequence information of PPO genes from the tea tree (*Camellia sinensis var. assamica*) published on NCBI website, CsPPO1 (Gene ID: MK977642.1), CsPPO2 (Gene ID: MK977643.1), CsPPO3 (Gene ID: MK977644.1), and CsPPO4 (Gene ID: MK977645.1), we downloaded the above sequences and optimized the amino acid codons for *E. coli* expression. With respect to CsPPO4 (Gene ID: MK977645.1), we downloaded the above sequences and optimized the amino acid codons for expression in *E. coli*, and submitted them to Beijing Tsingke's Biotechnology Co. (Beijing, China) for full gene synthesis, with an *NdeI* cleavage site at the 5′ end of the gene, and an *XhoI* cleavage site with a stop codon at the 3′ end. The gene with an *NdeI* cleavage site at the 5′ end and an *XhoI* cleavage site with a stop codon at the 3′ end was constructed into a pET-30a prokaryotic expression vector.

2.4. Prediction of N-Terminal Transit Peptides of PPO Proteins and Construction of Each Recombinant Expression Strain

According to the transit peptide online prediction software (http://www.cbs.dtu.dk/services/TargetP-2.0/) (accessed on 5 October 2022), transit peptides were predicted for the protein sequence of each PPO. Based on the predicted sequences, protein expression vectors were constructed. The N-terminal transfer peptides were removed from some vectors, while others underwent N-terminal fusion of the thioredoxin-reducing protein *TrxA*. The construction was achieved using the methods of large primers and loop PCR [23,24]. The primers used in this study can be found in Table 1. After correct sequencing analysis, the constructed vectors were transformed into *E. coli* expression cells using CaCl2 thermal excitation. The transformed cells were then plated on LB solid culture dishes containing 50 μg/mL kanamycin and cultured at 37 °C overnight. The positive clone containing the target gene was identified by growing a single colony on the culture plate.

Table 1. Large primers and primers used for loop PCR.

No.	Remarks	Primer Name	Primer Sequence (5′-3′)
1	N-terminal truncated expression primer	CsPPO1-F	TAAGAAGGAGATATACATATGAAAGCAATTGATGGTGACACCAAT
2		CsPPO1-R	ATTGGTGTCACCATCAATTGCTTTCATATGTATATCTCCTTCTTA
3		CsPPO2-F:	TTAACTTTAAGAAGGAGATATACATATGAGTAAAGCCAAAGATAGCGATCC
4		CsPPO2-R	GGATCGCTATCTTTGGCTTTACTCATATGTATATCTCCTTCTTAAAGTTAA
5		CsPPO3-F	TTAACTTTAAGAAGGAGATATACATATGGCACCGGTGACCGCACCGGATCTGGCA
6		CsPPO3-R	TGCCAGATCCGGTGCGGTCACCGGTGCCATATGTATATCTCCTTCTTAAAGTTAA
7	*TrxA* fusion expression primer	*TrxA*-F	AACTTTAAGAAGGAGATATACATATGAGCGATAAAATTATTCACCTGACTGACGAC
8		*TrxA*-CsPPO1-R	TTCATTGGTGTCACCATCAATTGCTTTGGCCAGGTTAGCGTCGAGGAA
9		*TrxA*-CsPPO2-R	CAGATTCGGATCGCTATCTTTGGCTTTGGCCAGGTTAGCGTCGAGGAA
10		*TrxA*-CsPPO3-R	TGCCAGATCCGGTGCGGTCACCGGTGCGGCCAGGTTAGCGTCGAGGAA

2.5. Shake Flask Fermentation Culture and SDS-PAGE Electrophoretic Analysis of PPO Recombinant Expression Strains

Single colonies were isolated from plates containing positive clones using a sterilized lance tip and then inoculated into triangular flasks containing 20 mL of LB liquid medium supplemented with 50 μg/mL kanamycin. The cultures were incubated overnight at 37 °C with shaking at 200 r/min. The following day, the LB culture was transferred to triangular flasks containing 100 mL of TB liquid medium at a 1% inoculum. The cultures were incubated at 37 °C with oscillation at 220 r/min. When the OD600 nm reached 1.5, lactose was added to a final concentration of 1% (m/v), and the incubation was continued at 25 °C with shaking at 220 rpm for 4–6 h. The incubation was then stopped, and the organisms were collected by centrifugation at 10,000 r/min for 5 min. The supernatant and precipitate of each organism after fragmentation were used for SDS-PAGE electrophoretic detection and analysis of protein expression, respectively. The strain containing the empty vector pET30a served as a negative control.

2.6. PPO Purification

The purification of PPO was conducted following the method proposed by Teng [16] with slight modifications. The procedure involved the addition of an equal volume of saturated ammonium sulfate solution to the crude enzyme solution, followed by thorough shaking and a 6 h standing period. The mixture was then centrifuged for 30 min at 4 °C and 9000 r/min. After dissolving the different saturated ammonium sulfate precipitates, the mixture was centrifuged again for 10 min. The saturation level of ammonium sulfate precipitates that exhibited the most suitable viability in the supernatant solutions was selected by comparing their specific activity. The ammonium sulfate precipitate with the optimal saturation was chosen, and the corresponding enzyme solution with the highest specific activity was dialyzed for 18 h.

The dialyzed enzyme solution was loaded onto a DEAE-Sepharose Fast Flow anion-exchange chromatography column. The sample was adsorbed by the ion-exchanger and eluted using NaCl buffer solutions with concentrations ranging from 0.1 to 0.5 mol/L. The elution was

performed in consecutive order, with 5 mL collected in each tube at a flow rate of 0.5 mL/min. The target enzyme solution was collected through elution in six volumes of each gradient. The collected liquid was then dialyzed, concentrated, freeze-dried, and set aside.

2.7. Determination of PPO Activity

The determination of PPO activity was based on the method of Mishra with slight modifications [25]. The specific steps were as follows: 0.3 g of each of the different types of tea polyphenol substrates (EC, EGC, ECG, EGCG) was weighed, then dissolved in 100 mL of 0.1 mol/L pH 5.0 citrate-phosphate buffer containing 1 mM Cu^{2+}, and preheated for 10 min at 30 °C in a water bath; 50 mL of the fermentation broth was taken from the fermentation flask and placed into a 50 mL centrifugal tube. Centrifugation at 10,000 r/min was performed for 5 min to collect the bacterial bodies. Then, it was resuspended with 25 mL of deionized water, and then centrifuged again after ultrasonic crushing, and the supernatant after centrifugation was the crude enzyme solution. Catalytic reaction: 4.5 mL of the preheated substrate polyphenol solution was added to 0.5 mL of the crude enzyme solution sequentially into the test tube, mixed well, and then placed on the shaking table at 30 °C for shaking and incubation. The reaction was accurately timed for 5 min, and at the end of the reaction, the reaction was terminated by adding an equal volume of 20% (*w*/*v*) trichloroacetic acid solution. Then, centrifugation was carried out and the supernatant was taken for absorbance value determination with the wavelength set at 420 nm. The reaction without enzyme solution was used as a negative control. Three determinations were made for all samples and averaged. A unit of enzyme activity was defined as a change in OD 420 nm of 0.001 per minute at 30 °C, pH 5.0: $Umin^{-1} \cdot mL^{-1}$.

2.8. Sequence and Structure Analysis of Four PPO Proteins

In order to further understand the functional differences between the isozymes of PPO, we analyzed their amino acid sequences. Meanwhile, using the existing crystal structure of PPO as a template, we carried out protein structure prediction and simulation analysis using the SWISS-MODEL homology modeling website (https://swissmodel.expasy.org/) (accessed on 7 October 2022). In this process, we selected histidine residues conserved in the copper ion binding region, performed interaction analysis, and analyzed and annotated the structure using PyMol 2.5. The wild-type Bmtyrc crystal structure was obtained from the Protein Data Bank (PDB ID: 3nm8), and the theoretical structural model of the mutant was constructed using the SWISS-MODEL protein server. The structure of EGCG was obtained using Chem 3D18.0 and the energy was minimized using Chem 3D18.0. The binding pocket of the active site was drawn using the GetBox plug-in for PyMol. Ligand docking analysis of EGCG and the receptor was performed using Lephar Research, Stockholm, Sweden, and then the results obtained were analyzed and visualized using PyMol.

2.9. PPO Enzyme Immobilization and Catalytic Synthesis of TFDG

The enzyme that catalyzes the best synthesis of TFDG was selected for cross-linking with polyethylene glycol, and the cross-linking reaction was performed in a 1.5 mL EP tube. First, 500 µL of polyethylene glycol was added to the tube, and then 50 µL of polyphenol oxidase solution (protein concentration: 2 mg/mL, enzyme viability unit: 100 U) was added, and the tube was rapidly placed at 30 °C for 60 min at 120 r/min for cross-linking, and after the completion of the cross-linking reaction, the tube was centrifuged at 12,000 r/min for 5 min, and the precipitate was collected, which was obtained as immobilized polyphenol oxidase, washed with an appropriate amount of buffer 2–3 times and prepared for use. In order to detect the activity of the immobilized enzyme and calculate the enzyme activity recovery, enzyme activity assay experiments were carried out. In the assay, a specific substrate is used to detect the activity of the immobilized enzyme and the enzyme activity recovery is calculated accordingly to see whether the enzyme activity is retained after the cross-linking reaction.

$$\text{Enzyme activity recovery (\%)} = \text{immobilized enzyme/total activity of free enzyme} \times 100\%$$

Immobilized enzyme and free enzyme were used to catalyze the synthesis of TFDG, and the reaction conditions were as follows: substrates EGCG and ECG were dissolved in 15 mL of 0.1 M pH 4.0 citrate-phosphate buffer with a molar ratio of 2:1 (4 mM:2 mM), a reaction temperature of 30 °C, a reaction pH of 4.0, a reaction time of 30 min, and a polyphenol oxidase activity level of 100 U [26]. The reaction was carried out using the immobilized and free enzymes. When the reaction was finished, the supernatant was centrifuged for TFDG concentration determination, and the filtered solid was reintroduced into the substrate solution for the next batch of the reaction. In this process, the immobilized enzyme and free enzyme will catalyze the reaction of substrates EGCG and ECG to produce TFDG products, respectively. The reaction conditions were set to ensure the high efficiency of the enzyme-catalyzed reaction and the stability of the product. Determination of the TFDG concentration based on the resulting supernatant allows the effectiveness of the catalyzed reaction to be assessed and the reaction can continue to the next batch as needed. By comparing the catalytic efficiency and stability of the immobilized enzyme and the free enzyme, the process conditions for the catalytic synthesis of TFDG can be optimized to provide an effective method for the production of theaflavins.

2.10. Data Analysis

All experiments were repeated at least three times, and the data were analyzed and plotted using the statistical analysis software GraphPad_Prism_9.5.1, and the one-way ANOVA and T-test were used to check whether there were any significant differences between the numbers, and different letters indicated that there were differences in significance ($p < 0.05$).

3. Results

3.1. Prediction of PPO Protein Transit Peptide Structure

The amino acid sequences of each PPO were examined using online prediction software for transit peptides on a website. The prediction results for these transit peptides contribute to the understanding of the localization and transport mechanisms of different CsPPO proteins, which is crucial for further investigations into their functions and enzyme catalytic properties. The results revealed that the predicted vesicle-like transit peptide of the CsPPO1 protein spanned the first 40 amino acids at its N-terminal end. For the CsPPO2 protein, the transit peptide consisted of the first 45 amino acids at its N-terminal end, while the CsPPO3 protein had a transit peptide of 95 amino acids at its N-terminal end. In contrast, CsPPO4 lacked a transit peptide (Figure 1). The molecular weight and protein size of each recombinant PPO gene fragment are presented in Table 2. Among them, CsPPO2-N45-*TrxA* exhibited the largest recombinant gene fragment (2013 bp) with a protein size of 71.76 KD, while CsPPO1-N405 had the smallest recombinant gene fragment (1641 bp) with a protein size of 1521 KD. CsPPO1 and CsPPO2 exhibit a single peak, while CsPPO3 exhibits other peaks, but it has little effect on purity. However, CsPPO4 did not show a peak and its protein was not expressed.

Table 2. Characteristics and molecular weight of each PPO recombinant protein.

NO.	PPO Protein	Gene Size (bp)	Protein Molecular Weight (KD)
CsPPO1	full length protein	1758	65.43
CsPPO1-N40	N-terminal truncated expression	1641	61.07
CsPPO1-N40-TrxA	N-terminal truncation + TrxA fusion expression	1965	72.86
CsPPO2	full length protein	1818	68.04
CsPPO2-N45	N-terminal truncated expression	1686	59.95
CsPPO2-N45-TrxA	N-terminal truncation + TrxA fusion expression	2013	71.76
CsPPO3	full length protein	1806	66.71
CsPPO3-N95	N-terminal truncated expression	1521	56.49
CsPPO3-N95-*TrxA*	N-terminal truncation + TrxA fusion expression	1848	68.3
CsPPO4	full length protein	474	17.41

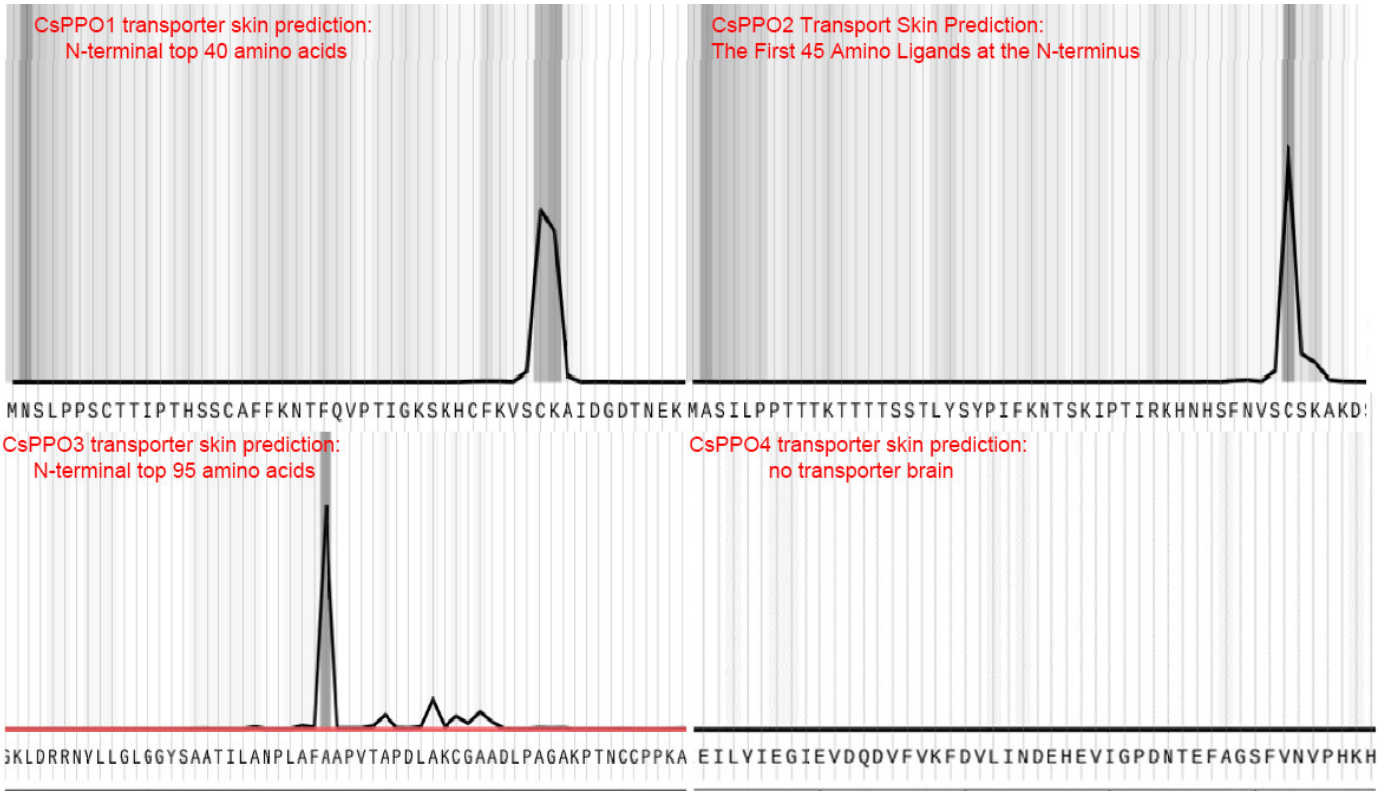

Figure 1. Predicted structure of each PPO protein transport peptide of tea tree origin.

3.2. SDS-PAGE Electrophoretic Detection of PPO Recombinant Protein

The expression of CsPPO proteins was analyzed for the same amount of bacteriophage, as shown in Figure 2. The results showed that the full-length sequences of CsPPO1, CsPPO2, and CsPPO3 were expressed, but they had less soluble expression and were mainly expressed in insoluble inclusion bodies. In contrast, CsPPO4 showed no detectable expression of the target protein either in the bacterial-lysed supernatant or in the precipitate. When expression experiments with N-terminal truncation were performed, it was found that the expression of inclusion body proteins was reduced, but the soluble expression did not increase significantly. In addition, in the expression experiments with N-terminal truncation plus *TrxA* fusion, the expression of inclusion body proteins was increased compared with N-terminal truncation, but the soluble expression was still not significantly increased. These results suggest that the expression of CsPPO1, CsPPO2, and CsPPO3 exists in the cell mainly in the form of inclusion bodies with less soluble expression. For CsPPO4, both full-length sequence and N-terminal truncated expression failed to obtain expression of the target protein. These findings suggest that the soluble expression of CsPPO proteins may face some challenges, and further optimization of the expression conditions and construction of ex-pression vectors may be required to improve their soluble expression. This is of great significance for a deeper understanding of the function and catalytic mechanism of the CsPPO protein, and provides useful guidance and information for the production and application of theaflavins.

CK- is the negative control, S is the supernatant after the bacteria are lysed, and P is the precipitate after the bacteria are lysed.

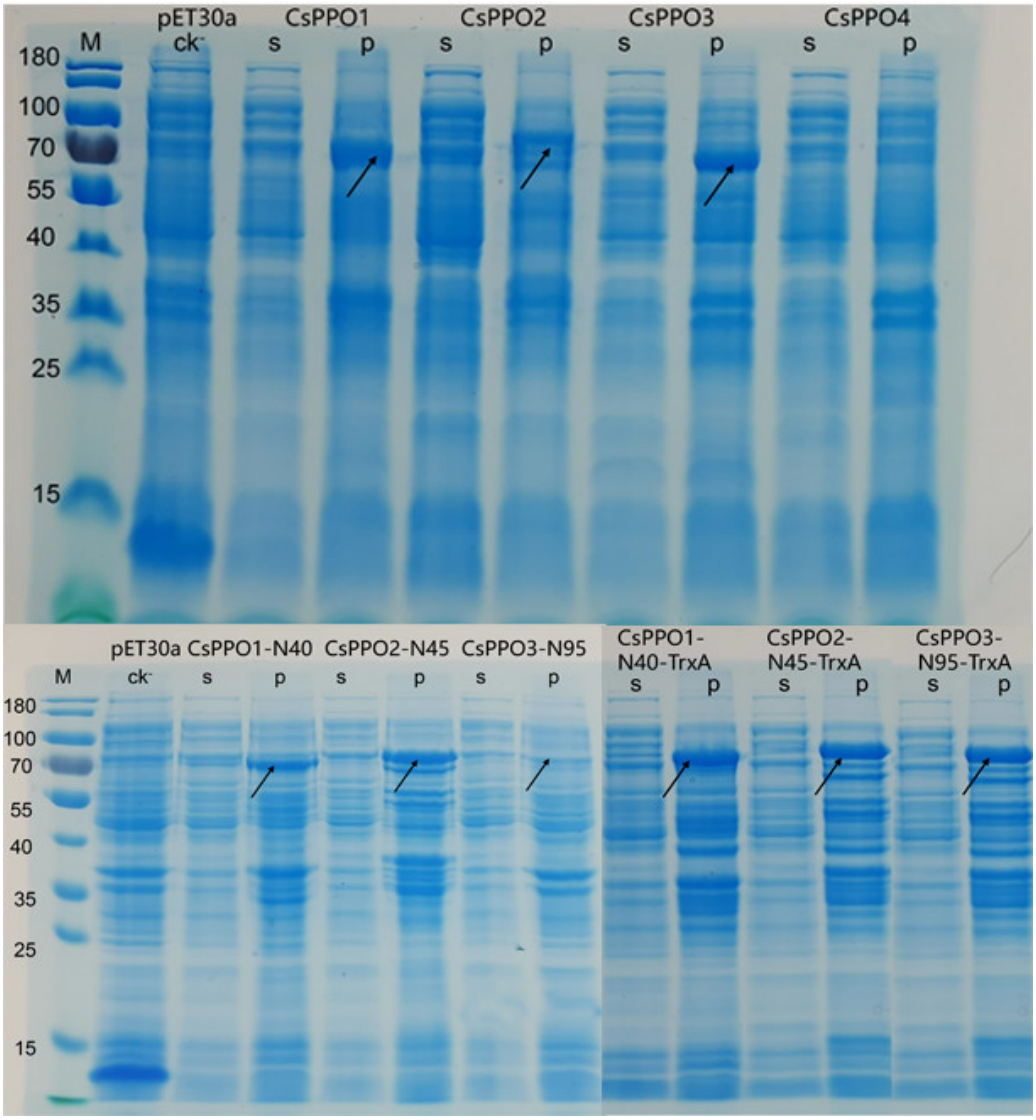

Figure 2. SDS-PAGE electrophoresis detection of each PPO protein. CK- is the negative control, S is the supernatant after the bacteria are lysed, and P is the precipitate after the bacteria are lysed. Arrow labeled as target protein.

3.3. Activity Analysis of PPO Recombinant Proteins

The activity of each recombinant protein was assessed using tea polyphenols as substrates. The activity of the recombinant proteins was determined with various polyphenols (EC, ECG, EGC, and EGCG) under the same bacterial volume, as depicted in Figure 3. The outcomes revealed a significant increase in the activity of the CsPPO2 protein towards EC and ECG following fusion expression of N-terminal truncation and *TrxA*. Conversely, the activity of the CsPPO2 protein towards EC and ECG decreased after fusion expression of N-terminal truncation and *TrxA*, albeit with a slight increase in activity towards EGC and EGCG. Additionally, the expression of N-terminal truncation and *TrxA* fusion led to a moderate increase in the activity of the CsPPO3 protein towards EC, ECG, EGC, and EGCG. Notably, the CsPPO3 protein displayed the most prominent increase in activity towards the EC substrate. The activity of CsPPO proteins was successfully modulated through N-terminal truncation and *TrxA* fusion expression. The catalytic efficiency of different CsPPO proteins varied for different substrates, potentially attributed to disparities in amino acid sequences and structures. Consequently, further investigations into the structure–function relationship of CsPPO proteins can aid in optimizing the efficacy and yield of the enzyme-catalyzed synthesis of theaflavin.

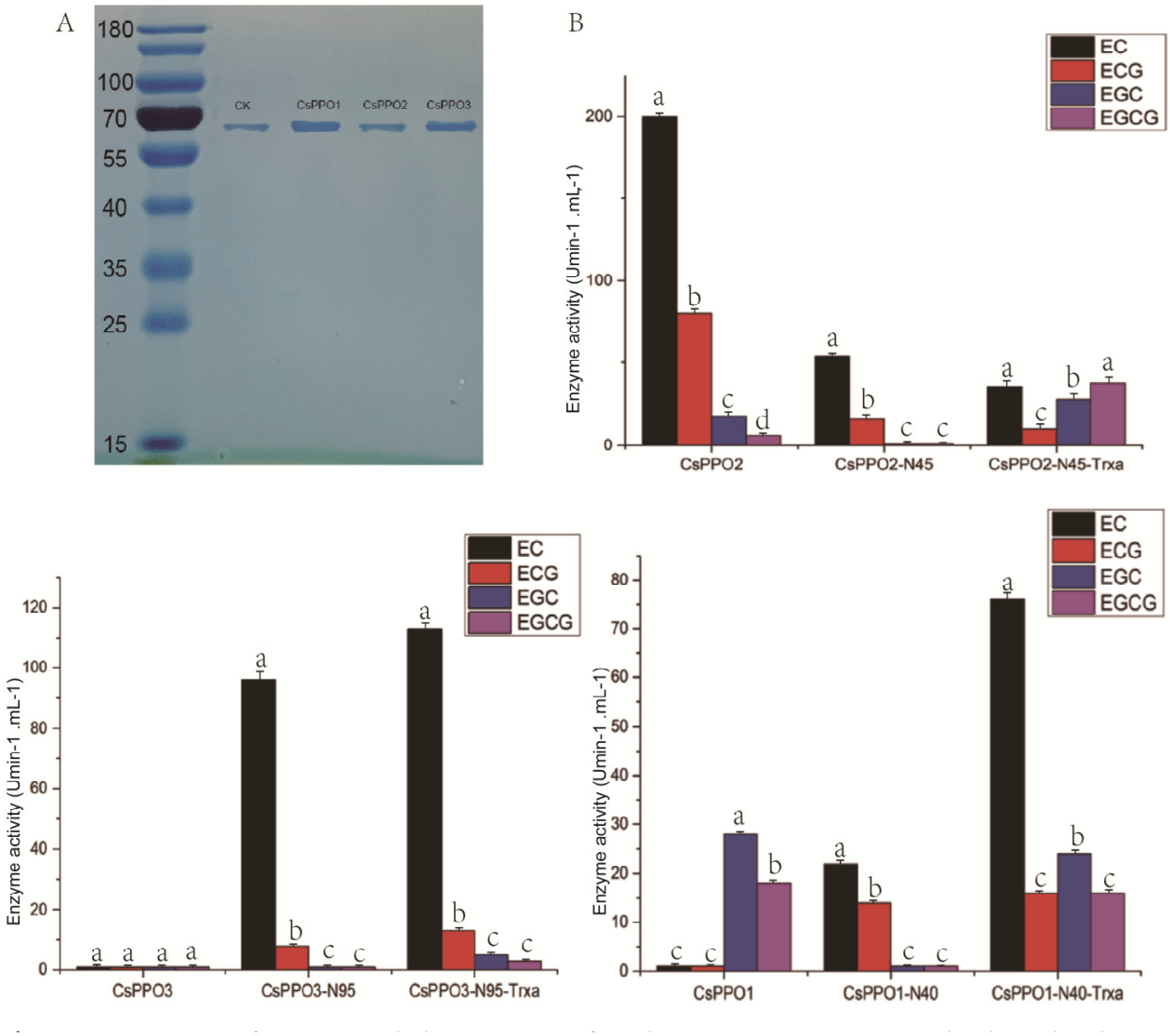

Figure 3. PPO purification and the activity of each isoenzyme on tea polyphenol substrates. (**A**): Purified protein. (**B**): The activity of various proteins on tea polyphenol substrates (different letters indicate significant differences $p < 0.05$).

3.4. Sequence and Structure Analysis of Four PPO Proteins

After performing structural simulation and prediction analysis on the four PPO isozymes, it was discovered that CsPPO1, CsPPO2, and CsPPO3 exhibited similar structures and all functioned as bicopper-ion-binding proteins. However, CsPPO4 showed no catalytic activity towards tea polyphenols and may work in conjunction with other isoenzyme proteins in tea plants. Examination of the amino acid residues surrounding the copper ions' active center revealed that conserved histidine residues encompassed the copper ions and formed the substrate catalytic activity center as well as the binding pocket. Nonetheless, the active centers and binding pockets of CsPPO1, CsPPO2, and CsPPO3 exhibited distinct conformations (refer to Figure 4), which might account for the varying activities of PPO proteins against different tea polyphenol substrates. These structural analyses yield critical insights into comprehending the catalytic mechanism and substrate specificity of PPO proteins. Understanding the correlation between PPO protein structure and function can facilitate the optimization of efficiency and yield in the enzyme-catalyzed synthesis of theaflavins.

The amino acid residues of the active centers of Csppo1, CsPPO2, and CsPPO3 are all histidine and exhibit distinct configurations. Figure 5 illustrates further analysis, highlighting the positions of active groups in the protein sequence. For CsPPO1, these groups are situated at positions 171, 192, 201, 357, 323, 356, and 327 of histidine. In the case of CsPPO2, the active groups can be found at positions 183, 204, 213, 370, 369, and 340 of histidine. As for CsPPO3, the active groups are located at positions 181, 202, 211, 337, 333, and 366 of histidine. The variation in amino acid positions leads to differences in the spatial structure of enzyme activity centers, resulting in varying enzymatic catalytic activities towards the substrates.

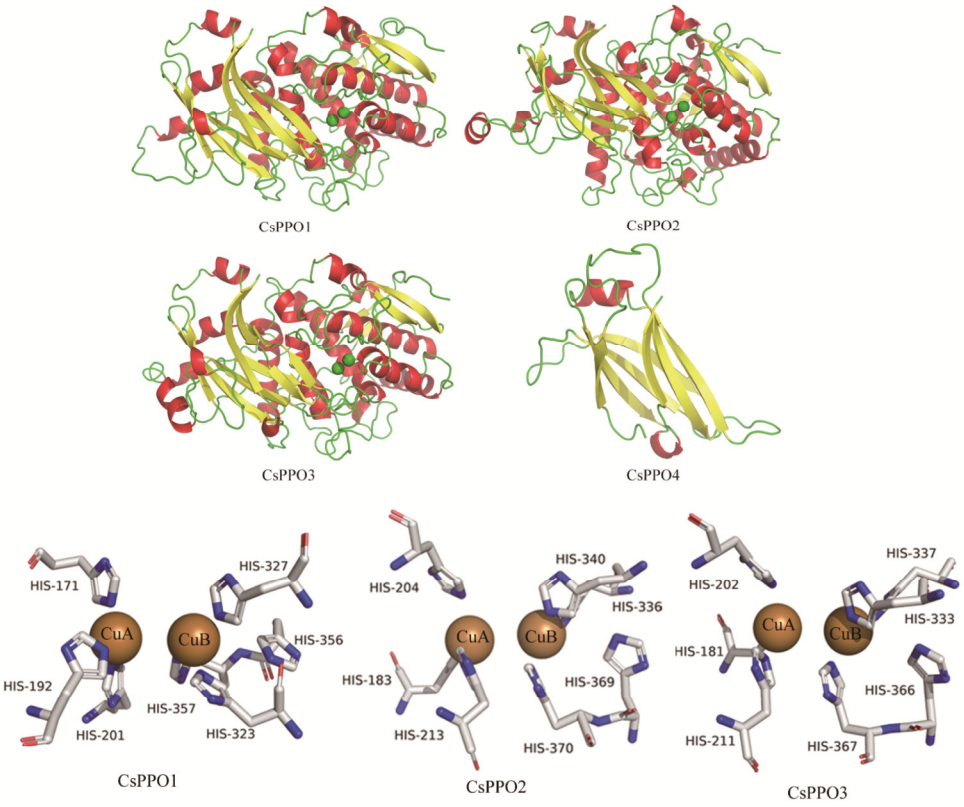

Figure 4. Structure prediction and active site analysis of each isoenzyme of PPO.

3.5. PPO Enzyme Immobilization and Catalytic Synthesis of TFDG

The ability of recombinant polyphenol oxidase and immobilized enzyme to catalyze the synthesis of TFDG was evaluated, and the results are presented in Figure 6. It was observed that both recombinant polyphenol oxidase and immobilized enzyme effectively catalyzed the synthesis of theaflavins from catechin substrates. However, the catalytic effect of the immobilized enzyme was slightly lower compared to the free enzyme. The concentration of TFDG in the system was approximately 102 μg/mL, and the concentration of the reaction product remained above 80 μg/mL in three consecutive batches, indicating excellent catalytic stability. Nevertheless, as the number of reaction batches increased, the product concentration exhibited a gradual decline. Two plausible explanations were analyzed: firstly, polyphenols might possess an inhibitory effect on the enzyme, resulting in decreased enzyme activity. Secondly, the polyphenol and the product could form more complex compounds with TFDG, making it challenging for the substrate to bind with the enzyme and ultimately decreasing the enzyme activity.

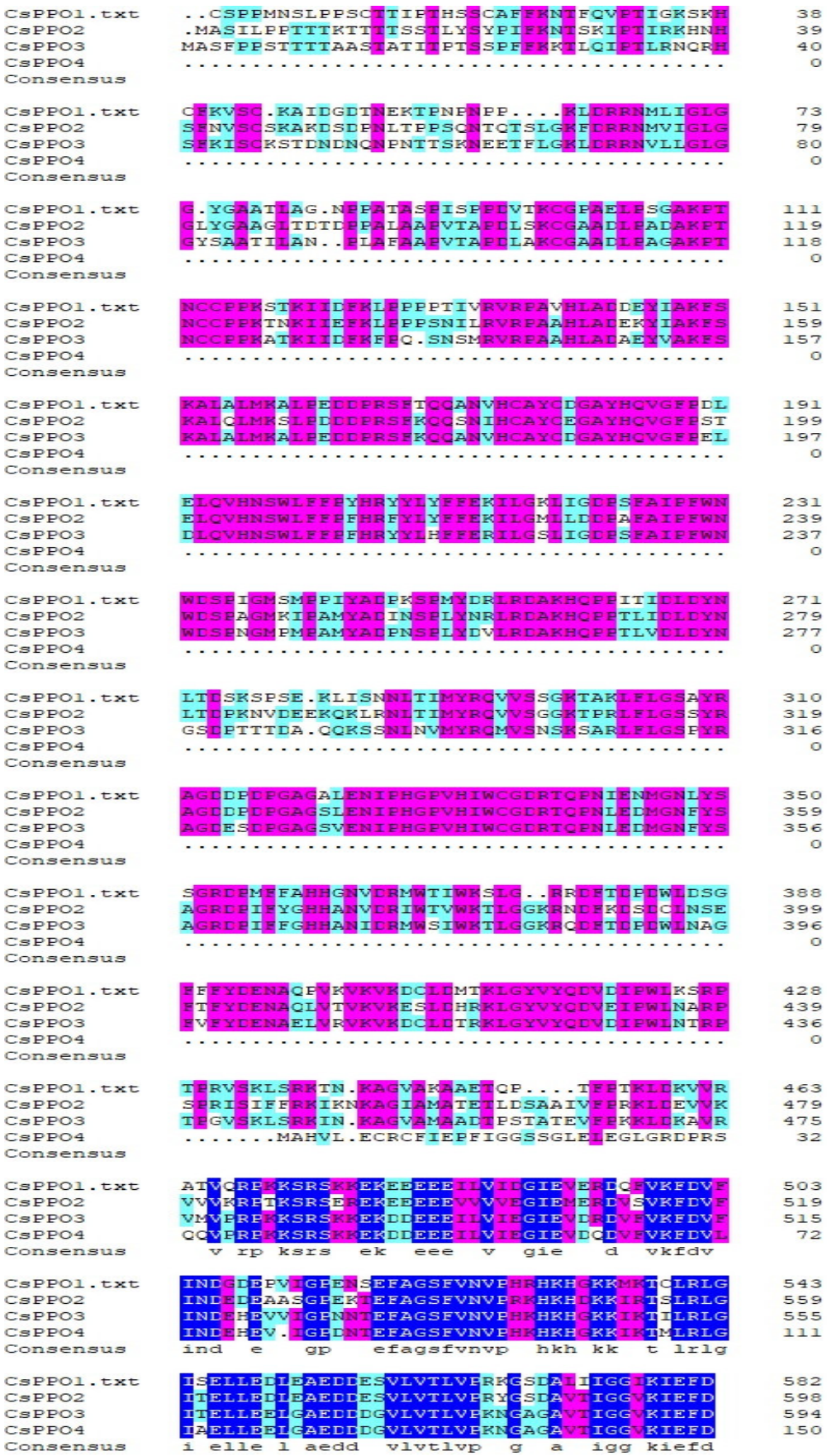

Figure 5. Amino acid sequence of 4 PPO enzymes.

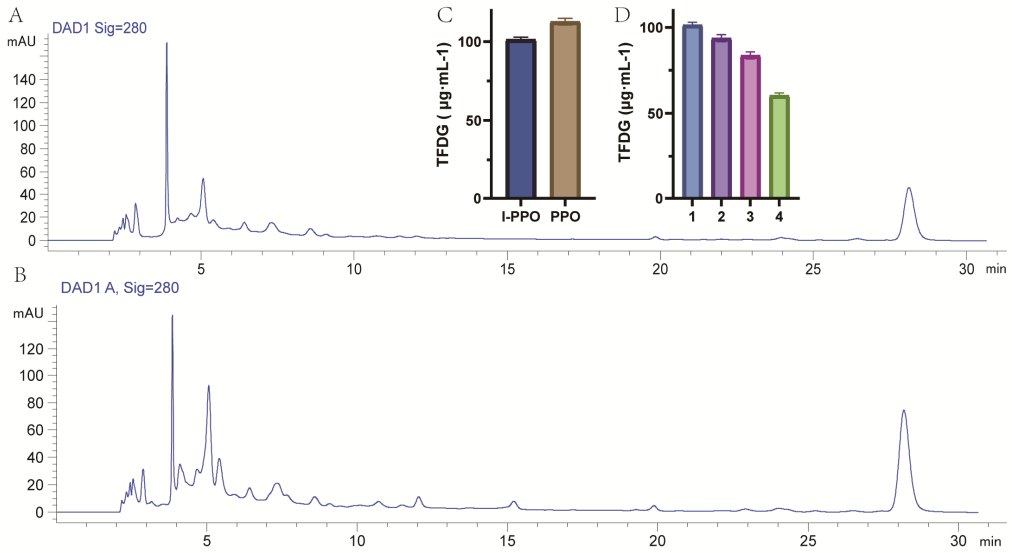

Figure 6. Catalytic synthesis of TFDG by CsPPO1. (**A**): Catalytic synthesis of TFDG by immobilized CsPPO1. (**B**): Immobilized CsPPO1 catalyzed the synthesis of TFDG. (**C**): CsPPO1 catalytic yield TFDG. (**D**): Dif-ferent batches of immobilized CsPPO1 catalyzed TFDG activity.

4. Discussion

The presence of multiple isozymes of PPO in the tea tree plays a crucial role in the formation of theaflavin, a key active ingredient in black tea [27]. Therefore, it is of great significance to develop the tea-tree-derived PPOs and use tea polyphenols as substrates for in vitro enzymatic synthesis of theaflavins [28]. However, the extraction of PPO from tea tree is complicated, costly and limited by raw materials, which restricts the possibility of its large-scale production. Since microorganisms are characterized by strong reproduction ability and many metabolites, transforming the tea tree PPO gene into prokaryotic organisms, such as *Escherichia coli*, and the high expression of the gene led to the production of a large number of polyphenol oxidases by the prokaryotic organisms. This is expected to solve the problems of complex processes, high costs, and raw material limitations in extracting PPO from the tea tree. Once the strains that can produce the desired PPO are successfully developed, large-scale production of theaflavins can be realized, thus providing an efficient and economical pathway for theaflavin synthesis. In this study, we obtained prokaryotic heterologous expression of full-length sequences, N-terminal truncated sequences, and N-terminal truncated + fused *TrxA* tags for four isozymes (CsPPO1, CsPPO2, CsPPO3, and CsPPO4) from tea tree sources. Our aim was to obtain a large quantity of soluble recombinant proteins for the enzymatic synthesis of theaflavins. We based our above-mentioned schemes on the following considerations: firstly, we excluded the N-terminal transit peptide, which is a 12–60 amino acid preamble sequence responsible for directing the import of proteins synthesized in the cytosol into mitochondria and chloroplasts [29]. The transit peptide ensures that the synthesized target proteins are properly localized within the appropriate organelles (membranes) and correctly folded with the assistance of molecular chaperone proteins. PPO proteins in plants are typically synthesized in the cytoplasm as precursor proteins containing a transit peptide, which is subsequently transported to the endosomal membrane with the involvement of the transit peptide, ultimately forming a mature peptide upon excision [30]. However, *E. coli* lacks the necessary excision and modification mechanisms, which is why we chose to truncate the predicted transit peptide protein for expression.

The experimental results revealed that the elimination of the transit peptide for CsPPO1 significantly increased the viability of EC and ECG substrates, while decreasing the viability of EGC and EGCG substrates. Conversely, the exclusion of the transit peptide for CsPPO2 did not yield favorable results. Secondly, we consider the addition of pro-soluble tags

to increase the soluble expression of target proteins. Prokaryotic expression results in the formation of a large number of inclusion body proteins, and the addition of a pro-soluble tag can increase the soluble expression of the target protein. Previous studies have demonstrated that the fusion of maltose-binding protein pro-soluble tag sequences can be effective in obtaining soluble proteins in recombinant expression of PPO. In this study, we chose the *TrxA* pro-soluble tag, considering its smaller molecular weight on the one hand, and on the other hand, we wanted to find other tags from different sources to try. The experimental results showed that the soluble protein expression was increased in the proteins after the addition of pro-soluble tags compared with the proteins without tags.

In terms of expression system selection, only prokaryotic expression vectors were chosen for *E. coli* expression studies of PPO in this study. However, since the PPO gene is of plant origin, its expression in *E. coli* suffers from imperfect folding and lack of post-translational processing modification of the protein, and even after fusion expression of N-terminal truncation + pro-soluble tag *TrxA* was performed, this did not have a significant effect. Compared to prokaryotic expression, Pichia yeast expression allows for post-translational modification of the protein, resulting in a higher efficiency of correct folding of the protein expression product. However, in the expression of tea tree PPO genes, the effect of microsystems expression is still poor, for example, Wang et al. [19] performed microsystems expression of PPO genes, and although active target proteins were obtained, they still could not meet the requirements of large-scale preparation.

PPOs is a copper containing redox metal enzyme with two copper atoms at its active site [31]. PPO is widely present in plants, humans, animals, microorganisms, and other organisms. The active center of PPO is located at the center of a hydrophobic pocket formed by four spiral beams, and each copper ion forms a coordination bond with N atoms in three He residues, forming a specific triangular pyramid shape [32]. CsPPO1 has a more complete structure compared to the other three enzymes (Figure 5), which may also be one of the reasons that CsPPO1 has the best catalytic effect.

In order to solve the problem of protein solubility, it is recommended to consider choosing other expression systems, such as Bacillus subtilis, tobacco cells, and mycobacteria to test in future studies. These expression systems may show better results in terms of proper protein folding and solubility. Combined experimental studies and analysis of the results indicated that none of the aforementioned expression routes effectively addressed the issue of soluble expression when expressing tea tree PPO in *E. coli*. The formation of a large number of inclusion bodies severely restricted the protein's activity and yield. Additionally, the addition of the *TrxA* pro-solubilization tag was ineffective, leading to low viability of the recombinant PPO to the tea polyphenols substrate. These findings align with previous research. Despite the low quantity of soluble proteins and the limited viability of recombinant PPO to the tea polyphenols substrate in *E. coli* expression, it is anticipated that this problem can be resolved through the heterologous expression of the tea PPO gene in alternative systems, like Bacillus subtilis or tobacco cells. Furthermore, exploring different pro-solubilization tags for fusion expression of PPO proteins or screening microbial sources of PPO genes with specific activity and catalytic efficiency for effective heterologous expression should be considered. These approaches hold potential to enhance the yield and activity of PPO proteins, thereby providing a more reliable foundation for further research.

Additionally, we evaluated the activities of three recombinant PPOs towards the tea polyphenols substrate. The results demonstrated that all three enzymes exhibited activity towards catechin monomers, with CsPPO2 showing the highest activity towards EGCG and ECG, which are precursors of TFDG. The utilization of free enzyme for industrial production presents limitations, such as single-use experiments and high costs. Improving free enzyme technology to enable multiple batch reuse can effectively reduce enzyme costs [33]. Immobilized enzymes are widely employed in industrial biocatalysis due to their stability, high recovery and reusability, and elevated catalytic activity [34]. In our study, the immobilized enzyme experienced a partial loss in enzyme activity during immobilization, but the immobilized PPO enzyme could be reused at least three times,

significantly improving catalytic efficiency and reducing the cost of theaflavin production. However, the immobilized polyphenol oxidase in our study still has limitations, such as the low efficiency of the recombinant PPO enzyme in catalyzing TFDG synthesis from the tea tree gene. Further research is required to identify a polyphenol oxidase with a higher catalytic efficiency.

5. Conclusions

In summary, there is significant potential in transforming the gene that governs the synthesis of PPOase in eukaryotes into prokaryotes, such as *E. coli*, and inducing high-level expression of this gene. This process allows prokaryotes to efficiently produce a substantial quantity of polyphenol oxidase and utilize immobilization technology to catalyze the synthesis of teosinte.

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