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Research advancements on the biomimetic generation and stability strategies of theaflavins: from mechanisms, synthesis influencing factors to encapsulation with nanomaterials

Beiqi Wu[a,b], Shan Wang[a,b], Chaoyang Ma[a,b], Wenping Lyu[a,b], Zaixiang Lou[a,b], Xinlin Wei[c], Hongxin Wang[a,b,*]

[a] State Key Laboratory of Food Science and Resources, Jiangnan University, Wuxi 214122, China
[b] School of Food Science and Technology, Jiangnan University, Wuxi 214122, China
[c] School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, China

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ABSTRACT

Theaflavins (TFs), natural orange-red tea pigments formed during tea fermentation and characterized by a benzophenone structure, are known for their multiple potential health benefits. However, their low content in black tea and instability pose challenges for the industrial production of high-purity TF products, limiting their broader application. A comprehensive understanding of current methods for synthesizing TFs is crucial for their industrialization. This review highlights the formation mechanism and the biomimetic synthesis of TFs, particularly the enzymatic approach and its various influencing factors. Additionally, recent advances in enhancing the stability of TFs are briefly introduced. Although significant breakthroughs have been realized in the *in vitro* enzymatic synthesis of TFs, enzymatic methodologies still face substantial challenges in scaling up for commercial production, while the directed synthesis of specific TF monomers remains technically constrained. As a result, exploring targeted synthesis processes for TFs is still a focal point for future research. Furthermore, the search for novel and efficient delivery systems to enhance the bioavailability of TFs is imperative for expanding the utilization and application scope of TFs.

1. Introduction

Theaflavins (TFs) were first discovered by Roberts in black tea and are natural orange-red pigments[1]. As distinctive polyphenolic compounds present in black tea, TFs are critical components that determine the quality and health effects of the tea. The chemical structure of TFs is relatively intricate, as they are formed from catechins during the fermentation of tea leaves through an oxidative polymerization reaction[2]. TF molecules contain a benzotropolone skeleton, linked by C–C and C–O bonds between 2 catechin molecules[3]. Based on the combinations and connectivity of the catechin units, TFs can be categorized into various types. The structural variations among these TFs confer unique biological activities to each variant[4].

TFs exhibit a variety of significant biological activities and health benefits. The structural characteristics of the phenolic hydroxyl groups in TFs confer exceptional antioxidant activity, allowing them to scavenge free radicals, inhibit lipid peroxidation, and protect cells from oxidative stress damage[5]. TFs have also demonstrated anticancer potential in various cancer studies, exerting antitumor effects by inhibiting cancer cell proliferation, inducing apoptosis, and blocking tumor angiogenesis[6]. In terms of cardiovascular health, TFs contribute to reducing the oxidation of low-density lipoprotein (LDL), decreasing vascular inflammation, and improving high-density

* Corresponding author at: School of Food Science and Technology, Jiangnan University, Wuxi 214122, China.
E-mail address: hxwang@jiangnan.edu.cn (H.X. Wang)
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lipoprotein (HDL) function, thereby helping to prevent atherosclerosis and related cardiovascular diseases[7]. TFs also play an important role in regulating blood glucose levels, improving insulin sensitivity, and influencing lipid metabolism, which may help in the prevention of diabetes and metabolic syndrome[8-9]. Furthermore, TFs display certain antibacterial and antiviral activities, effectively inhibiting the growth and spread of various pathogens[10].

The content of TFs naturally formed during the processing of black tea is extremely low, accounting for only 0.5%−2.0% of the dry weight of black tea[11]. This is primarily due to the low content of polyphenol oxidase (PPO) in black tea, resulting in limited enzyme activity, and to the fact that ideal conditions for its synthesis have not been met. Consequently, isolating high-purity TFs and their monomers directly from black tea is challenging and associated with high extraction costs. *In vitro* biomimetic synthesis of TFs, including chemical oxidation[12] and enzymatic oxidation methods[13], can facilitate their large-scale production. On the other hand, the bioavailability of TFs in the human body is very low, with subjects showing only 18.3 ng/mL of TFs in their blood after consuming black tea for 14 consecutive days[14]. The main reasons for this include: 1) The poor lipophilicity of TFs hinders cellular absorption[15]. 2) The instability of TFs, along with metabolic processes by intestinal microbiota, reduces their absorption[16]. 3) The presence of multiple hydroxyl groups in TFs allows them to form hydrogen bonds with cell membranes, altering membrane permeability and thus affecting their absorption and utilization[17]. These factors significantly impede the development of TFs based and TF-monomer based natural medicines and functional foods. Nanomaterial encapsulation technology, with its advantages of stimulus responsiveness, multimodal controlled release, and high mechanical properties, has been applied to enhance the stability of polyphenolic compounds[18].

This paper primarily reviews the mechanisms and synthetic methods of TFs *via* chemical and enzymatic approaches, as well as advancements in their stabilization studies. It discusses in detail the selection of different enzyme sources and examines various factors influencing enzymatic oxidation reactions, including enzyme type, pH, temperature, reaction time, oxygen, substrate composition, and concentration. The findings presented in this study may provide valuable insights for *in vitro* enzymatic synthesis of TFs and their stabilization, thereby improving their bioavailability, and serving as a theoretical basis and developmental pathway for their applications.

2. TFs formation mechanism

2.1 Formation mechanism of TFs during the processing of black tea

TFs are pigments generated during the processing of black tea and determine the quality of the tea. The main steps in black tea production include withering, rolling, fermentation, and drying[19]. After harvesting, the fresh tea leaves undergo a controlled withering process to reduce moisture content and enhance the activity of PPO, which is beneficial for the formation of TFs[20]. Proper management of temperature and time during the withering process can maximize PPO activity while minimizing excessive depletion of polyphenolic compounds. Following withering, the leaves are rolled to disrupt the cells and increase membrane permeability, allowing polyphenolic compounds to come into sufficient contact with PPO and undergo a series of enzymatic reactions, which is conducive to the subsequent fermentation process[21]. The activity of PPO increases dramatically during rolling, establishing the foundation for the distinct characteristics of black tea during fermentation. Fermentation is a critical stage in the development of black tea quality, marking the primary reaction through which the leaves transition from green to red[22]. Under the catalysis of PPO or peroxidase (POD), tea polyphenols are oxidized into a series of polymers, including TFs, thearubigins, and theabrownins. To achieve effective fermentation, it is necessary to control the temperature, humidity, time, and provide ample oxygen during fermentation, which is beneficial for the accumulation of TFs and reduces the formation of theabrownins[23]. The formation process of tea pigments during black tea processing was shown in Fig. 1.

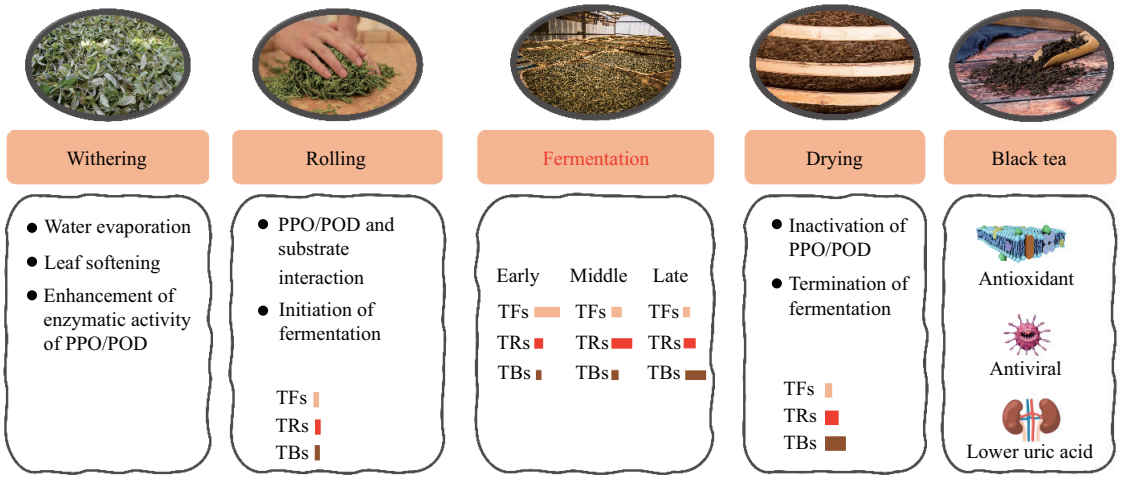

Fig. 1 Formation process of tea pigments during black tea processing. TFs are mainly formed during the fermentation process, and as the fermentation time increases, TFs decrease and are converted into thearubigins (TRs) and theabrownins (TBs).

2.2 Biomimetic synthesis mechanism of TFs

2.2.1 Chemical oxidation synthesis mechanism

The non-enzymatic oxidative synthesis of TFs, also known as chemical oxidative synthesis, involves the conversion of catechins into TFs using inorganic reagents as catalysts *in vitro*. This method eliminates PPO or oxygen, avoiding complications related to enzyme extraction and purification, enzyme instability, and difficulties in controlling reaction conditions. Commonly utilized chemical oxidants include $K_3Fe(CN)_6$, $FeCl_3$, $CuSO_4$, Ag_2O, PbO_2, and MgO_2. Researchers can manipulate reaction conditions to synthesize specific types of TFs[5]. The basic pathway of chemical synthesis involves the oxidation of catechins by chemical oxidants to form ortho-quinone intermediates. In the presence of water, these intermediates can participate in polymerization reactions with other catechin molecules. This process is a coupling reaction between phenolic hydroxyl groups, ultimately leading to the formation of TFs[4].

2.2.2 Enzyme oxidation synthesis mechanism

The enzymatic oxidation of TFs is a complex process that involves both enzymatic and non-enzymatic reactions. In essence, catechins undergo oxidation, polymerization, non-enzymatic condensation, and decarboxylation reactions mediated by PPO or POD to form TFs[24]. Catechins, as precursors to TFs, are natural polyphenolic compounds, with the primary contributors to TFs synthesis being catechol-type catechins (epicatechin (EC) or epicatechin gallate (ECG)) and pyrogallol-type catechins (epigallocatechin (EGC) or epigallocatechin gallate (EGCG))[3]. The structure of catechins has a decisive impact on the types of TFs ultimately produced[25]. Currently, TFs are isolated through purification or synthesized *in vitro* number up to 28 distinct varieties[24]. Among these, the 4 most common TFs found in tea are TF (or TF1), theaflavin-3-gallate (TF-3-G or TF2A), theaflavin-3′-gallate (TF-3′-G or TF2B), and theaflavin-3,3′-gallate (TFDG or TF3), which are formed from the polymerization of EC with EGC, EC with EGCG, ECG with EGC, and ECG with EGCG, respectively (Fig. 2).

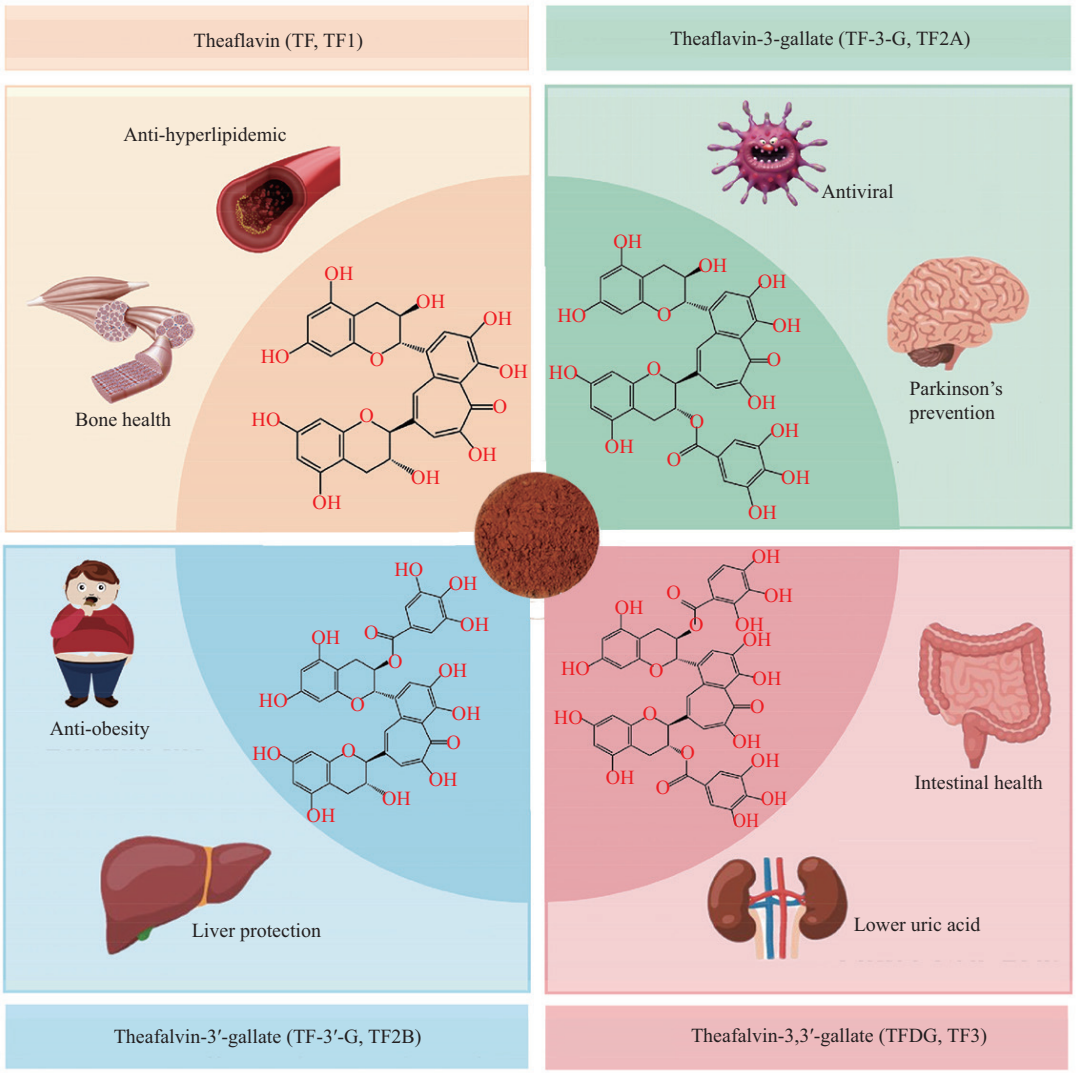

Fig. 2 Structures of the 4 major TFs and their biological activities.

At present, several pathways for the *in vitro* enzymatic synthesis of TFs have been reported. Understanding the mechanism of TFs formation is not only crucial for enhancing its yield but also essential for improving the quality of black tea. Yanase et al.[12] proposed that a catechin molecule is first oxidized by PPO or POD to form the corresponding catechin quinone, which then oxidizes a second catechin molecule to generate a second catechin quinone. This quinone subsequently undergoes Michael addition and carbonyl addition to form a bicyclo[3.2.1]octane-type intermediate (BOI), which is a critical step in the formation of TFs. Following this, in the presence of water, BOI is subject to rapid oxidation and decarboxylation reactions to form a benzotropolone skeleton, ultimately yielding TFs[26-27] (Fig. 3A). Takino et al.[28] proposed an alternative mechanism for the formation of the benzotropolone skeleton, where the B-rings of catechins self-assemble through intermolecular π-π interactions to form region-selective hydroquinone π-π complexes, and finally decarboxylation to generate the benzotropolone skeleton (Fig. 3B). Matsuo et al.[29] introduced a third mechanism, where catechol-type catechins are first oxidized by PPO to form catechol-type quinones, followed by a nucleophilic attack on the catechol-type quinone by pyrogallol-type catechins, and then condensation to form TFs (Fig. 3C). Currently, the first mechanism is widely accepted, and researchers are conducting *in vitro* synthesis studies based on the mechanism of TFs formation to increase the yield of TFs.

Moreover, studies have indicated that the polymerization of quinones also leads to the formation of thearubigins and theabrownins, resulting in a decrease in TFs content[7], which is also attributed to the presence of POD[30]. The unequal consumption rates of catechol and pyrogallol-type catechins can result in a reduction in total TFs levels and an increase in thearubigins levels[31]. The stable increase in TFs content is correlated with the average decrease of both groups of catechins. Other conditions of oxidative reaction conditions, such as pH and oxygen concentration, also significantly affect the efficiency of TFs production and the types of TF monomers formed[32]. Understanding the mechanism of TFs formation is not only crucial for enhancing its yield but also essential for improving the quality of black tea.

3. Recent progress in the biomimetic synthesis of TFs

To obtain more TFs, a biomimetic synthesis strategy has been developed based on their synthesis mechanisms. Catechins are extracted from tea leaves as key precursor substances for the synthesis of TFs. An appropriate catalyst is selected and the fermentation process of tea processing is simulated *in vitro* to synthesize TFs. Based on the type of catalyst, biomimetic synthesis can be classified into 2 main categories: enzymatic and non-enzymatic (i.e., chemical oxidation). Enzymatic oxidation employs natural oxidases as catalysts to synthesize TFs, while chemical oxidation relies on inorganic catalysts for their synthesis (Fig. 4).

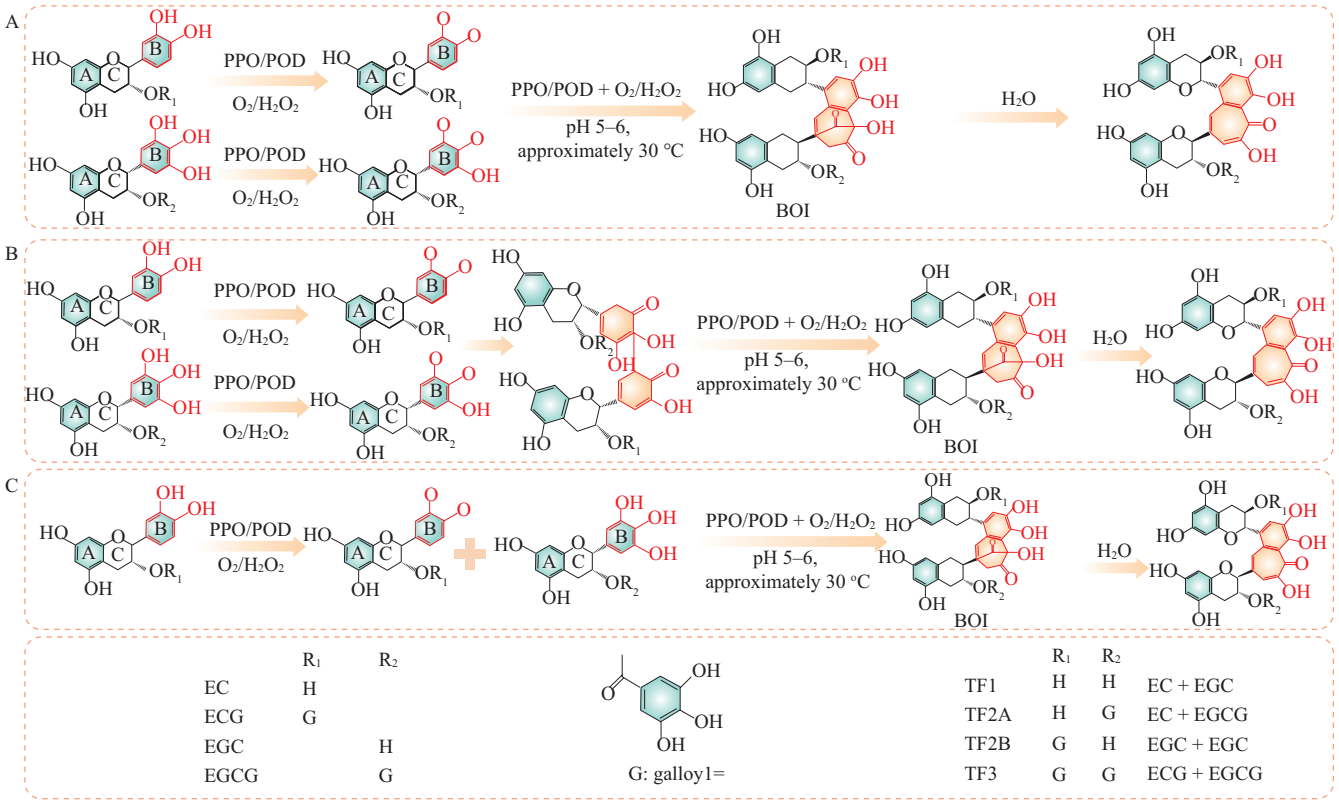

Fig. 3 The 3 potential mechanisms of TFs formation.

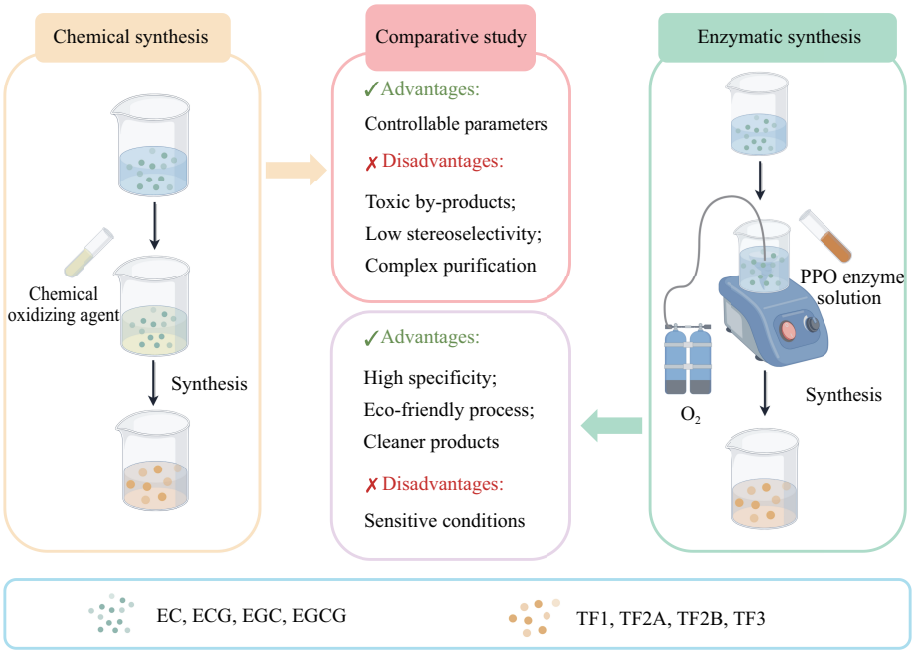

Fig. 4 Biomimetic oxidative synthesis of TFs.

3.1 Chemical oxidative synthesis

Chemical oxidation synthesis methods can be categorized into acidic oxidation and alkaline oxidation based on the pH of the oxidation system. Commonly used acidic oxidants include $K_3Fe(CN)_6$, $FeCl_3$, $Fe_2(SO_4)_3$, $Fe(NO_3)_3$, $CuSO_4$, Ag_2O, PbO_2 and so on. Alkaline oxidants typically include $K_3Fe(CN)_6$/$NaHCO_3$. Li[33] compared the differences in the composition of TFs synthesized under various oxidation systems and found that the proportion of TFs formed by chemical catalyst oxidation differs from that directly extracted from black tea infusion. The ester-type TFs were more abundant in the TFs extracted from black tea, while chemical oxidation TFs show a higher proportion of non-ester components. It was also pointed out that acidic oxidation products are primarily TF2A, while alkaline oxidation products are mainly TF1. Shan et al.[34] compared the variance in the synthesis of TFs using 3 different acidic oxidants, $FeCl_3$, $Fe_2(SO_4)_3$, $Fe(NO_3)_3$. The study showed that when $FeCl_3$ was used as the oxidant at a concentration of 3% of the total reaction volume, the highest yield of TFs (0.23%) was achieved. Wang et al.[35] compared the effects of acidic and alkaline oxidants on the synthesis of TFs and concluded that the alkaline oxidant $K_3Fe(CN)_6$/$NaHCO_3$ provided the more favorable oxidation results. Using EGCG as a substrate, the conversion rate of TFs reached 14.5%. Wang et al.[36] further explored the effects of the ratio of alkaline oxidant $K_3Fe(CN)_6$/$NaHCO_3$ to catechins and the concentration of catechins on TF formation. The results indicated that when the mixed catechin concentration was 10 mg/mL and a ratio of oxidant to catechin of 1:2, the final TF content reached 10.14%. In order to enhance the synthesis efficiency of TFs, Yanase et al.[12] directly synthesized the BOI using Fetizon reagent (Ag_2CO_3/Celite). Furthermore, to minimize undesired side reactions in chemical synthesis, it is necessary to protect and deprotect the hydroxyl groups of catechins during the oxidation process. Kawabe et al.[37] used 2-nitrobenzenesulfonyl (Ns) as a phenolic protecting agent to reduce side reactions of the electron-rich aromatic rings, thereby enabling the construction of a complex benzotropolone skeleton in a one-step oxidative coupling reaction. Meanwhile, $Pb(CH_3COO)_4$ was used as a catalyst, resulting in better stability of the catechin A ring, with a TFs synthesis yield of 5.7%. However, the inclusion of protecting agents in the catalytic reaction leads to the formation of other undesired by-products, making the reaction process more complex. To explore more possibilities for non-enzymatic synthesis of TFs, Matsuo et al.[29] conducted oxidation using 1,1-diphenyl-2-trinitrobenzene hydrazine (DPPH) as a catalyst without phenolic protectants, achieving a TFs synthesis yield of 20%.

Although chemical synthesis methods are simple and more easily controlled, they suffer from low product specificity and high by-product formation, which are not environmentally friendly. In addition, the oxidizing agents used in the chemical synthesis process may be toxic, and the safety of the products cannot be guaranteed.

3.2 Enzymatic oxidation of TFs

Enzymatic synthesis methods are favored in the preparation of TFs due to their high specificity, high yields, and minimal side reactions[25]. This method not only contributes to an in-depth understanding of the generation mechanism of TFs but also provides a technical basis for the large-scale production of TFs. To date, numerous researchers have focused on optimizing the synthesis conditions for the enzymatic oxidation of TFs, including enzyme sources, pH, temperature, substrate concentration and composition, reaction time, and oxygen[35-36]. These factors not only affect the efficiency of TF synthesis but also have an impact on the type of TF synthesized.

3.2.1 Enzymes for the synthesis of TFs

In TF synthesis, PPO offers a distinct advantage over POD. PPO catalyzes the oxidation of catechins to form highly reactive quinone intermediates. These intermediates subsequently polymerize to form the core structure of TFs[7,37]. Although POD can also catalyze the formation of ortho-quinones from catechins, leading to TFs production[38], it subsequently promotes the rapid polymerization of TFs into thearubigins and theabrownins, resulting in a decrease in TF yield[39-40]. The ability of pure PPO to produce TFs is significantly greater than that of pure POD[41]. Therefore, PPO is considered the most advantageous enzyme source for the production of TFs.

PPOs are a class of copper-containing metalloproteins with a molecular weight ranging from 60 to 95 kDa[42], widely distributed across various organisms such as tea plants, bananas, apples, and microorganisms[43-46]. PPOs can be classified into 3 groups based on catalytic properties and structural features, including tyrosinases (monophenol oxidases, EC 1.14.18.1), catecholases (diphenol oxidases, EC 1.10.3.2), and laccases (EC 1.10.3.1)[47-48]. Plant PPO precursor proteins generally comprise a transit peptide (which directs chloroplast import and is subsequently cleaved[49], a highly conserved Cu^{2+} binding region (containing 2 copper-binding domains that constitute the primary functional site)[50], and a hydrophobic region (critical for PPO folding and stability)[51]. Although the active site of PPO is conserved, the amino acid sequence exhibits significant variability across species, leading to the presence of multiple forms of PPO in most plants.

3.2.1.1 Crude PPO extract from different sources

To date, crude PPO enzymes are typically extracted using acetone solvent methods, aqueous[52] homogenization extraction[44], or a combination of both from various enzyme sources[45]. By these simple methods, a large quantity of crude PPO extracts can be obtained in a relatively short time, preparing the necessary amount of enzymes for industrial-scale application of TFs synthesis.

Due to structural variances, different sources of PPO have varying activities, giving rise to variation in the ability to catalyze TFs synthesis[13,53-55]. According to the source, PPO is categorized into endogenous PPO (from tea leaves) and exogenous PPO (from plants other than tea leaves and microorganisms). Robertson et al.[56] were the first to use crude PPO from tea leaves to simulate an enzymatic oxidation system *in vitro*. Their results demonstrated that PPO extracted from tea leaves and high-purity catechins could be used to prepare TFs. However, the concentration of endogenous PPO in fresh tea is relatively low and the method of direct extraction from fresh tea leaves was limited by the relatively short harvesting season of tea and tea varieties, which resulted in low yield and high cost of purified PPO and could not be applied to large-scale production of TFs[57-58].

As a result, researchers have been actively seeking new sources of PPO enzymes to replace tea PPO in catalyzing the formation of TFs in recent years. Microbial sources of PPO have low activity and considerable variability and are rarely used for TF synthesis[25]. On the other hand, PPOs from plants showed higher TFs synthesis capacity than those from microorganisms, which could be attributed to the strong substrate specificity of microbial PPOs and the demanding cultivation conditions required by various microbial strains, where changes in the living environment can easily lead to alterations in PPO enzyme activity and catalytic capacity[34,40,59]. The catalytic activity and substrate specificity of the enzyme vary greatly among PPOs from different plant sources due to differences in their structure and expression during the developmental period (Table 1). Kamabe et al.[37] evaluated the ability of 62 plant species to synthesize TF1, and the results showed that crude PPO extracts from 46 plant species were capable of synthesizing TF1, with loquat, Japanese pear, and blueberry demonstrating better activity than fresh tea leaves. Under optimal conditions, crude pear PPO increased TFs content in green tea beverages by 966.19 times[60], while PPO from Chinese yam showed TF1 yield of 30.66%[61]. There are also significant differences in the ability of PPO to catalyze the synthesis of TFs between different varieties from the same source, with PPO from Fengshui pear better in catalytic capacity than Gong pear, and Xiang pear due to higher amount of isozymes in the former[62], while PPO from yellow heart potato low in activity but higher in quantity than PPO from big bull horn potato, with TFs yield of 129.71 μg/mL in the former, among which TF3 content is significantly higher than other TF isomers[63].

Table 1
Comparison of enzymatic synthesis of theaflavins by PPO.

Enzyme sources	Substrate	Reaction conditions	Products	TFs content/ conversion rate	References
Tea purifying enzyme	Catechins (the total amount of EG, EGC, ECG and EGCG was 25 mg/mL)	2.5 mg/mL substrate, pH 6, 40 °C, 40 min	TFs	12 μg/mL	[59]
Apple crude enzyme	Catechins（≥ 98%）	2.5 mg/mL substrate, pH 5.6, 37 °C, 180 min	TFs	0.011 mg/mL	[70]
Chinese yam crude enzyme	EC, ECG, EGC, EGCG	5 mg/mL substrate, pH 5.6, 35 °C, 60 min	TFs	71.50%	[69]
Pear crude enzyme	Green tea powder	0.5 mg/mL substrate, pH 5.0, 40 °C, 100 min	TFs	2.47%	[68]
Eggplant crude enzyme	Green tea powder	2.5 mg/mL substrate, pH 4.5, 25 °C, 40 min	TFs	0.21 mg/mL	[86]
Pear purifying enzyme	Green tea powder	0.5 mg/mL substrate, pH 6.8, 90 °C, 10 min	TFs	14.68 mg/g DW	[87]
Pear crude enzyme	EC, EGC	0.7 mg/mL substrate, pH 5, 0 °C, 30 min	TF1	36%	[26]
Pear immobilized enzyme	ECG, EGCG	6 mg/mL substrate, pH 4.0, 30 °C, 40 min	TF3	42.23%	[81]
Potato purifying enzyme	Catechins (the total amount of EG, EGC, ECG and EGCG was 2.69 mg/mL)	6 mg/mL substrate, pH 5.5, 20 °C, 150 min	TFs	651.75 μg/mL	[32]
Mushroom commercial enzymes	ECG, EGCG	10 mmol/L substrate, pH 5.0, 40 °C, 60 min	TF3	80%	[13]
Immobilized laccase	Catechins	1.5 mg/mL substrate, pH 3.5, 40 °C, 180 min	TFs	30%−40%	[82]
Immobilized laccase	Catechins	1.5 mg/mL substrate, pH 3.5, 40 °C, 180 min	TFs	30%−40%	[82]

Although obtaining crude PPO is simple and cost-effective, a significant amount of impurities and multiple isoenzymes, which affect the purity and yield of the TFs and make it challenging to obtain specific TF monomers, are present in PPO crude extracts. Therefore, purification of the enzymes in combination with advanced biotechnology is necessary for obtaining PPO of higher purity, thus better synthesis efficiency of TFs, making PPO more feasible in industrial-scale applications[64].

3.2.1.2 Purified and modified PPO from various sources

The purification methods for PPO mainly include ammonium sulfate fractionation precipitation[65], ion exchange chromatography, gel filtration chromatography, affinity chromatography, hydrophobic chromatography, and reverse-phase chromatography[52,66]. Researchers first studied the purification of PPO from fresh tea leaves. By a combination of ammonium sulfate precipitation, Q-Sepharose Fast Flow anion exchange chromatography and Sephadex G-75 gel filtration chromatography, 2 types of PPO isoenzymes (PPO1 and PPO2), with PPO1, 12.90 times purer, capable of synthesizing TFs at the yield of 3.22 μg/mL under optimal conditions, were isolated[64,67]. Using isoelectric precipitation, ultrafiltration, and nanomembrane filtration, Li et al.[25] purified crude PPO extract from potatoes and synthesized 214.3 μg/mL of TFs.

Direct isolation and purification of PPO from plant tissues is a complex and costly process because of its reliance on a large quantity of raw materials, making it difficult to scale up in production and currently only suitable for small-scale applications and for high-added-value products. To lower the cost of industrialized PPO production, Li et al.[25] recovered PPO from the wastewater of sweet potato starch to successfully synthesize TFs, developing an economical, environmentally friendly, and simple method for the large-scale production of TFs. Commercial PPO, which is readily available on the market at a relatively low price, can also be used for the synthesis of TFs, by which the complex purification process can be avoided for TF producers. Yabuki et al.[13] compared the effects of different commercial enzymes, including tyrosinase (T3824) from mushrooms, laccase (Y120) from *Trametes* sp., and bilirubin oxidase from *Myrothecium* sp., on the rate of TF3 synthesis. They found that tyrosinase effectively synthesized TF3, with a conversion rate of up to 80%, while laccase and bilirubin oxidase only achieved conversion rates of 5.2% and 5.5%, respectively. It has been reported that PPOs within plants cannot simultaneously oxidize catechol-type and pyrogallol-type catechins, resulting in generally low yields of TFs, whereas tyrosinase oxidation can produce both types of quinones, accelerating the condensation of oxidation products and increasing the production of TFs[68]. Therefore, tyrosinase is a promising catalyst for the efficient synthesis of TFs[40]. While these methods represent potentially cost-effective alternatives for the large-scale production of TFs, a rigorous economic feasibility study is required, accounting for specific supply chain constraints and prevailing market conditions.

With the advent of modern biotechnology, the synthesis of TFs has become more economical and straightforward. Cloning and recombinant expression of PPO with high TF yields is another promising approach. The 4 potential PPO sequences namely CsPPO1, CsPPO2, CsPPO3, and CsPPO4, were identified from the whole genome of tea and they are highly conserved among tea tree varieties[54]. Among these, CsPPO1 has a better catalytic effect due to its more complete structure[69]. In addition, CsPPO isoenzymes (HjyPPO1 and HjyPPO3) from black tea were cloned and their catalytic efficiency studied, with results demonstrating that HjyPPO3 was twice as efficient as HjyPPO1, which could be attributed to the broader involvement of hydrogen bonds at the active site[70]. Zeng et al.[71] cloned PPO genes from nine species and successfully expressed them in *Escherichia coli.* The results proposed that the PPO enzymes from apple (Md2), pear (Pp4), and loquat (Ej2) exhibited higher enzymatic activity and TFs synthesizing capability than the crude enzyme extract. The tyrosinase from *Bacillus megaterium* was also expressed in *E. coli* and utilized in improving TF3 yield[72]. Coupled with the advantage of genetic engineering in production scaling-up, recombinant PPO expression is currently the optimal strategy for TF production on an industrial scale. Meanwhile, directed evolution of PPO and continuous flow bioreactor technology would be a future-oriented solution for large-scale green production of TFs.

3.2.2 Immobilization technology on the synthesis of TFs

PPO extracted from fruit and vegetables lacks reusability within enzymatic reaction systems and exhibits instability under certain harsh conditions. This necessitates additional production costs to separate the substrate from the enzyme. Immobilization technology enhances the stability and adaptability of enzymes, improving their utilization rate, thereby demonstrating commercial viability in terms of both efficiency and costs for the biosynthesis of TFs (Table 1). Zeng et al.[71] immobilized apple PPO on mesoporous silica, and the results showed that its activity could reach twice that of free enzymes, with a broader pH tolerance range (pH 4−6) and better thermal stability (10−40 °C). Tu et al.[73] immobilized crude tea PPO extract in a sodium alginate matrix, which demonstrated good stability (with activity maintained at 73% after 75 days of storage) and reusability (retaining 80% activity after 80 uses). Out of economic considerations, Sharma et al.[74] used more cost-effective cellulose as a matrix to immobilize crude tea PPO, and compared to free PPO, the conversion rate of TFs increased by 14 times, reaching 85%, and the enzyme activity retention rate was 83.58% after 14 cycles of reuse. As immobilization materials, new magnetic nanoparticles demonstrated advanced stability and acidity tolerance. Lei et al.[75] immobilized purified pear PPO on Fe_3O_4/chitosan nanoparticles, effectively synthesizing TF3 with a yield of 42.23%. The immobilized enzyme maintained approximately 85% activity after continuous use in 8 cycles. In addition to the enzyme, other TF boosting catalysts can be immobilized simultaneously on the same matrix to better increase TF conversion rate. Li et al.[76] utilized functionalized sodium alginate as a carrier to co-immobilize 2,2-diazodi-3-ethylbenzothiazolin-6-sulfonic acid (ABTS) and laccase on the carrier, catalyzing the synthesis of TFs, with a conversion rate ranging between 30% and 40%. The co-immobilized enzymes maintained more than 50% relative activity after 10 cycles of reuse.

Driven by consumer trust in natural compounds and the growing emphasis on sustainability, future synthesis of TFs is anticipated to progressively integrate enzymatic catalysis with immobilization technologies, thereby enabling industrial-scale production. The marriage of novel materials with advanced immobilization methodologies is expected to yield high-performance immobilized PPO, which is poised to become an effective strategy for efficient TFs synthesis in the years to come.

3.2.3 pH

The pH of the reaction system is crucial for the formation of TFs. It not only directly affects the activity of PPO but also influences the stability of TFs[77]. PPO exhibits optimal activity at specific pH values. For instance, the PPO from pear fruits demonstrates maximum catalytic activity at pH 5.5[78], while potato PPO has an optimal pH of 6.0, at which enzyme activity is at its highest[63]. TF monomers are more stable under acidic conditions, but they become unstable and prone to degradation and autoxidation into other compounds under neutral and alkaline conditions, with degradation rates increasing as pH rises[36]. Yabuki et al.[13] studied the dynamic changes in TFs synthesis with varying pH levels. They found that the content of TFs was extremely low at pH 3.0, significantly increased at pH 4.0, reached the highest synthesis efficiency at pH 4.5−5.0, and began to decrease as the pH rose within the range of 6.0−7.0. Additionally, pH affects different TF monomers differently. Acidic conditions favor the synthesis and accumulation of TF3, while neutral conditions are more conducive to the formation of TF1, TF2A, and TF2B[26]. Furthermore, pH directly alters the redox potential of catechins, thereby influencing the substrate conversion rate. When pH < 4, the electron-donating ability of catechins decreases, the oxidation rate of catechol-type catechins slows down, while the oxidation rate of pyrogallol-type catechins is less affected, which increases the formation of TFs overall. The consumption of catechins increased with the rise in pH, but no significant increase in the content of TFs was observed[79]. It is crucial for the synthesis of TFs to identify an appropriate pH value that can meet the optimal pH for PPO while ensuring the stability of TFs and preventing its oxidation.

3.2.4 Temperature

Temperature primarily influences the generation of TFs in catechin enzymatic reactions by affecting the activity of PPO and the activation energy of the substrate. At low temperatures (< 20 °C), PPO activity is inhibited. As the temperature rises, enzyme activity increases, but the oxygen demand of the reaction system also escalates. Excessively high temperatures (> 40 °C) lead to insufficient oxygen supply in the reaction system, thereby reducing the yield[80]. At the same time, high temperatures can denature the protein structure of PPO, resulting in decreased enzyme activity[67]. Elevated temperatures also accelerate the consumption of catechins, shortening the fermentation time required to reach maximum TF content[7]. Furthermore, the substrate in an enzymatic reaction requires appropriate activation energy, and when the thermal energy (manifested by temperature) reaches the activation energy needed by the substrate, the rate of the enzymatic reaction is accelerated[70]. In most researchers, the optimal temperature for PPOs to synthesize TFs is between 20−40 °C[62,81-82]. It has also been found that at lower temperatures, the total maximum level of TFs increases, while higher temperatures lead to the formation of other substances, such as thearubigins, which in turn decreases TF levels[83]. Jian et al.[26] investigated the impact of different reaction temperatures on the selective synthesis efficiency of TF1, finding that under ice bath conditions (2−3 °C), the by-products derived from EGC and EC were greatly reduced, which favored the synthesis of TF1, and the content of TF1 in the final system reached 220 μg/mL. In summary, the reaction temperature has a substantial impact on the formation of TFs, and optimal temperatures should be selected based on the specific source of PPO.

3.2.5 Composition, concentration and feeding methods of substrate

3.2.5.1 Substrate composition

The types and ratios of the main catechins in the substrate have a notable effect on both the oxidative coupling direction of catechins and the formation of TFs[84]. Yabuki et al.[13] found that when the substrate composition has a molar ratio of pyrogallol-type to catechol-type catechins greater than 3.0, the synthesis of TFs is more efficient. Hua et al.[41] highlighted that the ratio of EGC to EGCG is crucial for the formation of TFs during the enzymatic oxidation process. When the EGC/EGCG ratio is 1:2, it accelerates the oxidation rate of other substrates (EC, ECG), which is beneficial for the synthesis of TFs. Similarly, researchers have noted that appropriately increasing the proportion of EGCG in the substrate favors the formation of TF2A and TF3[85]. However, when sufficient EGCG is present in the system, the concentration of EGC becomes the key to determining the amount of TFs formed. This is because the composition ratio of the substrate catechins interacts with PPO activity, and a mixed substrate system (EC, EGC, EGCG, and ECG) is more favorable for the PPO-mediated catalytic reaction towards the synthesis of TFs[39].

3.2.5.2 Substrate concentration

Substrate concentration is also one of the key factors affecting the enzymatic synthesis of TFs. Lei et al.[75] investigated the effect of substrate concentration on the synthesis of TF3 and found that when the substrate concentration was below 0.5 mg/mL, the conversion rate of TF3 increased significantly. However, as the substrate concentration increased to 1.0 mg/mL, the TF3 content no longer increased and even declined. Additionally, when the substrate concentration was below 0.2 mg/mL, the catalytic efficiency of PPO decreased, and the synthesized TFs continued to be converted into other compounds such as thearubigins and theabrownins[80]. Wang et al.[86] used pear PPO to synthesize 645 mg of TFs per product at a substrate concentration of 5 mg/mL, achieving the highest synthesis yield. When the substrate concentration exceeded 10 mg/mL, the TF yield decreased rapidly. In another study, the enzymatic synthesis of TFs using potato PPO achieved a TFs yield of 651.75 μg/mL under the optimal substrate concentration conditions (6 mg/mL)[36]. Therefore, to avoid the inhibitory effect caused by excessive substrate concentration, a batch feeding approach for the substrate can be used[25]. Catechins, as substrates for PPO-catalyzed synthesis of TFs, also act as precipitants and denaturants for the enzyme. High concentrations of catechins can inhibit enzyme activity, thus affecting the synthesis efficiency of TFs[67].

3.2.5.3 Substrate feeding methods

Different substrate feeding methods allow better control of substrate concentration. Currently, the reported methods for substrate feeding are generally categorized into single-stage feeding, batch feeding, and continuous batch feeding. Li et al.[25] found that both batch feeding and continuous batch feeding methods significantly enhanced the synthesis of TFs, with the continuous batch feeding method enhancing the TFs content by 45.3%. Hua et al.[41] investigated various substrate feeding methods and discovered that the batch feeding method resulted in a

faster and more complete oxidation of catechins compared to single addition, maintaining higher levels of TF products over an extended period. Therefore, the batch feeding method presents a promising and efficient approach for the synthesis of TFs.

3.2.6 Time

The synthesis of TFs is a dynamic process characterized by the continuous transformation and accumulation of products and by-products. Therefore, reaction time plays a crucial role in the synthesis of TFs. The yield of TFs produced will increase with extended reaction time; however, if the reaction time is prolonged excessively, the TFs will be further oxidized and polymerized to produce therubigins and theabrowinn resulting in a decrease in TFs concentration[87]. Wang et al.[86] determined the optimal reaction time for synthesizing TFs is 40 min through orthogonal experiments. In another study, it was found that the majority of catechin substrates were consumed within 60 min of the reaction, yielding 0.41 mg/mL of TFs[78]. Besides, different types of TF monomers form at varying rates, and the optimal reaction times for synthesizing different TF monomers also differ. The formation rates of TF and TF2B are relatively fast, whereas the generation of TF2A and TF3 requires a longer reaction time[3]. Li et al.[36] reported that the maximum yields of TF1, TF2B, and TF3 catalyzed by potato PPO were achieved after 120 min, while the maximum yield of TF2A required 180 min. Precise control of the synthesis time not only facilitates the efficient generation of TFs but also enables the maximization of the yield of TF monomers through targeted synthesis, thereby meeting diverse requirements in food production.

3.2.7 Oxygen

The oxidation of catechins by PPO to form the corresponding quinones requires the presence of oxygen. Insufficient oxygen levels may cause incomplete substrate oxidation, while excessive oxygen may result in 2 adverse outcomes: on one hand, TFs are further oxidized to thearubigins, and on the other hand, excessive bubble formation leads to poor contact between the substrate and PPO, causing incomplete reactions[23]. Gu et al.[88] set the oxygen supply at 0.4 L/min in a 500 mL reaction system, achieving a maximum TFs content of 43.33%. In industrial production, an increase in oxygen levels dramatically boosts the yield of TFs catalyzed by PPO. Wang et al.[89] designed a continuous preparation system for TFs using a 100 mL packed-bed bioreactor, achieving a catechin conversion rate of 20.99% at an oxygen flow rate of 55 mL/min, providing a reference for the industrial production of TFs. Li et al.[25] conducted a scaled-up enzymatic synthesis of TFs in a 3 L fermenter and found that oxygen is a key factor in the enzymatic process; within the range of 0−300 L/h of air flow rate, the yield of TFs increased with the increase of air flow rate.

To conclude, the enzymatic synthesis efficiency of TFs is regulated by multiple factors in a synergistic manner, among which the enzyme type and dosage, the substrate concentration and composition, as well as pH, are the core regulators that significantly influence the yield and selectivity of the TFs. Temperature and reaction time also need to be optimized and in alignment with these core factors. In the future, by screening for high-activity enzymes, dynamically controlling substrate feeding, and identifying the pH-sensitive range, it will be possible to simultaneously improve the yield and selectivity of TFs, providing a theoretical basis for industrial production.

4. Progress on the stabilization of TFs

4.1 Instability and low bioavailability of TFs

The structure of TFs contains multiple phenolic hydroxyl groups, ester structures, and multiple benzene structures connected by single bonds, which render the molecule inherently unstable. Numerous factors during food processing, such as temperature, pH, and ion concentration, can disrupt the structure of TFs[90]. As the temperature increases, the single bonds within TFs are prone to break, leading to decreased stability with rising temperature. The polyhydroxy structure imparts a weak acidity to TFs, and under alkaline conditions, the enolic structures within TFs are readily converted to ketonic structures, thereby reducing their content[91]. It has been reported that the degradation rate of TFs after being cultured at pH 7.4 for 8 h is 34.8%, and when the pH is increased to 8.5, the degradation rate reaches 78.4% within 2 h[92]. Moreover, the stability of different TF monomers varies, ranked from most to least stable as TF3 > TF1 > TF2A > TF2B. The presence of metal ions, such as Fe^{3+}, can react with phenolic hydroxyl groups, disrupting the inherent stable structure of TFs[93]. The instability of the TF structure directly contributes to its low bioavailability. Research has reported that after administering a high dose of TFs (up to 700 mg, equivalent to approximately 30 cups of black tea) to healthy volunteers, the peak levels of TFs in plasma and urine were only 1.0 and 4.2 μg/mL, respectively[94]. In a separate study, mice treated for 2 weeks with caffeine-free black tea (50 mg/g diet) showed TF3 levels in tissue samples below 1 nmol/g tissue[95].

The digestion, absorption, distribution, metabolism, and excretion of TFs in the human gastrointestinal tract are significant factors contributing to the low bioavailability. Researchers have found that the hydroxyl and galloyl groups of TFs interact with the catalytic residues in the center of digestive enzymes through hydrogen bonding and π-π interactions, which reduces their activity and affects the digestibility of TFs[96]. Meanwhile, TFs can cover the active sites of these enzymes, resulting in relatively low digestibility of the proteins and providing increased resistance to proteolysis[97].

It has been shown that TFs are metabolized into small molecules by the intestinal microbiota after passing through the gastrointestinal tract to the colon, which is one of the main reasons for their low bioavailability[98]. The absorption of TFs in both the upper and lower gastrointestinal tracts was found to be undetectable by fecal culture and *in vitro* feeding. Furthermore, the structure of TFs can resist degradation by colonic bacteria. After 24 h of incubation of feces from mice ingesting TFs, the recovery rate of TFs was 67%, while 21 phenolic and aromatic metabolites were generated[15], suggesting that the metabolic reaction of TFs is mainly a process of conversion of complex TFs to simple TFs. Additionally, using a Caco-2 monolayer model to simulate the transport of TFs revealed that efflux transport proteins, such as P-glycoprotein and multidrug resistance associated protein, actively pump TFs out of intestinal cells and back into the intestinal lumen, thereby reducing their opportunity for absorption[99]. Overall, structural instability, cellular metabolism, and efflux transport proteins all contribute to the low bioavailability of TFs.

4.2 Strategies for stabilization of TFs

The instability of TFs limits their application in the food and pharmaceutical sectors, highlighting the necessity for stabilization studies on TFs. Common methods employed to enhance the bioavailability of polyphenols include structural modification, material encapsulation, and interactions with biomacromolecules. Encapsulation strategies have been successfully employed for numerous polyphenols. This approach utilizes diverse protective wall materials to safeguard bioactive substances *via* physical adsorption, chemical interactions, or phase separation techniques[100]. The primary objectives are to protect the bioactive compounds and control their release at targeted sites, thereby enhancing their bioaccessibility. Based on the composition of the wall material, these strategies can be classified into polysaccharide-based, protein-based, and lipid-based encapsulation[101]. Furthermore, depending on the requirements of a specific food application system, they can be categorized as emulsion-based systems or particulate-based systems[100]. By identifying suitable components for encapsulation and delivery systems to encase and transport TFs, it is possible to achieve sustained and controlled release, thereby maximizing the bioavailability of TFs. Currently, research focused on improving the stability and bioavailability of TFs is relatively scarce.

Different types of carriers are considered as promising TF delivery systems (Fig. 5), where nanoparticles and nanoemulsions possess a large specific surface area, which facilitates increased contact area between active ingredients and biological membranes, thereby prolonging their retaining time in the small intestine. Jiang et al.[102] synthesized chitosan-casein phosphopeptide nanocomplexes (CS-CPP) for encapsulating TF3, demonstrating through Caco-2 monolayer cell models that CS-CPP nanoparticles significantly enhanced the stability of TFs and the intestinal permeability of TF3. Although nanoparticles confer superior storage stability and transportability across diverse food matrices, nano-emulsions exhibit markedly enhanced delivery performance for the poorly water-soluble TFs. Chitosan-based nano-emulsions for delivering TFs achieved an encapsulation efficiency of 80.04%[103]. Observation by fluorescence microscopy revealed that the chitosan-TF nano-emulsions underwent a hydrolysis process in the gastrointestinal tract. In the stomach, the emulsion droplets exhibited flocculation; however, this flocculation disappeared after entering the small intestine. Ultimately, the bioavailability reached 9.60%, providing a valuable reference for improving the stability of TFs[103]. To overcome the storage and transport instabilities inherent to conventional nano-emulsions, Lu et al.[104] developed a W/O Pickering emulsion that elevated TFs' bioaccessibility to 57.8%. Pickering emulsions, stabilized by solid particles, exhibit exceptional physical stability. The concomitant reduction in surfactants renders these systems non-irritating and non-toxic, enabling their seamless deployment in food-grade applications .

Lipid-based delivery systems employ nanostructures primarily composed of natural phospholipids. These phospholipids self-assemble into phospholipid bilayer vesicles, encapsulating an aqueous core. This unique architecture enables the simultaneous encapsulation of both hydrophilic and hydrophobic therapeutic actives. Owing to their structural homology with cellular membranes, which allows for fusion with cellular membranes and thus improves the permeability

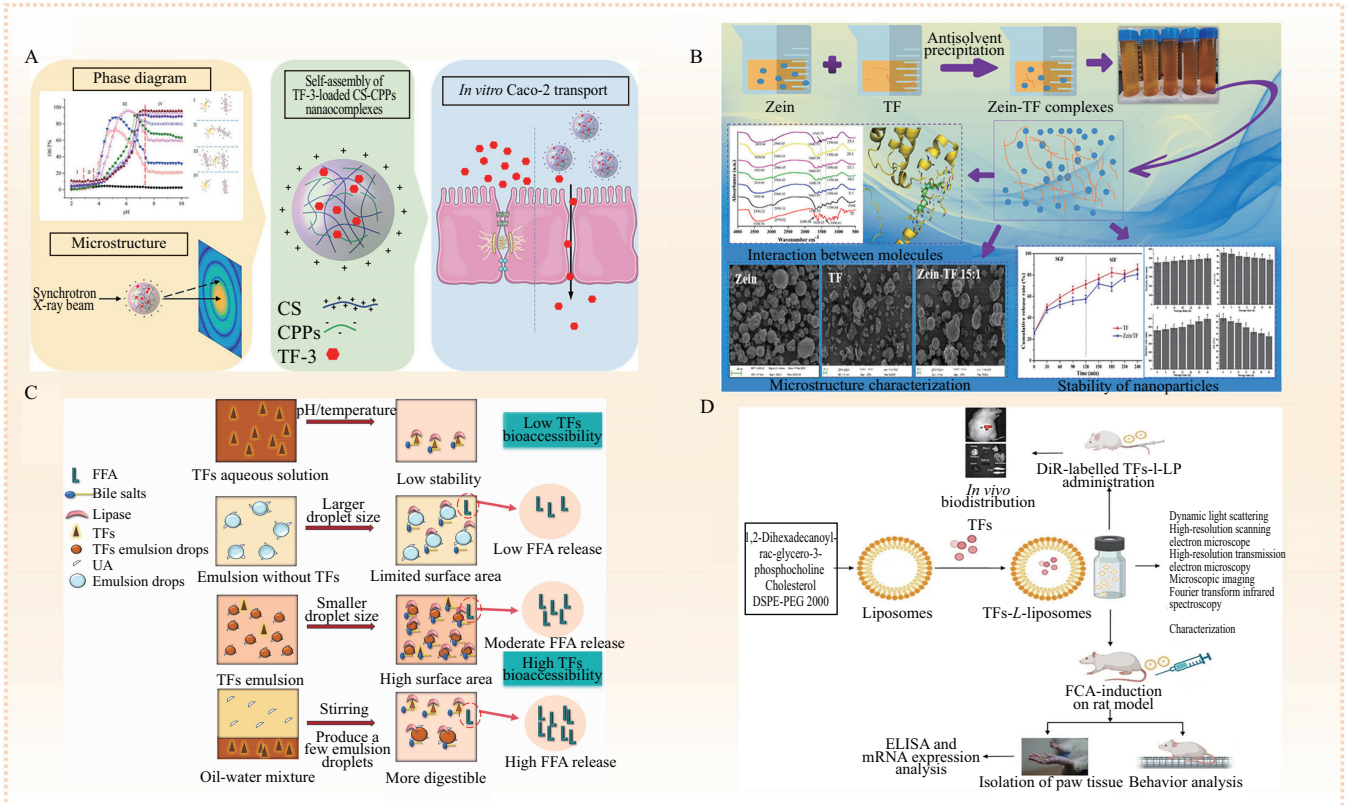

Fig. 5 Different delivery carriers for polyphenols. (A) Polysaccharide-based nanoparticles; (B) Protein-based nanoparticles; (C) Pickering emulsions; (D) Liposome[102,104,110,115].

of drugs across the cell membrane[105]. Key representatives of this system including liposomes, solid lipid nanoparticles (SLNs), and nano-structured lipid carriers (NLCs)[106]. Additionally, lipid carriers exhibit excellent biocompatibility and safety, which helps to mitigate immune responses and cytotoxicity during the delivery process, and thus they are widely used to encapsulate a variety of active substances[107]. Guri et al.[108] evaluated the ability of SLNs to deliver polyphenolic compounds in co-culture systems of Caco-2 and HT29-MTX cells. Compared to free curcumin, the delivery of the drug in SLNs was enhanced without affecting the integrity of cell junctions. Ding et al.[109] successfully prepared TF3 nanoliposomes with a particle size of 60 nm using the ethanol injection method combined with dynamic high-pressure microfluidization, which remarkably enhances the stability of TF3. Salim et al.[110] used liposomes prepared by thin-film hydration to encapsulate TFs and achieved a 68% encapsulation efficiency, along with a 43% *in vitro* release rate.

The application of liposomes is restricted by their inherent limitations, including storage instability, ineffective targeting, and the dependence on complex chemical modifications. Conversely, polyphenol-coated magnetic nanoparticles can achieve targeted delivery of bioactive agents to tumor sites, thereby eliciting potent anti-tumor efficacy with minimal adverse effects[111]. Meanwhile, carrier-free polymer nanoparticles capable of accommodating polyphenolic cargo circumvent the need for heterogeneous delivery systems, thereby eliminating the potential introduction of any contaminants or toxic by-products during encapsulation and preserving the intrinsic bioactivity of polyphenols[112]. Dendrimers further enable the co-encapsulation of amphiphilic polyphenols, facilitating their sustained release and, notably, their preferential accumulation within cancer cells[113]. Compared with liposomes, carbon nanomaterials offer superior specific surface area and higher drug-loading capacity, coupled with enhanced targeting efficiency and prolonged circulation half-life, positioning them as promising platforms for the delivery of bioactive compounds[114]. Collectively, these innovative delivery methods hold significant future potential for the stable delivery of TFs, which may enhance their bioavailability and controllable release capabilities, thereby further promoting their application in the health food and pharmaceutical sectors.

5. Conclusion and future perspectives

In this comprehensive review, we systematically elucidate the formation mechanisms of TFs, with particular emphasis on the PPO-mediated enzymatic oxidation pathway. We provide an in-depth analysis of the key influencing factors and their regulatory mechanisms within this synthetic route. The primary objective is to offer researchers and industrial practitioners comprehensive theoretical guidance and practical references for developing efficient, green, and economical *in vitro* synthesis strategies for TFs. Traditional chemical synthesis methods for TFs face persistent significant challenges. These include low substrate specificity, resulting in poor selectivity for the target products, complex reaction pathways generating structurally complex by-products that are challenging to separate, and reliance on energy-intensive processes or hazardous reagents, leading to environmental unfriendliness. By contrast, the PPO-catalyzed enzymatic oxidation approach presents compelling advantages congruent with the principles of green synthesis. Nevertheless, the efficiency of enzymatic TFs synthesis and the resulting product profile are contingent upon multiple variables, including enzyme type, pH, substrate concentration, temperature, and reaction duration. Precise modulation and optimization of these parameters enable the attainment of high-yield, high-selectivity TFs synthesis, thereby establishing a robust foundation for scalable production.

Nevertheless, the current sources of PPO primarily rely on crude extracts derived from plant tissues (such as tea leaves) or necessitate complex, time-consuming, and costly purification procedures to obtain enzyme preparations with high catalytic potency. Crude enzyme extracts suffer from inherent instability in enzymatic activity and significant interference from impurities. Furthermore, conventional purification methods are hampered by low enzyme recovery yields, resulting in an overall process with poor economic viability. These limitations severely constrain the industrial-scale implementation and cost competitiveness of the enzymatic synthesis of TFs. Consequently, the development of enzymes exhibiting high specificity and effective catalytic capacity remains a paramount research focus within this field. Future advancements should prioritize the development of high-performance immobilization carriers, the engineering of highly stable recombinant PPO, and the integration of these technologies with packed-bed bioreactor processes. This integrated approach aims to construct an efficient, stable, reusable, and cost-controllable enzymatic catalytic system. Such a system is anticipated to significantly enhance the economic feasibility and sustainability of the enzymatic synthesis of TFs, thereby accelerating the transition of enzymatic oxidation synthesis for TFs from laboratory-scale to industrial-scale production.

Moreover, the inherently low chemical stability and limited intestinal permeability of TFs severely compromise their oral bioavailability. Various nanoencapsulation systems hold significant potential to enhance the bioavailability and augment the bioactivity of TFs. For oral administration, the harsh pH conditions and presence of digestive enzymes within the gastrointestinal tract impose stringent requirements on the structural integrity and protective capabilities of the encapsulating wall materials used in these systems. Consequently, future research efforts should prioritize enhancing the gastrointestinal stability of nanocarrier wall materials and comprehensively characterizing their structural behavior under these challenging conditions. Beyond oral delivery, exploring alternative administration routes, including intravenous administration, nebulized inhalation, or targeted drug delivery, presents a viable strategy to circumvent the limitations associated with poor oral bioavailability. This diversification in delivery approaches could maximize the therapeutic and functional utility of TFs across diverse applications and potentially enable the development of novel therapeutic strategies and functional food products. Additionally, comprehensive investigations into the molecular interactions between TFs and food-derived or co-formulated compounds are warranted. Elucidating synergistic or antagonistic effects will provide a mechanistic foundation for the rational design of TF-based dietary supplements and pharmaceutical formulations, thereby facilitating robust efficacy assessment and guiding subsequent translational research.

This paper provides scientific rationale and strategies for the efficient *in vitro* synthesis of TFs and for improving their bioavailability. Continuous research by subsequent investigators is essential for further developing the synthesis and application of TFs.

Declaration of competing interest

The authors declare no conflict of interest.

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