Biochemical characterization of *Dimocarpus longan* polyphenol oxidase provides insights into its catalytic efficiency

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The “dragon-eye” fruits produced by the tropical longan tree are rich in nutrients and antioxidants. They suffer from post-harvest enzymatic browning, a process for which mainly the polyphenol oxidase (PPO) family of enzymes is responsible. In this study, two cDNAs encoding the PPO have been cloned from leaves of *Dimocarpus longan* (Dl), heterologously expressed in *Escherichia coli* and purified by affinity chromatography. The prepro-DIPPO1 contains two signal peptides at its N-terminal end that facilitate transportation of the protein into the chloroplast stroma and to the thylakoid lumen. Removal of the two signal peptides from prepro-DlPPO1 yields pro-DlPPO1. The prepro-DlPPO1 exhibited higher thermal tolerance than pro-DlPPO1 (unfolding at 65 °C vs. 40 °C), suggesting that the signal peptide may stabilize the fold of DlPPO1. DlPPO1 can be classified as a tyrosinase because it accepts both monophenolic and diphenolic substrates. The pro-DlPPO1 exhibited the highest specificity towards the natural diphenol (–)-epicatechin (kcat/KM of 800 ± 120 s⁻¹ mM⁻¹), which is higher than for 4-methylcatechol (590 ± 99 s⁻¹ mM⁻¹), pyrogallol (70 ± 9.7 s⁻¹ mM⁻¹) and caffeic acid (4.3 ± 0.72 s⁻¹ mM⁻¹). The kinetic efficiencies of prepro-DlPPO1 are 23, 36, 1.7 and 4.7-fold lower, respectively, than those observed with pro-DlPPO1 for the four aforementioned diphenolic substrates. Additionally, docking studies showed that (–)-epicatechin has a lower binding energy than any other investigated substrate. Both kinetic and *in-silico* studies strongly suggest that (–)-epicatechin is a good substrate of DlPPO1 and ascertain the affinity of PPOs towards specific flavonoid compounds.

Polyphenol oxidases (PPOs) are type-III dicopper metalloenzymes present in bacteria1, fungi2–3, archaea4, plants5, insects6,7, and animals including humans8–9. The PPO family contains tyrosinases (TYRs)10–13 and catechol oxidases (COs)14,15. TYRs catalyze the ortho-hydroxylation of monophenols to o-diphenols (monophenolase activity, EC 1.14.18.1) and subsequently the oxidation of the corresponding o-diphenols to o-quinones (diphenolase activity, EC 1.10.3.1), whereas COs can only perform the latter diphenolase activity13,16. The resulting quinones are highly reactive compounds and rapidly polymerize non-enzymatically, thereby forming yellow to black complex pigments known as melanins17,18.

In vivo, plant PPOs are translated as a precursor protein (prepro-PPO), corresponding to the translation of ~ 600 amino acids with approximately 60–75 kDa19,20. A prepro-PPO contains three distinct domains; an N-terminal signal sequence, the catalytically active domain that harbors the Cu-Cu site and a C-terminal domain shielding the active domain5,20,21 (Figure S1). The signal sequence (~ 80–100 amino acids) directs the protein to its sub-cellular destination while it is itself being cleaved off22,23. Following translocation across the thylakoid membrane the remaining passenger protein is processes to a latent form (pro-form) which is generally reported to be 45–69 kDa14. The binuclear copper center, where each copper is individually coordinated by three histidine residues24,25 located in the active domain, remains shielded by the C-terminal domain, effectively preventing the exposure of the active site to candidate substrates. The latency becomes lifted when the C-terminal domain is detached from the active site. In vitro, the disruption may be affected by proteases (e.g. trypsin, proteinase K)25, acidic or basic pH24, fatty acids26, and artificial detergents (e.g. sodium dodecyl sulfate, SDS)27–29. Although the natural proteolytic mechanism is unknown30, it has recently been reported that plant PPOs are capable of self-activation which can autoproteolytically separate the active enzyme from the C-terminal domain34–36.
Longan (Dimocarpus longan, DL) belongs to the soapberry Sapindaceae family and is native to the subtropical Southeast Asia region. The fruit is enriched with several nutritional components, especially the pericarp contains high amounts of phenolic compounds such as (-)-epicatechin, 4-methylcatechol, pyrogallol, caffeic acid, gallic acid, quercetin, vanillic acid and ferulic acid (Figure S2) that exhibit strong anti-inflammatory, antioxidant and anticancer activities. Despite the health benefits obtained from the phenolic compounds, longan pericarp can deteriorate rapidly and develop a brown color two to three days after harvest under ambient temperature. The presence of the resulting brown complexes substantially lowers the quality of the fruits.

Physical approaches including fungicide dips, wax, chitosan coating, and sulfur fumigation have been applied to extend the storage life of longan, however, the browning reaction has remained the main problem of the longan industry. Though PPOs have been extensively investigated as the main cause of post-harvest browning in many agricultural products, only few reports are available on the biochemical properties of longan PPO. The enzyme was extracted from the natural source and was purified by ammonium sulfate precipitation and successive column chromatography (Sephadex G-200 and Phenyl Sepharose) or dialysis. The optimal pH and temperature for the extracted longan PPO were reported to be 6.5 and 35 °C, respectively. The enzyme was active towards pyrogallol, 4-methylcatechol and catechol. A further report also described (-)-epicatechin as the optimal endogenous substrate for longan. A longan PPO gene was also cloned but has not been heterologously expressed. The enzyme classification of the longan PPOs remains unknown and their specificity and kinetic efficiency towards phenolic substrates have not been well-studied so far.

Here, two cDNAs containing PPO genes have been cloned from Dimocarpus longan leaves. The pro-DIPPO1 encodes a latent form, while the prepro-DIPPO1 encodes a precursor of the latent form that additionally contains a signal sequence. The signal sequence consists of a transit peptide which facilitates transport into the chloroplast stroma and a thylakoid-transfer signal that designates the thylakoid as the protein’s final destination. It was observed that the copper contents of pro-DIPPO1 were previously detected in longan extracts by HPLC while standard substrates are those commonly used (tyramine, tyrosine, dopamine and DOPA) (Figure S3). Natural substrates refer to phenolic compounds that were previously detected in longan extracts by HPLC while standard substrates are those commonly used to characterize PPOs (which may or may not be present in longan fruits). Moreover, docking studies applying a model structure of DIPPO1 were employed to investigate the enzyme-substrate interactions. The present study aimed at elucidating the biochemical impact of the presence of its signal sequence on DIPPO1 and was designed to provide the fundamental information of substrate acceptance and specificity of DIPPO1 that is necessary to devise new sustainable methods for ameliorating the post-harvest browning of this commercially important tropical fruit.

Results and discussion

Cloning, heterologous expression and purification of pro-DIPPO1 and prepro-DIPPO1. The PCR products on the complementary DNA of DL longan with primers enfoming the ORF for prepro-DIPPO1 and pro-DIPPO1 yielded distinct bands of approximately 1800 bp and 1500 bp, respectively (Figure S4). After the cloning into the pGEX-6P-SG vector, Sanger sequencing confirmed that the pro-DIPPO1 is composed of an ORF of 1797 nucleotides and encodes for 599 amino acids which are 100% identical to the amino acid sequence deduced from the published DIPPO1 (UniProt entry: A0A0K0NPU9) (the pro-DIPPO1 contains an ORF of 1512 nucleotides encoding 503 amino acids (Table S1) and has 99.60% identity with the previously published DIPPO1 sequence resulting from five altered amino acids (Pro46Arg, Glu226Asp, Gln451His, Ile455Met, and Asn486Tyr, Figure S5). The pro-DIPPO1 and prepro-DIPPO1 enzymes were heterologously expressed in E. coli with an N-terminal glutathione-S-transferase tag from Schistosoma japonicum (see Materials and Methods). The temperatures upon the isopropyl-β-D-1-thiogalactopyranoside (IPTG) induction during the expression were varied (37 °C, 28 °C and 17.5 °C) in order to investigate a condition favorable for high level production of soluble protein. Figure S6 shows that the most efficient expression temperature for both pro-DIPPO1 and prepro-DIPPO1 was 17.5 °C, while 37 °C resulted in the lowest expression level of both soluble and non-soluble forms. Effective productions of soluble PPO enzymes under low temperature expressions were also reported for TYRs from Juglans regia (20 °C), Agaricus bisporus (20 °C), Solanum lycopersicum (20 °C), Malus domestica (20 °C), Streptomyces avermitilis (18 °C), Streptomyces sp. ZL-24 (19 °C) and Larrea tridentata (25 °C). The C-terminal domain of eukaryotic PPOs has been reported to be necessary for the folding of the catalytic domain. So far, the expression of active grape PPO is the only example of successful production of a plant PPO without its C-terminal domain.

Through the purification by affinity chromatography applying the affinity of glutathione immobilized on cross-linked agarose, the expressions yielded 35.3 and 60.0 mg per liter of expression culture of GST-fused pro-DIPPO1 and GST-fused prepro-DIPPO1, respectively (Figure S7). Removal of the GST-fusion tag by HRV3C protease resulted in 5.7 and 7.0 mg of the latent pro-DIPPO1 and prepro-DIPPO1, respectively (Table 1). The prepro-DIPPO1 was successfully purified with 13.5% recovery of the total protein activity and a specific activity of 7.09 ± 0.05 U mg⁻¹, while the pro-DIPPO1 was retrieved with 19.6% recovery of the total protein activity and a specific activity of 28.3 ± 0.04 U mg⁻¹. The copper contents of the DIPPO1 variants were measured photometrically by reducing Cu(II) to Cu(I) with ascorbate and following the complexation of Cu(I) with 2,2′-biquinoline at 546 nm. It was observed that the copper contents of pro-DIPPO1 and prepro-DIPPO1 were 1.3 ± 0.018 and
2.0 ± 0.030 copper ions per active site, respectively. The freshly purified proteins eluted in 50 mM Tris–HCl and 250 mM NaCl at pH 7.5 and were immediately exchanged to 100 mM MES and 200 mM NaCl at pH 6.5, and glycerol was applied at 30%(v/v) for storage of both forms at − 80 °C until use. The purity of the final pro-

**Dl**
PPO1 and prepro-

**Dl**
PPO1 preparations was confirmed by SDS-PAGE (Fig. 1 and Figure S8 for the full-length gels).

**Molecular mass determination of pro-

**Dl**
PPO1 and prepro-

**Dl**
PPO1.** Gel electrophoresis demonstrated a prominent band of the final purified pro-

**Dl**
PPO1 and prepro-

**Dl**
PPO1 at approximately ~ 57 kDa and ~ 67 kDa, respectively, which suggested the latent (pro-

**Dl**
PPO1) and precursor (prepro-

**Dl**
PPO1) forms of longan PPO1 were obtained. The theoretical masses of pro-

**Dl**
PPO1 and prepro-

**Dl**
PPO1 were calculated from the respective protein’s sum formula using the atomic weight and isotopic composition of the constituent elements74 considering the presence of two conserved disulfide bonds (− 4H or − 4.032 Da) and one thioether bridge (− 2H or − 2.016 Da) as shown in Table 2. To confirm the actual molecular weights of the enzyme electrospray ionization—mass spectrometry (ESI–MS) was applied. The pro-

**Dl**
PPO1 yielded 56,845 Da (Fig. 2) which is in excellent agreement with the calculated mass of the protein starting from the vector-derived GlyProMet (marked as − 3, − 2, − 1 in Figure S5) up to the end of the latent PPO’s sequence (Ala 1 → Asp 503). This confirms that pro-

**Dl**
PPO1 was expressed as a latent PPO form with two disulfide bonds and one thioether bridge. In addition, prepro-

**Dl**
PPO1 was determined to have a mass of 67,285 Da, containing also one thioether bridge and two disulfide bonds (Fig. 2). The mass matches perfectly with the complete sequence of the prepro-polypeptide (Met 1 → Asp 599) plus the three amino acids GlyProMet (− 3, − 2, − 1) arising from the expression vector.

**Table 1.** Purification table for pro-

**Dl**
PPO1 and prepro-

**Dl**
PPO1 from 1 L of bacterial culture. *1 unit (U) is the amount of enzyme that catalyzes the conversion of 1 µmol of substrate per minute under the experimental conditions described in Materials and Methods. The enzymatic activity was measured with 8 mM of the monophenolic substrate tyramine and 0.25 mM SDS as activator.
Thermal shift assay of pro-DIPPO1 and prepro-DIPPO1. The thermal shift assay has been used to assess the thermal stability of recombinant proteins. Here, the thermal stability of the purified pro-DIPPO1 and prepro-DIPPO1 proteins at pH 6.5 (storage buffer condition) was tested. As shown in Fig. 3, for prepro-DIPPO1 two melting temperatures ($T_m$) at 69.5 °C and 77.5 °C were measured, while pro-DIPPO1 exhibited three $T_m$ values (54.5 °C, 70.5 °C, and 81.5 °C) until the complete denaturation of the enzymes. The unfolding of pro-DIPPO1 began at much lower temperature (~ 40 °C) (Fig. 3, Figure S9) than for prepro-DIPPO1 (~ 65 °C). The resulting first $T_m$ of pro-DIPPO1 is much lower than the other $T_m$ values observed for both variants of the enzyme. This strongly indicates that the latent pro-DIPPO1 exhibits some degree of limited unfolding at significantly lower temperature than its precursor prepro-DIPPO1. As the pro-DIPPO1 contains only domains that are also present in the prepro-DIPPO1, it seems that the inclusion of the signal peptides does actually increase the overall thermal stability of DIPPO1 and the signal peptides help to maintain protein stability.

Activation of pro-DIPPO1. Effect of SDS on pro-DIPPO1 activity. The activation of pro-DIPPO1 was monitored via the conversion of tyramine (Figure S3) to verify the proper SDS concentration for overcoming the enzyme’s latency. The reaction was examined using 0.5 µg of pro-DIPPO1 and 8 mM tyramine as substrate in 50 mM MES at pH 7.0 with various SDS concentrations (0 to 1.5 mM). As displayed in Figure S10, an increase in SDS concentration from 0.00 to 0.25 mM caused a significant enhancement of the pro-DIPPO1 activity, where the highest activation efficiency was achieved at 0.25 mM SDS (28.3 ± 0.04 U mg$^{-1}$). At higher SDS concentrations, the enzymatic activity was gradually reduced. The loss in activity by half of the optimum was observed at

Table 2. Molecular masses of pro-DIPPO1 and prepro-DIPPO1, determined by ESI–MS.

| PPO         | Calculated M (-6H) [Da] | M (measured) [Da] | Δ/Da |
|-------------|-------------------------|-------------------|------|
| pro-DIPPO1  | 56,844.05               | 56,844.92 ± 0.96  | +0.87|
| prepro-DIPPO1 | 67,284.68             | 67,285.04 ± 0.96  | +0.37|

Figure 2. ESI–MS of the precursor prepro-DIPPO1 and latent pro-DIPPO1. The entire mass spectra of (A) pro-DIPPO1 and (B) prepro-DIPPO1 are shown, whereas the inset represents the magnified view of the two most prominent charge stages. The calculated and measured masses of the two states of the enzyme are listed in Table 2.
degrees of activity in this pH range. The highest activity was exhibited at pH 7.0 (28.3 ± 0.04 U mg⁻¹) in MES
strate and 0.25 mM SDS for activation of the enzyme. Figure S11 demonstrates that the enzyme shows different
PPO1, 8 mM tyramine as sub-
50 mM MES and TRIS buffers ranging from pH 5.5 to 8.5 using 0.5 µg of pro-
Dl
PPO1 activity, the enzyme reaction was examined through
optimize the pH of the reaction buffer for the pro-
Dl
PPO1 resulted in
and
values of 54.5 °C, 70.5 °C and 81.5 °C. The raw fluorescence intensities are shown in
Figure S9.

Figure 3. Thermal stability of the DlPPO1 variants by thermal shift assays. The melting temperature values
(\(T_m\)) were determined from the derivative of fluorescence intensity (I, in arbitrary units) with respect to the
temperature increment. The prepro-DlPPO1 (blue line) resulted in \(T_m\) values of 69.5 °C and 77.5 °C. The pro-
DlPPO1 resulted in \(T_m\) values of 54.5 °C, 70.5 °C and 81.5 °C. The raw fluorescence intensities are shown in Figure S9.

an SDS concentration of 1.25 mM. Thus, the optimal SDS concentration for the activation of pro-DlPPO1 was
defined to be 0.25 mM and this level of added activator was applied in all subsequent experiments. A qualita-
tively identical activation behavior was seen for prepro-DlPPO1 (Figure S10).

Most enzymes lose their biological activities under treatment with SDS, while PPOs are resistant to SDS to
some extent, probably in part due to the existence of disulfide bonds in the structure. SDS initiates the
activation of PPOs by introducing conformational changes of the N- and C-termini of the latent form which
allow for exposure of the active site and promote substrate access. PPO activation is generally achieved at low
SDS concentrations, while higher concentrations of the anionic detergent significantly inhibit the enzymatic
activity, which is consistent with the SDS activation profile observed for DlPPO1. The use of SDS for the
activation of latent PPOs has been reported to be the best activation method in vitro as it almost completely
eliminates the latency of plant PPOs and provides an activity closely resembling mature PPO (after the removal
of the C-terminal domain). The degree of SDS activation varies greatly with plant extracts; for examples,
0.13 mM SDS was required to activate latent potato PPO (Solanum tuberosum), 0.35 mM SDS for mushroom
PPO (Agaricus bisporus), 0.69 mM SDS for table beet PPO (Beta vulgaris), 2 mM SDS for peach PPO (Prunus
persica cv. Paraguay) and 6.93 mM SDS for mango PPO (Mangifera indica). The necessary concentration of
detergent for activation of dandelion PPO has been shown to be determined primarily by the amino acids
forming the interface between the catalytic and the C-terminal domain. The use of 0.25 mM SDS for the pro-
DlPPO1 activation is at the lower end when compared to other heterologously expressed PPOs such as walnut
JrPPO1 (2 mM SDS), tomato SIPPO1 and SIPPO2 (1.5 mM SDS), mushroom AbPPO4 (2 mM SDS) and for
apple MdPPO1 (3 mM), MdPPO2 (2 mM) and MdPPO3 (4 mM).

Effect of pH on the pro-DlPPO1 activity. The pH is one of the parameters that affects a PPO's activity. To
optimize the pH of the reaction buffer for the pro-DlPPO1 activity, the enzyme reaction was examined through
50 mM MES and TRIS buffers ranging from pH 5.5 to 8.5 using 0.5 µg of pro-DlPPO1, 8 mM tyramine as sub-
strate and 0.25 mM SDS for activation of the enzyme. Figure S11 demonstrates that the enzyme shows different
degrees of activity in this pH range. The highest activity was exhibited at pH 7.0 (28.3 ± 0.04 U mg⁻¹) in MES
buffer whilst the activity slightly decreased at pH 6.5 by 17% and was significantly reduced at pH 6.0 and 5.5 by
61% and 86%, respectively. Likewise, the activity significantly dropped at higher pH (pH 7.5–8.5). A decrease by
43% was observed at pH 7.5 and by 74% at pH 8.0. The activity was almost depleted at pH 8.5 with a 96% activity
loss from the optimum. A pH 7.0 optimum was previously reported for the tyrosinases in apple (MdPPO1 and
MdPPO3) and mushroom (AbPPO4). A slightly higher optimal pH at 7.5 was observed in MdPPO3. JrPPO1 and
JrPPO2 from walnut had lower pH optima of pH 6.0, whereas the bacterial tyrosinase from a Streptomyces sp.
from peatland (SzTYR) revealed an unusually high pH optimum of pH 9.0 as a result of adaptation to its
natural pH environment.

Substrate specificity and enzyme kinetics. The catalytic performance of the pro-DlPPO1 was first examined
using four standard substrates (Figure S3): two monophenols (tyramine, D- and L-tyrosine) and two diphenols
(dopamine and L-DOPA) were applied at the enzyme's optimal pH 7.0 and an SDS concentration of 0.25 mM.
The pro-DlPPO1 does accept all four standard substrates from the monophenolic and diphenolic categories, and
consequently can be classified as a TYR (EC 1.14.18.1). \(K_m\) and \(k_{cat}\) values were calculated by nonlinear regres-
sion and the substrate specificity was estimated using the \(k_{cat}/K_m\) ratio. Data reported on the substrate specificity
for pro-DlPPO1 are shown in Table 3 and Figure S13. The diphenolic substrate dopamine is processed faster \(k_{cat}^{"}\)
260 ± 22 s⁻¹) than l-DOPA (kcat: 98 ± 8.5 s⁻¹), and the monophenol tyramine (kcat: 35 ± 1.9 s⁻¹) is hydroxylated faster than l- or d-tyrosine. Moreover, prepro-DIPPO1 has a lower KM value for dopamine (KM: 2.0 ± 0.35 mM), thus resulting in the highest catalytic efficiency (kcat/KM) among the four tested standard substrates. The kcat/KM ratio for dopamine is 4.6-, 16-, 336- and 504-fold higher than that of l-DOPA (28 ± 3.9 s⁻¹ mM⁻¹), tyramine (8.2 ± 0.91 s⁻¹ mM⁻¹), and l-tyrosine (0.26 ± 0.016 s⁻¹ mM⁻¹), respectively (Table 3). The prepro-DIPPO1 also accepts both standard monophenols (tyramine and tyrosine) and diphenols (dopamine and l-DOPA), but with different degrees of catalytic efficiency (Table 3 and Figure S14). The prepro-DIPPO1 binds dopamine with a similar KM value (KM: 2.3 ± 0.27 mM) to that observed for the pro form (KM: 2.0 ± 0.35 mM); however, the catalytic efficiency of prepro-DIPPO1 is reduced 7.5-fold compared to pro-DIPPO1 with a kcat/KM of 17 ± 2.4 s⁻¹ mM⁻¹. The conversion of the diphenol l-DOPA by prepro-DIPPO1 (kcat/KM of 12 ± 1.1 s⁻¹ mM⁻¹) was 2.4-fold slower due to the lower catalytic activity (kcat: 40 ± 2.1 s⁻¹) compared to pro-DIPPO1. A similar observation was noted for the monophenolics l- and d-tyrosine demonstrated decreased kcat/KM ratios, which are 1.3- and 1.6-fold lower than those observed for pro-DIPPO.

To gain insights into the kinetic behavior of DIPPO1 in its natural environment, a series of natural phenolic substrates ((–)-epicatechin, 4-methylcatechol, caffeic acid, pyrogallol, gallic acid, quercetin, vanillic acid and ferulic acid (Figure S2)), all of which have been detected in longan peel, pulp and seed39–41, were assessed. Data reporting the natural substrate specificity of pro-DIPPO1 are shown in Table 3 and Figure S15. Through the kinetic studies it was revealed that pro-DIPPO1 is active towards the polyphenols (–)-epicatechin, 4-methylcatechol, caffeic acid, pyrogallol, gallic acid, quercetin, vanillic acid and ferulic acid (Figure S2)), all of which have been detected in longan peel, pulp and seed39–41, were assessed. Data reporting the natural substrate specificity of prepro-DIPPO1 are shown in Table 3 and Figure S15. Through the kinetic studies it was revealed that pro-DIPPO1 is active towards the polyphenols (–)-epicatechin, 4-methylcatechol, caffeic acid, pyrogallol, gallic acid, quercetin, vanillic acid and ferulic acid, while the enzyme was completely inactive with the rest of the tested substrates. The highest efficiency of pro-DIPPO1 was observed towards (–)-epicatechin (kcat: 270 ± 15 s⁻¹) with a KM of 0.35 ± 0.052 mM, resulting in kcat/KM of 800 ± 120 s⁻¹ mM⁻¹. This evidence suggests that (–)-epicatechin is highly likely to be one of the physiologically substrates of DIPPO1 in longan. The rates of 4-methylcatechol, pyrogallol and caffeic acid conversions were lower than for (–)-epicatechin with resulting kcat/KM values of 590 ± 99 s⁻¹ mM⁻¹, 70 ± 9.7 s⁻¹ mM⁻¹ and 4.3 ± 0.72 s⁻¹ mM⁻¹, respectively. However, the catalytic efficiency towards these natural substrates significantly decreased in the prepro-DIPPO1. The kinetic examination with (–)-epicatechin resulted in a higher KM value (1.1 ± 0.12 mM), indicating that the precursor has lower specificity towards this substrate.

| Enzyme      | Substrate   | Km (mM)   | kcat (s⁻¹) | kcat/KM (s⁻¹ mM⁻¹) |
|-------------|-------------|-----------|------------|-------------------|
| pro-DIPPO1  | tyramine    | 4.3 ± 0.42| 35 ± 1.9   | 8.2 ± 0.91        |
|             | l-tyrosine  | **        | **         | 0.26 ± 0.016      |
|             | d-tyrosine  | **        | **         | 0.39 ± 0.016      |
|             | l-DOPA      | 3.4 ± 0.37| 98 ± 8.5   | 28 ± 3.9          |
|             | dopamine    | 2.0 ± 0.35| 260 ± 22   | 130 ± 25          |
|             | (–)-epicatechin | 0.35 ± 0.052 | 270 ± 15 | 800 ± 120         |
|             | 4-methylcatechol | 0.57 ± 0.091 | 330 ± 17 | 590 ± 99          |
|             | caffeic acid | 0.70 ± 0.11| 3.0 ± 0.19 | 4.3 ± 0.72        |
|             | pyrogallol  | 2.7 ± 0.34 | 190 ± 11   | 70 ± 9.7          |
|             | gallic acid  | No activity |           |                   |
|             | quercetin    |           |           |                   |
|             | vanillic acid |         |           |                   |
|             | ferulic acid |           |           |                   |
| prepro-DIPPO1 | tyramine    | 15 ± 1.0  | 24 ± 1.4  | 1.6 ± 0.14        |
|             | l-tyrosine  | **        | **         | 0.20 ± 0.032      |
|             | d-tyrosine  | **        | **         | 0.24 ± 0.036      |
|             | l-DOPA      | 3.3 ± 0.26| 40 ± 2.1  | 12 ± 1.1          |
|             | dopamine    | 2.3 ± 0.27| 40 ± 2.4  | 17 ± 2.4          |
|             | (–)-epicatechin | 1.1 ± 0.12 | 36 ± 2.0 | 33 ± 4.1          |
|             | 4-methylcatechol | 4.4 ± 0.37 | 73 ± 4.0 | 17 ± 1.7         |
|             | caffeic acid | 0.71 ± 0.046| 1.76 ± 0.084 | 2.5 ± 0.20        |
|             | pyrogallol  | 4.1 ± 0.28 | 61 ± 3.0  | 15 ± 1.2          |
|             | gallic acid  | No activity |           |                   |
|             | quercetin    |           |           |                   |
|             | vanillic acid |         |           |                   |
|             | ferulic acid |           |           |                   |

Table 3. Kinetic parameters of pro-DIPPO1 and prepro-DIPPO1. **The substrate solubilities were too low for a determination of the kinetic parameters using the Michaelis–Menten model. Linearization was subsequently implemented considering substrate concentration < < KM, yielding kcat/KM for l- and d-tyrosine as displayed in the Table.
than pre-DlPPO1 (K_m = 0.35 ± 0.052 mM). Thus, the catalytic efficiency of prepro-DlPPO1 was observed to be only 33 ± 4.1 s^-1 mM^-1, which is 23-fold lower than the value of pro-DlPPO1. Likewise, the specificity of the prepro-DlPPO1 to 4-methylethanol, caffeic acid and pyrogallol is less than the pro-DlPPO1 as indicated by the higher K_m values in comparison to the pro-DlPPO1. Data reporting the natural substrate specificity of prepro-DlPPO1 are shown in Table 3 and Figure S16. The resulting k_cat/K_m values of 17 ± 1.7 s^-1 mM^-1 (4-methylacetol), 2.5 ± 0.20 s^-1 mM^-1 (caffeic acid) and 15 ± 1.2 s^-1 mM^-1 (pyrogallol) indicate that the catalytic efficiency of the prepro-DlPPO1 decreased 36, 1.7 and 4.7-fold, respectively, from the pro-DlPPO1. Thus, the signal peptide component could be one of the factors responsible for the reduced rate of PPOs in catalyzing the oxidation and hydroxylation of phenolic compounds.

With concentrations of (–)-epicatechin in excess of 2 mM the enzymatic conversion was slower than the rate seen at lower substrate levels for both prepro-DlPPO1 and pro-DlPPO1 (Figures S15A and S16A). Including competitive inhibition by the substrate itself ("substrate inhibition", Equation S3) in the Michaelis–Menten model allowed to expand the modelled concentration range up to 3 mM for pro-DlPPO1 and up to 5 mM for prepro-DlPPO1. Changing the mathematical model of enzymatic conversion causes major changes to the obtained kinetic parameters. For pre-DlPPO1 (K_m = 4.6 ± 2.0 mM, k_cat = 1500 ± 590 s^-1, K_i = 0.56 ± 0.26 mM) as well as for pro-DlPPO1 (K_m = 6.1 ± 3.7 mM, k_cat = 140 ± 72 s^-1, K_i = 1.2 ± 0.79 mM) both K_m and k_cat are significantly increased (compare Table 3) to compensate for the newly introduced inhibiting effect of the substrate itself (K_i) while the variances of all fitted parameters are seriously inflated relative to the simple Michaelis–Menten model. For pre-DlPPO1 and dopamine (Figure S13C; K_m = 1.2 ± 0.11 mM, k_cat = 250 ± 15 s^-1, K_i = 30 ± 7.3 mM), with a much higher K_i indicating less efficient substrate inhibition, the inflation of the parameter's variances is less severe and both K_m and K_i are slightly lower than with the simple Michaelis–Menten model.

The binuclear copper center of PPOs was reported to function enantioselectively81. The enantioselectivity of the catalytic reaction of prepro-DlPPO1 on tyrosine was investigated. The enzymes showed a preference for d-tyrosine with a slightly higher catalytic efficiency of 0.39 ± 0.016 s^-1 mM^-1 over the l-enantomer (0.26 ± 0.016 s^-1 mM^-1). 1 mM of d-tyrosine is converted at a rate of 0.11 ± 0.01 U mg^-1, which is 1.1-fold faster than the rate of l-tyrosine conversion by pro-DlPPO1. The effect of substrate chirality was also observed in mushroom AbPPO4 where l-tyrosine was slightly more preferable than the d-counterpart81. The tyrosinase produced by Streptomyces sp. REN-21 reacted 35.9-fold faster on l-tyrosine than d-tyrosine, which is the strongest enantioselectivity effect on the substrate tyrosine among PPOs that has been reported87. The preference towards a d-stereoisomer substrate was also reported in a PPO purified from eggplant (Solanum melongena), in which the conversion of 0.1 mM d-DOPA was 1.8-fold faster than l-DOPA88. Another example of the enantiospecific reaction in plant PPOs is found in larreatricin hydroxylase from Larrea tridentata (LIPPO) which exhibits a pronounced preference (23-fold) for (+)-larreatricin in comparison to the (–)-enantomer, whereas in the same study AbPPO4 reveals an opposite effect by preferring (–)-larreatricin (13-fold)89.

Formation of the oxy-form in prepro-DlPPO1 and pro-DlPPO1. The prepro-DlPPO1 and pro-DlPPO1 were spectrophotometrically examined using H_2O_2 to assess the formation of the oxy-state of the enzyme in the presence of oxygen. Addition of H_2O_2 to the prepro-DlPPO1 leads to an increase of the specific absorption band (~ 345 nm), which is indicative of the oxygen-induced oxy-form characteristic for type-III copper center enzymes and has been attributed to the charge transfer transition of O2^- (π^-) → Cu(H)4,3,4,49. A similar observation was made with pro-DlPPO1, where the oxy adduct formation resulted in the formation of a pronounced absorption band at 345 nm (Fig. 4). The absorption at 345 nm became saturated when the titration reached ~ 150 equivalents of H_2O_2 for prepro-DlPPO1 and ~ 175 equivalents of H_2O_2 for pro-DlPPO1, respectively. The resulting absorption coefficients are ~ 9000 M^-1 cm^-1 per mol enzyme for the prepro-DlPPO1 and ~ 10,300 M^-1 cm^-1 per mol enzyme for the pro-DlPPO1. The formation of the oxy-form by H_2O_2 has previously been shown for PPOs extracted from natural sources36,38,39. Catechol oxidases purified from lemon balm (Melissa officinalis)88, sweet potato (Ipomoea batatas)39 and walnut leaves (Juglans regia)39 resulted in a full saturation of the oxy form when two equivalents of H_2O_2 were added. In gypsywort (Lycopus europaeus) catechol oxidase, 6 equivalents of H_2O_2 were required to saturate the λ_{max} band, while in black poplar (Populus nigra)80 80 equivalents of H_2O_2 were needed. Heterologously expressed SIPPO1 and SIPPO2 from Solanum lycopersicum26 were reported to generate the fully saturated oxy form at 25 and 11 equivalents of H_2O_2 addition, respectively. Moreover, the addition of H_2O_2 induced the wildtype CqAUS from Coreopsis grandiflora that was heterologously expressed in E. coli to fully form an oxy-adduct at 24 equivalents of H_2O_2.93 The present study is the first to investigate the oxy-form formation of a heterologously expressed prepro-PPO which still has its signal peptides attached.

Molecular docking simulations of DlPPO1. Molecular docking studies were performed to provide more insight into the enzyme–substrate interactions of the pro-DlPPO1. The simulation studies were carried out using the AutoDock Vina software84. The structural model of pro-DlPPO1 was prepared as described in Materials and Methods and was used for the computational calculations. Binding poses were calculated for all the investigated substrates (Figures S17 and S18) and validated according to the TYR structure of Bacillus megaterium which contains the substrate λ-tyrosine bound to the Zn-substituted active site (PDB: 4P6R). The results provided detailed information about the putative position of the investigated substrates and the binding energies of these poses around the active center (Table S3). The natural substrate (–)-epicatechin exhibited the strongest binding affinity among the investigated substrates as strongly suggested by the measured K_m (K_m = 0.35 ± 0.052 mM) and the most favorable binding energy (Table S3). The higher catalytic efficiency in combination with the similar K_m values of 4-methylethanol and caffeic acid indicates that 4-methylethanol forms a more stable complex with DlPPO1 than caffeic acid does. Pyrogallol features the highest K_m among the tested natural substrates, suggest-
ing weak binding interactions between pro-DIPPO1 and the substrate. The higher catalytic efficiency than the one observed for caffeic acid is due to the much higher $k_{cat}$ value of pyrogallol (Table 3).

Further analysis with LiqPlot + of (–)-epicatechin docked to the active center of the DlPPO1 revealed the specific interactions of the substrate with the amino acids around the dicopper active center. Specifically, one of the two hydroxy groups on the (–)-epicatechin’s $o$-diphenolic ring (Fig. 5) establishes two hydrogen bonds; one with the imidazole nitrogen of the conserved copper-coordinating His243 (2.8 Å) and the second one with the carboxylic backbone group of Met257 (2.8 Å). The second hydroxy group of the same ring interacts with the two copper ions and is well-positioned for the oxidative reaction (CuA: 4.1 Å and CuB: 3.7 Å, respectively). Moreover, nine hydrophobic interactions with the residues His87, Asn109, Ala231, Glu235, Asn236, Gly258, Asn259, Phe260 and Ala263 cover the whole body of the substrate (Fig. 5). Similar to other plant PPOs the conserved Phe260 is the gatekeeper residue, turns to the phenolic ring of the (–)-epicatechin and forms sandwich type $\pi-\pi$ interactions. The high affinity binding of (–)-epicatechin showcases the preference of plant PPOs for phenolic secondary metabolites and strongly suggests (–)-epicatechin to be a physiological substrate of DlPPO1. Similar results have been presented for the tomato PPO (SIPPO1) which has a high affinity towards the dihydrochalcone phloretin17 and for the aurone synthase from Coreopsis grandiflora for which butein was proposed as its natural substrate51,95.

**Conclusion**

In this work, prepro-DIPPO1 from D. longan was expressed, purified and characterized in comparison with pro-DIPPO1. This constitutes the first report on the biochemical differences between a PPO expressed in a prokaryotic system both with and without its N-terminal signal sequence. Titration with H$_2$O$_2$ monitored by UV–Vis spectroscopy verified that DIPPO1 contains a type-III copper center. After addition of ~150 and ~175 equivalents of H$_2$O$_2$, the absorption band around 345 nm indicating the oxy-form was fully developed in prepro-DIPPO1 and pro-DIPPO1, respectively (Fig. 4). Kinetic experiments showed that both enzymes accept monophenolic substrates, both forms of DIPPO1 were thus classified as tyrosinases (EC. 1.14.18.1). The pro-DIPPO1 exhibited lower $K_M$ and higher $k_{cat}$ values, and consequently much higher kinetic efficiencies, towards all tested substrates than prepro-DIPPO1 did (Table 3). The N-terminal signal peptides seem to provide an efficient barrier for substrate binding to the active site of DIPPO1. Inclusion of the supposedly unfolded signal peptides resulted in a substantial thermal tolerance of prepro-DIPPO1 that even surpasses the thermal stability of pro-DIPPO1 (Fig. 3). In combination with the signal peptides’ impeding effect on the catalytic function of DIPPO1, the increased
thermostability they confer to DlPPO1 may indicate a more ordered and compact structure of these peptides as currently envisioned. (–)-Epicatechin exhibited the lowest $K_m$, the highest catalytic efficiency (Table 3), the most favorable binding to DlPPO1 (Table S3 and Fig. 5) and also the most severe substrate inhibition among all investigated substrates. The results suggest (–)-epicatechin as a physiological substrate of DlPPO1. This work provides insights into the biochemical and catalytic properties of longan PPO that will be useful for future post-harvesting interventions for effective management of costly fruit browning reactions.

Materials and methods

Unless specified differently, all used chemicals have been purchased from Sigma-Aldrich (Vienna, Austria) or Carl-Roth (Karlsruhe, Germany) and were at least of analytical grade. Enzymes for the manipulation of nucleic acids were from New England Biolabs (NEB; Frankfurt am Main, Germany) or Thermo Fisher Scientific (Waltham, MA, USA).

Plant material, cloning, and sequencing of DlPPO1. Longan leaves were collected in Lamphun (18.481174° N, 99.176014° E), located in the northern part of Thailand, immediately transported to the laboratory and stored at −80 °C. Permission to collect longan leaves for research purposes was obtained from the local proprietor. Healthy leaves were ground under liquid nitrogen and the total RNA was isolated using the Rapid CTAB method according to Gambino et al. with an extraction buffer containing 2%(m/v) cetyltrimethylammonium bromide, 2.5%(m/v) polyvinylpyrrolidone, 2 M NaCl, 100 mM Tris–HCl (pH 8.0), 25 mM ethylenediaminetetraacetic acid (EDTA, set to pH 8.0 with NaOH) and 2%(v/v) β-mercaptoethanol. Subsequently, cDNA was synthesized using a poly-T primer (5’-T 25VN-3’) and Moloney Murine Leukemia Virus reverse transcriptase. Specific primers for the longan PPO (prepro-DlPPO1 and pro-DlPPO1) gene were designed (Table S2). The primers were used to amplify the PPO genes from the cDNA template using the Q5 High-Fidelity DNA polymerase (NEB). PCR products were cloned into the pGEX-6P-SG expression vector by the following procedure: The pGEX-6P-SG vector (1 fmol) was incubated with 5 fmol of the purified prepro-DlPPO1 or 5 fmol of pro-DlPPO1 amplicon, 6 mM adenosine triphosphate (ATP), 4 units of Esp3I (Thermo Fisher Scientific), and 160 units of T4 DNA ligase (NEB) in a total volume of 6 μL 1 × CutSmart buffer (NEB) for 90 min at 30 °C, followed by enzyme inactivation at 65 °C for 20 min. The obtained plasmids were transformed into chemically competent SHuffle T7 Express cells (NEB). Transformants were selected on LB agar plates supplemented with 100 mg L⁻¹ ampicillin by incubation at 37 °C overnight. Positive clones were detected with colony PCR and verified using Sanger-sequencing, carried out by the Microsynth GmbH (Vienna, Austria).

Heterologous expression and purification of recombinant prepro-DlPPO1 and pre-DlPPO1. In the recombinant plasmids, the prepro-DlPPO1 and pro-DlPPO1 genes are N-terminally fused with the GST-tag of the pGEX-6P-SG vector. The human rhinovirus 3C protease (HRV3C) recognition sequence (LEVLFQ|GP) is located between the GST-tag and the target gene, enabling the controlled proteolytic dissociation of the tag from the protein of interest upon purification. The two fusion genes (GST-prepro-DlPPO1 and GST-pro-DlPPO1) were efficiently overexpressed using the synthetic tac promoter of the pGEX-6P-SG vector. The SHuffle T7 Express E. coli strain was grown in a modified 2xYT medium (1.6%(m/v) tryptone-peptone, 1%(m/v) yeast extract, 1%(m/v) NaCl, 0.5%(m/v) NH₄Cl, 0.5%(v/v) glycerol, 2 mM MgCl₂, 1 mM CaCl₂; set to pH 7.5) in
the presence of ampicillin (100 mg/L). Overnight cultures were grown at 37 °C. The expression batches were inoculated with the saturated overnight cultures and grown (starting OD<sub>600</sub> of 0.05) at the same temperature for ~4 h until the OD<sub>600</sub> reached a value of 0.6–0.8. To optimize the protein quantity and simultaneously to avoid inclusion body formation, the temperature was reduced to 17.5 °C for expression. The cultures were induced with 0.5 mM IPTG, and 2.0 mM CuSO4 were added. The expression cultures remained at 17.5 °C under shaking for 48 h. When the OD<sub>600</sub> reached a value of 7–10, the cultures were collected by centrifugation at 6500 × g for 25 min at 4 °C.

Cell lysis was carried out implementing the freeze–thaw technique using liquid nitrogen. The pellets were resuspended in lysis buffer (50 mM Tris–HCl pH 7.5, 200 mM NaCl, 1 mM EDTA and 50 mM sucrose). Lysozyme (0.5 g/L) and protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine) were added to lyse the bacterial cell walls and to prevent protein degradation by endogenous proteases, respectively. The suspensions were shaken and incubated on ice for 45 min. The mixtures were then subjected to five cycles of freezing in liquid nitrogen, alternating with thawing in a 25 °C water bath. After the 5th round of the freeze–thaw procedure, 2 mM MgCl₂ and 0.02 g/L DNaseI were added to remove the viscosity of the solutions caused by the DNA that was released from the bacteria cells and to improve protein extraction. The lysates were subsequently centrifuged at 7500 × g for 1 h at 4 °C.

Chromatographic purifications were carried out using an ÄKTA Purifier (GE Healthcare) placed in a refrigerator at 4 °C. The filtered lysates were placed in a 50 mL injection loop and applied onto a prepacked 5 mL GSTrap FF column using a solution of 50 mM Tris–HCl, 250 mM NaCl pH 7.5 as the binding buffer. The target GST-fusion proteins were trapped onto the column, whereas the unbound proteins were flushed out by the binding buffer applied at a flow rate of 1 mL/min and chaperone washing buffer (50 mM Tris–HCl, 250 mM NaCl, 10 mM MgCl₂, and 5 mM Na₂-ATP set to pH 8; applied for 15 column volumes) to remove binding chaperones from the target fusion proteins. Eventually, the desired proteins were eluted with 50 mM Tris–HCl, 250 mM NaCl and 15 mM reduced glutathione set to pH 7.5. The GST-fusion protein fractions were pooled and concentrated using a Vivaspin ultracentrifugation device with a 30 kDa molecular weight cut-off (Sartorius; Göttingen, Germany). To remove glutathione, the buffer was then exchanged to 50 mM Tris–HCl pH 7.5 and 250 mM NaCl. Subsequently, the samples were mixed with an in-house prepared GST-HRV3C protease at a mass ratio of 1:50 (protease: GST-fusion protein). The proteolysis was carried out over 48 h at 4 °C. Afterwards, the cleaved proteins were again applied onto a 5 mL GSTrap FF column. At this time, the GST-protein and the GST-tagged protease were trapped in the column while the PPOs were able to flow through the column and were immediately eluted. The protein fractions of prepro-DlPPO1 and pro-DlPPO1 were collected, concentrated and stored in 100 mM MES and 200 mM NaCl pH 6.5 with 30% (v/v) glycerol at -80 °C. The protein concentrations were determined using the Beer-Lambert law based on the absorption at 280 nm and the extinction coefficient provided by ExPASy ProtParam. The predicted molecular mass of the expressed proteins were estimated from the atomic weight and standard isotopic composition of the constituent elements. The copper contents of the prepro-DlPPO1 and pro-DlPPO1 enzymes were assessed photometrically after acidification with acetic acid and reducing Cu(II) to Cu(I) with ascorbate, which allowed for the chelation of Cu(I) by 2,2′-biquinoline according to the method published by Hanno et al. Mol

**Molecular mass determination by ESI-LTQ-Orbitrap-Velos.** Mass determination of prepro-DlPPO1 and pro-DlPPO1 were performed by an ESI-LTQ-Orbitrap Velos (Thermo Fisher Scientific Bremen, Germany) mass spectrometer with a mass range of 200–4000 m/z and a mass accuracy close to 3 ppm with external calibration. Prior to MS 100 μg of the protein solutions were exchanged into 5 mM sodium acetate (pH 7.0) buffer. The protein solutions were diluted 100-fold in a mixture of 80% (v/v) acetonitrile and 20% (v/v) formic acid immediately before being applied to the mass spectrometer.

**Gel electrophoresis.** Denaturing SDS-PAGE was performed as described by Laemmli et al. in a mini gel apparatus (Mini-PROTEAN Tetra Cell, Bio-Rad). The prepro-DlPPO1 and pro-DlPPO1 were diluted in gel loading buffer that contains 30 g/L SDS, 6% (v/v) glycerol, 75 mM Tris, 2.5% (v/v) 2-mercaptoethanol and 50 mg/L bromophenol blue set to pH 6.8. The samples were heated at 99 °C (Thermomixer comfort, Eppendorf) for 5 mins to aid in the denaturation. The samples were then applied on the 5% stacking and 11% resolving polyacrylamide gels along with a molecular weight standard (Precision Plus Protein Standard Dual Color, Bio-Rad) to provide information on the size and purity of the proteins. The electrophoresis was run at 120 V. Gels were stained in dye solution (200 mg/L Coomassie brilliant blue G-250, 50 g/L Al₂(SO₄)₃; 16 H₂O, 10% (v/v) ethanol and 20 g/L H₃PO₄) and were subsequently destained with 10% (v/v) ethanol and 20 g/L ortho-phosphoric acid. Photographs of the gels were taken using the BioRad Gel Doc XR Imaging System.

**Thermal shift assay of prepro-DlPPO1 and pro-DlPPO1.** The thermal shift assay was conducted to measure the melting points of the two isoenzymes in the storage buffer (50 mM MES pH 6.5 and 200 mM NaCl) in order to determine the stability of the two states of the enzyme. The assay was performed thrice using PCR tubes (Axygen, INC Corning) in a real-time PCR instrument (mastercycler ep-realplex, Eppendorf). The reaction solutions contained 10 μM enzyme and 4× SYPRO Orange (Sigma-Aldrich) in the storage buffer. The samples were gradually heated with an increment of one K per minute from 4 °C to 94 °C in the PCR machine, while the change in fluorescence intensity was monitored at 560 nm following excitation at 470 nm. The resulting fluorescence intensities of the solutions containing each enzyme were then plotted against the temperature for stability analysis.
Enzyme activity assays. The DIPPO1 activities were spectrophotometrically determined by monitoring the increase in absorbance at 480 nm upon the conversion of tyramine by the enzyme. The standard measurements were performed using 0.5 μg of the enzyme and 8 mM of tyramine in a total reaction volume of 200 μL with the determined optimal SDS concentration (0.25 mM) to activate prepro-DIPPO1 or pro-DIPPO1. In the absence of SDS no useful absorption-time curves were obtained for the tested substrates (Figure S12). Absorption curves and spectra were recorded at 25 °C in a 96 well microplate applying a TECAN infinite M200 (Tecan). The activities were determined from the slope of the initial linear part of the experimental curves (absorbance vs. time) and expressed as U/min. One unit of enzymatic activity (U) was defined as the amount of enzyme that catalyzed the formation of 1 μmol of quinones per minute (1 U = 1 μmol/min). All assays were performed in triplicate. Optimizations of the SDS concentration and pH of the reaction buffer towards the pro-DIPPO1 activity are as follows (vide infra).

Activation of pro-DIPPO1. Effect of SDS on pro-DIPPO1 activity. The measurements (200 μL) were performed in the presence of various SDS concentrations (0.00, 0.05, 0.10, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mM) using 0.5 μg pro-DIPPO1 and 8 mM tyramine in 50 mM MES at pH 7.0. The optimal SDS concentration was subsequently used in all other following studies.

Effect of pH on pro-DIPPO1 activity. A series of MES and Tris buffers over a pH range of 5.5–8.5 were used as the reaction buffer to determine the most favorable pH that provides the best enzyme activity for further kinetics studies. Each reaction (200 μL) was carried out with 50 mM buffer concentration using 0.5 μg pro-DIPPO1, 8 mM tyramine and the optimal SDS concentration obtained from the previous experiment.

Substrate specificity and kinetic efficiency determination. Substrate specificity of prepro-DIPPO1 and pro-DIPPO1 was probed using various phenolic substrates. The substrates that resulted in decent activities were subsequently selected to determine kinetic parameters. Activities were evaluated spectrophotometrically on two standard monophenolic substrates (tyramine, l- and d-tyrosine), two standard diphenolic compounds (dopamine and l-DOPA) and four natural phenolic substrates ((−)-epicatechin, 4-methylcatechol, pyrogallol and caffeic acid). The absorption curves of the colored products were monitored on a TECAN infinite M200 for different molarities of the respective substrate. The reaction mixture (total volume of 200 μL) contained variable amounts of the enzyme (see Table S4, stored in 100 mM MES and 200 mM NaCl at pH 6.5) and 0.25 mM SDS in the reaction solution of 50 mM MES buffer at pH 7. The molar absorption coefficients (εmax) of the formed chromophores are reported in Table S4 and Figure S19. To perform the kinetic experiments, about 7–8 substrate concentrations around a preliminary Km value were chosen if the limited substrate solubility allowed to do so (Figures S13–S16). Km and vmax values were calculated by nonlinear regression using the Levenberg–Marquardt algorithm100 that was supplied with initial values for the model parameters derived from applying the Hanes–Woolf linearization101 of the Michaelis–Menten equation (Equation S1). The maximal turnover rate (kcat, Equation S3) was evaluated by dividing the total number of substrate molecules that were converted per min by the total number of enzyme molecules present in the reaction volume. For combinations of enzyme and substrate showing substrate inhibition the Michaelis–Menten equation was extended to include inhibition by the substrate (Equation S3). Formation of the oxy-adduct. The prepro-DIPPO1 and pro-DIPPO1 were spectrophotometrically investigated using H2O2 that induces the formation of the characteristic oxy-adduct. Formation of the oxy-adduct.

Homology modelling of pro-DIPPO1. The amino acid sequence of the pro-DIPPO1 (Ala1-Asp503) was submitted to the SWISS-MODEL103,104 server. At the beginning, the pipeline searched for an appropriate template of the investigated sequence (OU702517) based on BLAST105 and HHblits106. The search resulted in PPO166, specifically from the putative structure of the pro-DIPPO1 the C-terminal domain (Pro336-Asp503) was removed and only the active domain (Ala1-Val335) was used for the docking studies. Moreover, the gatekeeper residue (Phe260) was defined as a flexible residue, as its flexibility has
been verified for plant PPOs. The structure of all the substrates were downloaded from the PubChem server, then translated to the 3D pdb format and converted into pdbqt files using AutoDockTools (ADT, v. 1.5.6). For all substrates the highest possible number of active torsions was chosen. Polar hydrogens were assigned to the structures by ADT and were also saved in the substrate pdbqt files. In all the experiments, the search grid was centered in the middle of the two active site copper ions and spread over a cube (12 x 12 x 12 Å³, grid point spacing: 1 Å) with edges parallel to the three coordinate axes of the target DIPPO1 model. Then AutoDock/Vina was used for docking by supplying the investigated enzymes and inhibitors along with the grid box properties in the configuration file. For all the measurements the energy range was set to 5, while the exhaustiveness was set to 50. Upon docking, the binding poses were evaluated by superimposing the co-crystallized substrate L-tyrosine from the BmTyr crystal structure (PDB: 4P6R). Poses that grossly deviated from the binding pose of L-tyrosine in the BmTyr structure (i.e. substrate poses with the phenolic ring not interacting with the dicopper center) were characterized as 'unreasonable' poses. Reasonable docking configurations (the phenolic ring linked to the copper ions similar to BmTyr (PDB: 4P6R)) were plotted as a 3D image using the visualization software PyMol Molecular graphic system (Schrödinger, LLC).

Generation of interaction plots. After the docking study, reasonable binding poses of interest were saved as pdb files and further visualized using the LigPlot + software to evaluate the interactions between the ligand (~)-epicatechin and the interacting amino acid residues around the active center. Runtime parameters were defined as suggested by the software. Specifically, hydrogen-bonds were allowed up to a maximal range of 2.70 Å between a hydrogen and a hydrogen acceptor and also up to a maximal range of 3.35 Å between a hydrogen donor and a hydrogen acceptor. Non-bonded contact parameters were defined as maximal distances of 2.90 Å between the hydrophobic carbons of the ligand and the protein and of 3.90 Å between sulfur atoms of the ligand and the protein. Ethical approval. All plant experiments described in this study complied with the relevant institutional, national, and international guidelines and legislation.

Data availability
The sequences generated and analyzed during the presented study are available from the European Nucleotide Archive (ENA) repository under the accessions OU702517 (partial pro-DIPPO1 gene) and OU702518 (prepro-DIPPO1 gene).

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Competing interests

The authors declare no competing interests.

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