Umbelliferone and Esculetin: Inhibitors or Substrates for Polyphenol Oxidases?

Francesca Sollai,[a] Paolo Zucca,[a] Enrico Sanjust,[a,b] Daniela Steri,[a] and Antonio Rescigno*,[a,b]

[a] *Department of Biomedical Science and Technology, University of Cagliari; Cittadella Universitaria; 09042 Monserrato, Sardegna, Italy: and* [b] *Center for Colloid and Surface Science (CSGI); 50019 Sesto Fiorentino, Italy.*
Received July 15, 2008; accepted August 19, 2008; published online September 10, 2008

Recently, an interesting debate arose about the nature (substrate *versus* inhibitor) of esculetin, a coumarin derivative, for mushroom polyphenol oxidase (PPO). The present study examined the behavior of PPOs preparations from fungal and plant origin towards esculetin as a substrate. Both enzymes were able to oxidize esculetin though at a slow rate. A higher sensitivity was reached when the assay was performed in the presence of 3-methyl-2-benzothiazolinone hydrazone (MBTH) even with a lower amount of PPO. These observations unambiguously confirmed that esculetin has to be considered a substrate for mushroom polyphenol oxidase. The oxidation of esculetin was also demonstrated for the first time by a fungal laccase. This should be taken into account because some mushroom PPO preparations could exert contaminant laccase activity. In addition, a PPO preparation from *Ferula communis* was demonstrated to use esculetin as a substrate. Umbelliferone, the monophenolic precursor of esculetin along the phenylpropanoid pathway, behaved as a competitive inhibitor for the monophenolase activity of mushroom PPO with a K_i value=0.014 mM. This is worth a mention because only a few couples of mono- and corresponding *o*-diphenol show such opposite behavior towards PPO. A possible role of PPO in the esculetin fate along biosynthesis pathway of coumarin derivatives is also discussed.

Key words polyphenol oxidase; tyrosinase; laccase; esculetin; umbelliferone; *Ferula communis*

Hydroxycoumarins (*ar*-hydroxy-1,2-benzopyrones) are lactone derivatives of 4-coumaric (*p*-coumaric) acid. In higher plants these molecules are synthesized *via* the phenylpropanoid pathway[1] beginning from an unusual hydroxylation in the *ortho* position of the acid side chain (Chart 1). The next step is the *trans*/*cis* isomerization of the double bond of this 2,4-dihydroxycinnamic acid. The subsequent lactonization leads to the formation of 7-hydroxycoumarin, trivial name umbelliferone. Other coumarin derivatives such as 6,7-dihydroxycoumarin (trivial name esculetin) and 7-hydroxy-6-methoxycoumarin (scopoletin) seem to originate from the addition of oxygenated substituents to the aromatic ring of umbelliferone rather than from a cinnamic acid derivative.[2] Coumarins are widely distributed in higher plants and are especially abundant in Umbelliferae and Rutaceae families.[3]

In the past few years, coumarins received much attention for their diverse bioactivities (reviewed in Borges *et al.*).[4] Some coumarins from natural sources have been also used as therapeutic agents in humans.[5]

Polyphenol oxidase (PPO) is a widespread copper-enzyme, containing two copper ions, that catalyzes the *ortho*-hydroxylation of monophenols to the corresponding *ortho*-diphenols (catechols) and the oxidation of catechols to the corresponding *ortho*-quinones.[6] Enzyme nomenclature differentiates between tyrosinase, phenolase, phenol oxidase, polyphenol oxidase, and catechol oxidase, depending on the particular source and also on the authors who have described any particular enzyme. The term tyrosinase is usually adopted for the microorganism, animal, and human enzyme, and refers to the "typical" substrate, tyrosine, while PPO mostly refers to the plant enzyme. PPO is perhaps the most suitable general

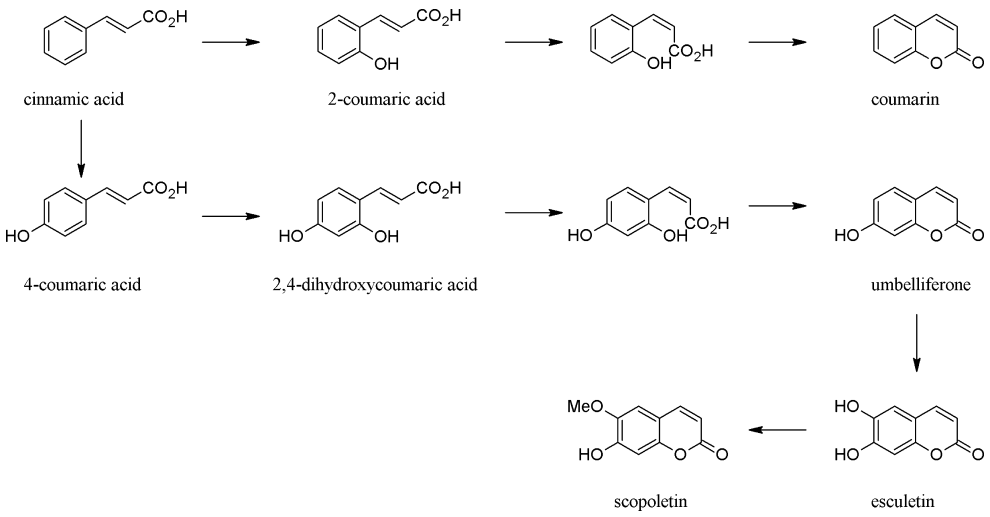

Chart 1. Biosynthesis of Hydroxycoumarins

Also the biosynthesis of unsubstituted coumarin is shown for reasons of clarity.

* To whom correspondence should be addressed. e-mail: rescigno@unica.it

definition, and will be used throughout this paper.

The enzyme is involved in many biological functions such as melanogenesis, insect development and metamorphosis, and in vegetable browning,[7—9] so the understanding of its physiopathological expression, activity, regulation, and inhibition has been and still is the focus of a huge number of studies. For this purpose a lot of inhibitors have been studied, important for potential or actual practical applications.[10,11]

Laccase is another copper-enzyme, containing four copper ions, that oxidizes a wide range of substrates, including mono-, di-, and tri-phenolic compounds, with poor specificity by means of one electron abstraction thus forming a radical.

Recently, an interesting controversy arose over whether esculetin should be considered a mushroom PPO substrate rather than an inhibitor of that enzyme.

In particular, Masamoto *et al.*[12] investigated the structure–activity relationship of 18 coumarins for their inhibitory activity on mushroom PPO. Among these compounds, esculetin exhibited the strongest inhibitory activity (IC_{50}= 0.043 mM). For these experiments a mushroom PPO commercial preparation (1000 U/ml, Sigma Chemical Co.) was used.

Additionally, *in vitro* inhibitory effect of esculetin on melanogenesis was demonstrated in cultured B16 melanoma cells. In their work the authors concluded that a likely mechanism of competitive inhibition of 3,4-dihydroxy phenyl alanine (DOPA) oxidase activity as previously reported[13] together with an antioxidative activity of esculetin[14] during melanin formation in melanocytes could explain the overall effect observed. On the other hand, Masamoto and coworkers highlighted the need of further studies to shed light on the inhibitory mechanism of esculetin.

Not long afterwards, Munoz *et al.*[15] characterized kinetically the oxidation of esculetin, as a possible PPO substrate rather than an inhibitor. In that study, the authors concluded that esculetin is not a true inhibitor of PPO. Rather, esculetin acted as an alternative substrate to the physiological substrates L-tyrosine and L-DOPA. Therefore esculetin probably directed the melanogenesis pathway towards quinonoid derivatives other than *o*-dopaquinone and dopachrome. Also for these experiments a mushroom PPO from a commercial preparation (1530 U/ml, Sigma Chemical Co.) was used. In addition, that preparation was further purified, but a possible contamination by laccase was not taken into consideration.

Recently, some investigators have stressed the presence, in mushroom PPO commercial preparations, of contaminating enzymatic activities different from PPO activity.[16,17] Contamination of commercial PPO by laccase is especially important because laccase can use some of the same substrates as PPO does (*e.g.*, *o*-diphenolics as esculetin) and may, therefore, interfere with determination of PPO activity.

In the present study we examined the behavior of preparations of both *Agaricus bisporus* PPO (Ab-PPO) and *Pleurotus sajor-caju* laccase towards esculetin. The aim of the present work was to investigate the possible participation of laccase in the oxidation of esculetin. Additionally, the esculetin role as PPO substrate or inhibitor and the effect of umbelliferone on the activity of PPO were also studied. Finally, a partially purified PPO from *Ferula communis* (Fc-PPO), a plant belonging to Apiaceae (also referred to as giant fennel), was tested so as to hypothesize a possible role of plant PPOs in the oxidation of esculetin.

MATERIALS AND METHODS

Materials L-Tyrosine, umbelliferone, esculetin, and syringaldazine were purchased from Sigma-Fluka-Aldrich (Milan, Italy). All other chemicals were of the best grade available and used without any further purification.

Laccase from *Pleurotus sajor-caju* Laccase was partially purified from a culture medium of the fungus *Pleurotus sajor-caju* and any potential PPO contamination excluded. The culture medium containing the extracellular laccase was submitted to two consecutive chromatographic steps with Macro-Prep DEAE and Sephacryl HR-200 (Pharmacia, Sweden). Laccase activity was measured spectrophotometrically by monitoring absorbance increases at 525 nm when 50 μM syringaldazine was present in a final volume of 1 ml of a buffered (50 mM potassium phosphate, pH 6.0) solution. One laccase unit is defined as the amount of the enzyme capable of forming 1 μmol of product per minute.[18]

PPO from *Agaricus bisporus* Mushroom PPO used in these experiments was partially purified as previously described.[19] In some experiments an additional mushroom PPO coarse preparation (c-Ab-PPO) was used. Such enzyme preparation still retained traces of contaminant laccase because it was not subjected to chromatographic steps as described above. Ab-PPO activity was estimated in the presence of given units of enzyme, 50 mM potassium phosphate buffer, pH 6.5, and 0.33 mM L-tyrosine, in a final volume of 1 ml. A stock solution of 2 mM L-tyrosine was prepared. For kinetic measurements of enzyme inhibition by umbelliferone, L-tyrosine concentration ranged from 0.021 to 1.630 mM. In this paper one Ab-PPO unit corresponds to the amount of enzyme that increases the absorbance at 305 nm by 0.001 per minute in the assay conditions.[17]

PPO from *Ferula communis* Aerial parts of the plant were harvested in spring in the Macomer countryside (Sardinia, Italy) and stored at −80 °C within 4 h from the harvesting. In a typical extraction step, the leaves (100 g) were homogenized in a blender in the presence of 250 ml of cold (−50 °C) acetone. The homogenate was filtered through a Buchner funnel equipped with fast flow filter paper and washed with 2 l of cold acetone until the filtrate was colorless. The solid residue was dried by an air flow and ground with a mortar and pestle. The obtained powder was divided in 5-g aliquots and stored at −20 °C.

When necessary one aliquot was resuspended in 100 ml of 100 mM potassium phosphate buffer, pH 6.5, containing 2 mM reduced glutathione (GSH), under gentle stirring at 4 °C for 30 min. The insoluble matter was separated by centrifugation (20000×***g*** for 20 min) and discarded. The supernatant was then subjected both to a diafiltration and concentration procedure in a Vivaflow 200 apparatus (Vivascience AG, Hannover, Germany) equipped with one Hydrosart membrane module (nominal M_r cut-off=10000) and a Masterflex L/S system pump (Cole-Parmer, U.S.A.). Throughout this step, a total volume of 2000 ml of 15 mM potassium phosphate buffer, pH 6.5, containing 1 mM GSH was added to the enzyme solution. The final enzyme solution, withdrawn in a final volume of 84 ml, contained 670 total units of enzyme activity calculated as previously described.[20]

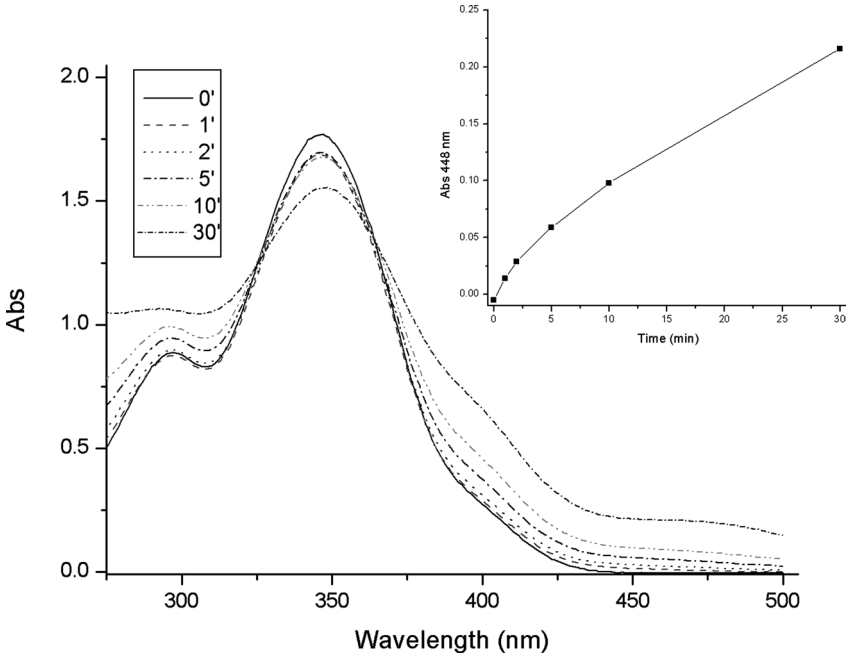

Fig. 1. Spectrophotometric Scans of Esculetin Oxidation by Laccase as a Function of Time

The reaction mixture contained 100 μM esculetin in the presence of laccase (14 U/ml). Other experimental conditions were 100 mM potassium phosphate buffer (pH 6.5), 1 ml as the final volume. Absorption spectra were recorded at prearranged times as shown in the label. Inset shows the kinetic evolution of the esculetin oxidation product measured at 448 nm.

Native-Polyacrylamide Gel Electrophoresis (PAGE) of Mushroom PPO Preparation and Activity Staining Experiments with Ab-PPO were performed with the Mini Protean III apparatus (Bio Rad Italiana, Milan, Italy), following the manufacturer's instructions for gel preparation. PAGE was carried out in 4% stacking gel and 10% separating polyacrylamide gel (0.75 mm thickness). When electrophoresis was completed, the gel was subjected to activity staining for laccase and PPO (see legend to the figure for more details).

Spectrophotometric Measurements of PPO and Laccase Activity Scans and kinetic measurements were carried out with an *Ultrospec 2110 pro* UV/Vis spectrophotometer (Amersham Biosciences, Milan, Italy). Since PPO and laccase catalyze a reaction between two substrates, molecular oxygen and a phenolic, the assays were carried out in air-saturated solutions.

Statistical Analysis and Software All experiments were run at least in triplicate. Origin 7.0 (Origin Corporation, Northampton, MA, U.S.A.) was used for statistical analysis. Spectrophotometric data were plotted with the same program. Lineweaver–Burk data were analyzed with GraFit 4.0.21, Erithacus Software Ltd., U.K.

RESULTS AND DISCUSSION

Oxidation of Esculetin by Fungal Laccase The first goal was to assess whether esculetin could be considered a substrate for laccase. Figure 1 depicts spectrophotometric scans of esculetin oxidation as a function of time. The inset of Fig. 1 shows enzymatic kinetics of the *o*-quinone formation derived from the esculetin oxidation by laccase. To our knowledge, the oxidation of esculetin by a laccase has not yet been reported. Owing to the natural occurrence of esculetin as a secondary metabolite in many plants, this finding could be of interest if plants laccase should do the same. That event seems highly probable since laccases generally show poor substrate specificity.

Native-PAGE of Mushroom PPO Preparation and Activity Staining with Esculetin Fostered by that observation, an Ab-PPO coarse preparation, c-Ab-PPO, was investigated by means of non-denaturing PAGE. As mentioned above, enzyme preparations of PPO are sometimes contaminated by laccase activity. The two enzymes are difficult to separate with usual protein purification protocols[17]; fortunately, however, they show different electrophoretic mobility. After electrophoretic run, the polyacrylamide gel was longitudinally sliced in different strips. Strips were used for activity staining of laccase and PPO. Figure 2 plainly shows that c-Ab-PPO contained a small amount of contaminating laccase when the gel-strip was incubated with syringaldazine (Fig. 2A). Syringaldazine is a typical substrate for fungal laccases[21] and its oxidation by this enzyme gives rise to a pink product with a high molar extinction coefficient ($65000\ M^{-1}\,cm^{-1}$); thus even traces of laccase can be emphasized.

As previously described,[15] the quinone from esculetin oxidation is quite unstable and its formation could be difficult to assess. This is the reason why a second strip was stained with esculetin plus 3-methyl-2-benzothiazolinone hydrazone (MBTH) a well known potent nucleophilic reagent. The coupling reaction between quinones and MBTH is often used for measuring PPO activity.[22,23] Also the quinone produced from esculetin oxidation reacted with MBTH leading to a better appearance of the spot. In Fig. 2B two bands of enzyme activity are evident, located corresponding to laccase and PPO, respectively. This demonstrates that both PPO and laccase are able to oxidize esculetin under the same experimental conditions. That esculetin oxidation by PPO occurred was corroborated by the unambiguous detection of PPO activity as shown in Fig. 2C, which shows the result of the incubation

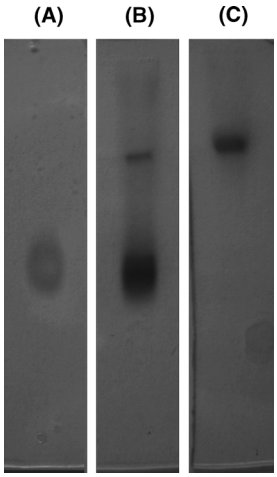

Fig. 2. Non Denaturing-PAGE of Mushroom PPO Preparation and Activity Staining with Esculetin

Non-denaturing PAGE of an Ab-PPO coarse preparation: three samples containing approximately 15 enzyme units were loaded. After electrophoresis run, the gel was soaked in 0.2 M potassium phosphate buffer (pH 6.5) at room temperature for 10 min to exchange the buffer system. Then, the gel was longitudinally cut into three strips and each strip was withdrawn and stretched in a separate glass Petri dish. One strip was used for activity staining of laccase using syringaldazine as substrate (A). The second strip was incubated with 5 ml of 1 mM esculetin plus 0.5 mM MBTH in the soaking buffer for 5 min (B). Another strip was used for monophenolase activity by staining with 5 mM tyramine plus 0.5 mM MBTH (C).

with tyramine plus MBTH. Tyramine is a PPO monophenolic substrate that is converted to dopamine and then to the corresponding *o*-quinone, which in turn can react with MBTH to give a chemical adduct with a higher extinction coefficient than dopaminequinone alone.[24]

Spectrophotometric Determination of PPO Diphenolase Activity on Esculetin in the Presence of MBTH Owing to the chemical instability of the quinone formed,[15] esculetin oxidation by a partially purified Ab-PPO both in the absence and the presence of MBTH was carried out and monitored over time. Superimposed spectra, recorded in the range 0—30 min, showed a sensitive increase of absorbance centered at 448 nm when a high amount of PPO was used in the absence of MBTH (Fig. 3A and inset).

A higher sensitivity was reached when the assay was performed in the presence of MBTH. In that case a maximum of absorbance at 478 nm arose (Fig. 3B). In Fig. 3B, inspection of the inset indicates that the linear pattern of enzyme activity is more extended in the presence of MBTH. By contrast, in its absence, the enzymatic oxidation of esculetin shows the typical enzyme hyperbolic kinetics (Fig. 3A). It is well known that PPOs might undergo inactivation by the quinone produced during catalysis. Thus continuous removal of the quinone preserves the enzyme catalytic properties.

However, our observations unambiguously confirm the work of Munoz (Munoz *et al.*, 2007)[15] that esculetin has to be considered a substrate for Ab-PPO.

Umbelliferone as Substrate for Monophenolase Activity of PPO Since Ab-PPO exerted its diphenolase activity towards esculetin, it seemed interesting to ascertain whether its monophenolase activity could be exerted with umbelliferone as a substrate. As showed in Chart 1, umbelliferone could be the monophenolic precursor of esculetin. Studies carried out on *Cichorium intybus* suggested 6-hydroxylation of umbelliferone, probably by the action of a P450 monooxygenase.[25] On the other hand, conversion of caffeic acid to esculetin was accomplished *in vitro* with extracts of *Saxifraga stolonifera*, but has not been confirmed *in planta*. Thus the enzymatic hydroxylation step of umbelliferone needs to be further investigated. With this in mind, further experiments with both native-PAGE and spectrophotometric assay—as described in Figs. 2 and 3, respectively—were carried out with umbelliferone in place of esculetin as the enzyme substrate. Surprisingly, the hydroxylation of umbelliferone had to be excluded under the assay conditions, also in the presence of MBTH.

Umbelliferone as Inhibitor for Monophenolase Activity of PPO The observation that umbelliferone failed as a substrate for PPO was quite interesting. Indeed only a few couples of mono- and corresponding *o*-diphenols show different behavior towards PPO. For example, *p*-coumaric acid is oxidized as monophenol substrate analogue at an extremely slow rate by Ab-PPO[26] whereas caffeic acid is a good diphenolic substrate for diphenolase activity of the enzyme.[27] Mostly, such couples of compounds are both good or poor substrates for PPOs. Thus it seemed an intriguing task to ascertain the exact role for umbelliferone. The effect of umbelliferone on monophenolase activity of Ab-PPO in the presence of L-tyrosine was then measured. K_m value for L-tyrosine was found to be 0.281 mM (0.0268 S.E.). Results examined with the Lineweaver–Burk plot clearly showed that umbelliferone behaved as a true competitive inhibitor for the enzyme with a K_i value $=0.014$ mM (0.001 S.E.).

Oxidation of Esculetin by a Polyphenol Oxidase from *Ferula communis* The perspective that plant PPO could play a role in esculetin metabolism prompted us to investigate the ability of such enzyme to oxidize esculetin. A partially purified PPO from *Ferula communis* (Fc-PPO) was obtained and used for the experiments discussed below. *Ferula* is a genus of umbelliferous plants (both names Apiaceae or Umbelliferae are allowed by the ICBN) widely present also in the Mediterranean area and its PPO activity has been studied in detail in some species.[28] With no detectable monophenolase activity against 6 common monophenolic compounds, including umbelliferone (data not shown), Fc-PPO was tested for its diphenolase activity. The results showed that the enzyme behaved as a catechol oxidase rather than a true PPO. Thus our attention was addressed to study the ability of Fc-PPO to transform esculetin in the corresponding quinone. An enzyme preparation of *F. communis* was assayed in the presence of MBTH, and monitored over time. Superimposed spectra, recorded in the range 0—15 min, showed significant oxidation of esculetin. A linear increase of absorbance was centered at 478 nm (Fig. 4). It is worthy of remark that the use of MBTH is strongly advised when low enzyme amounts are used. Thus the present results show clear capability of Fc-PPO to use esculetin as a substrate.

CONCLUSION

Accordingly with the results of Munoz *et al.*,[15] data here presented clearly show that esculetin is to be considered an Ab-PPO substrate rather than an inhibitor. This is in contrast with the findings of Masamoto *et al.*,[12] which found that esculetin exhibited the strongest inhibitory activity among 18 coumarin derivatives under investigation. Unfortunately, in

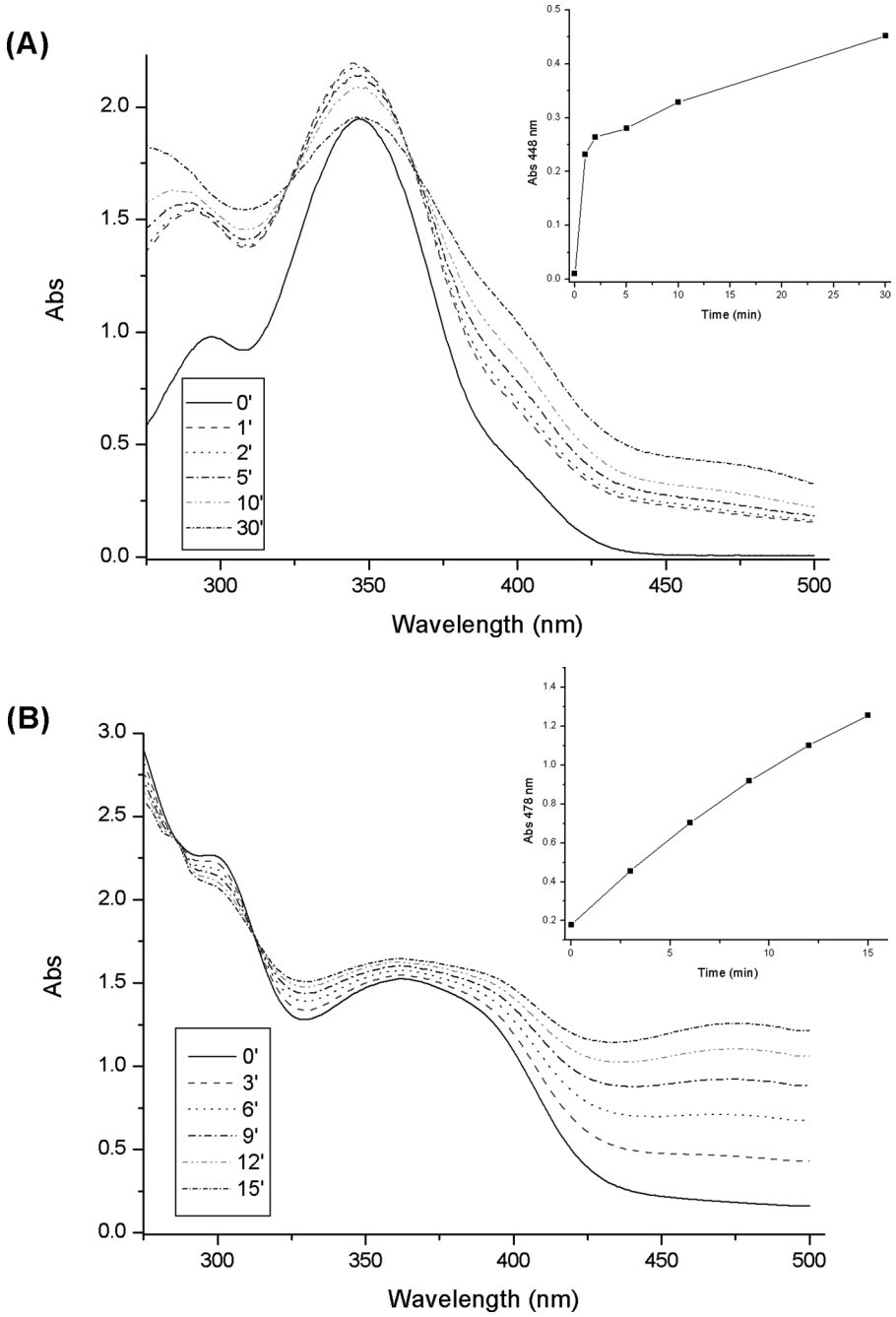

Fig. 3. Spectrophotometric Scans of Esculetin Oxidation by Mushroom PPO as a Function of Time

The reaction mixture contained 100 μM esculetin in the presence of Ab-PPO (130 U/ml). Other experimental conditions were 100 mM potassium phosphate buffer, pH 6.5, 1 ml as the final volume. Absorption spectra were recorded at prearranged times as shown in the label. Inset shows the kinetic evolution of the esculetin oxidation product measured at 448 nm (A). Analogous experimental conditions were used when the assay was performed in the presence of 0.5 mM MBTH. In that case a maximum of absorbance at 478 nm arose (B).

that case, the effect of esculetin on Ab-PPO was monitored by means of dopachrome formation at 475 nm. Rarely, if ever, such a spectrophotometric measurement is suitable for inhibition studies of diphenolase activity of PPOs. The concurrent presence of $\geq$2 substrates (in this case esculetin and DOPA) in the same reaction mixture could lead to competition between substrates for the catalytic site of PPO, thus explaining a misleading inhibition. In addition, cross reaction between esculetin and/or DOPA oxidized products cannot be excluded. More detailed results could be gained by measuring reactant concentrations with more specific methods, *i.e.* by HPLC techniques.

On the other hand, the inhibitory effect of esculetin on melanin biosynthesis in murine B16 melanoma cells was also found by Masamoto and co-workers. That effect could be well explained by the general antioxidant effect of esculetin and other coumarin derivatives, as suggested by these authors. Indeed such an antioxidant effect has been previously reported and recently confirmed for some coumarin derivatives.[14,29,30] We also observed that esculetin is a good substrate for a fungal laccase. Since some PPO preparations could be affected by laccase contamination, we suggest that some precautions should be taken to overcome this problem.

However, owing to the instability of the quinone formed as

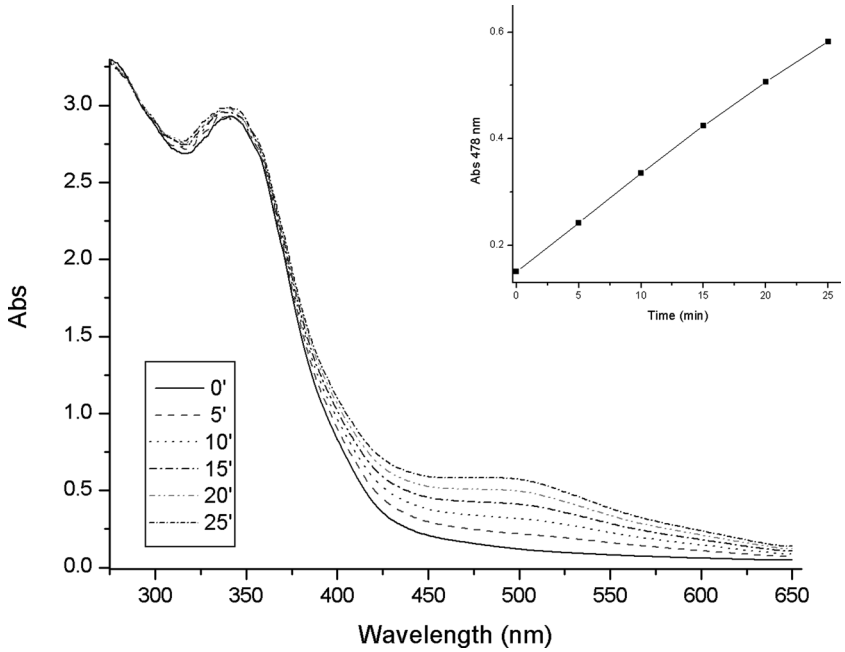

Fig. 4. Spectrophotometric Scans of Esculetin Oxidation by a Polyphenol Oxidase from *Ferula communis* as a Function of the Time

The reaction mixture contained 100 μM esculetin in the presence of Fc-PPO (1.2 U/ml) and 0.5 mM MBTH. Other experimental conditions were 100 mM potassium phosphate buffer (pH 6.5), 1 ml as the final volume. Absorption spectra were recorded at prearranged times as shown in the label. Inset shows the kinetic evolution of the esculetin oxidation product measured at 478 nm.

a consequence of esculetin oxidation, the use of MBTH as the coupling agent is strongly advised in an enzymatic assay. Interestingly, umbelliferone, the natural precursor of esculetin *via* the phenylpropanoid pathway, was found a good inhibitor for Ab-PPO with a K_i=0.014 mM showing a competitive-type inhibition. The low K_i value makes umbelliferone a promising target for further studies of melanin biosynthesis. Such studies are in progress.

Lastly, a PPO preparation from *F. communis* was observed to use esculetin as a substrate. That observation opens the perspective to further investigate a possible role for plant PPO in esculetin metabolism. Indeed, despite the huge number of studies dealing with PPO, the function of the enzyme in plants still remains uncertain. Plant PPOs probably carry out different functions in different species (for an exhaustive review see Mayer, 2006). This would explain, among other matters, why monophenolase activity is undetectable in many, albeit not all plant PPOs. Thus in *Ferula* genus, one might assume that PPO oxidation of esculetin has physiological significance. On the other hand, it is well known that extraction procedures of plant material release PPO and diphenolic substrates, which in turn can react with each other. Such a process has been related with at least a partial role of PPO in defense mechanism against herbivores. In favor of such a hypothesis would come the observation that most of the reports on identification of metabolites found in *Ferula* spp. describe several coumarin derivatives, including umbelliferone, but oddly enough not esculetin.[31—34] However, further detailed studies are necessary to strengthen such a hypothesis.

Acknowledgements This research on plant PPOs is supported by a grant (Prot. 1061/2008.0504) from Fondazione Banco di Sardegna, Sassari, Italy.

REFERENCES

1) Harborne J. B., "Classes and Functions of Secondary Products from Plants," Imperial College Press, London, 1999.
2) Murray R. D., *Nat. Prod. Rep.*, **12**, 477—505 (1995).
3) Bourgaud F., Hehn A., Larbat R., Doerper S., Gontier E., Kellner S., Matern U., *Phytochem. Rev.*, **5**, 293—308 (2006).
4) Borges F., Roleira F., Milhazes N., Santana L., Uriarte E., *Curr. Med. Chem.*, **12**, 887—916 (2005).
5) Mueller R. L., *Best Pract. Res. Clin. Haematol.*, **17**, 23—53 (2004).
6) Solomon E. I., Sundaram U. M., Machonkin T. E., *Chem. Rev.*, **96**, 2563—2605 (1996).
7) Halaouli S., Asther M., Sigoillot J. C., Hamdi M., Lomascolo A., *J. Appl. Microbiol.*, **100**, 219—232 (2006).
8) Mayer A. M., *Phytochemistry*, **67**, 2318—2331 (2006).
9) Pyne S. G., Truscott R. J. W., Maxwell K., Morales M. C., Walsh B. C., Wynn B. L., *Tetrahedron*, **46**, 661—670 (1990).
10) Friedman M., *J. Agric. Food Chem.*, **44**, 631—653 (1996).
11) Rescigno A., Sollai F., Pisu B., Rinaldi A., Sanjust E., *J. Enz. Inhib. Med. Chem.*, **17**, 207—218 (2002).
12) Masamoto Y., Murata Y., Baba K., Shimoishi Y., Tada M., Takahata K., *Biol. Pharm. Bull.*, **27**, 422—425 (2004).
13) Masamoto Y., Ando H., Murata Y., Shimoishi Y., Tada M., Takahata K., *Biosci. Biotechnol. Biochem.*, **67**, 631—634 (2003).
14) Payà M., Halliwell B., Hoult J. R., *Biochem. Pharmacol.*, **44**, 205—214 (1992).
15) Munoz J. L., Garcia Molina F., Varon R., Rodriguez-Lopez J. N., Garcia Canovas F., Tudela J., *Biosci. Biotechnol. Biochem.*, **71**, 390—396 (2007).
16) Flurkey A., Cooksey J., Reddy A., Spoonmore K., Rescigno A., Inlow J., Flurkey W. H., *J. Agric. Food Chem.*, **56**, 4760—4768 (2008).
17) Rescigno A., Zucca P., Flurkey A., Inlow J., Flurkey W. H., *Enzyme Microb. Technol.*, **41**, 620—627 (2007).
18) Leonowicz A., Grywnowicz K., *Enzyme Microb. Technol.*, **3**, 55—58 (1981).
19) Marongiu B., Piras A., Porcedda S., Tuveri E., Sanjust E., Meli M., Sollai F., Zucca P., Rescigno A., *J. Agric. Food Chem.*, **55**, 10022—10027 (2007).
20) Rescigno A., Sanjust E., Pedulli G. F., Valgimigli L., *Anal. Lett.*, **32**, 2007—2017 (1999).
21) Harvey B. M., Walker J. R. L., *J. Biochem. Mol. Biol. Biophys.*, **3**,

45—51 (1999).
22) Olianas A., Sanjust E., Pellegrini M., Rescigno A., *J. Comp. Physiol. B*, **175**, 405—411 (2005).
23) Winder A. J., Harris H., *Eur. J. Biochem.*, **198**, 317—326 (1991).
24) Espin J. C., Morales M., Varon R., Tudela J., Garcia-Canovas F., *Anal. Biochem.*, **231**, 237—246 (1995).
25) Brown S. A., *Can. J. Biochem. Cell Biol.*, **63**, 292—295 (1985).
26) Kubo I., Nihei K. I., Tsujimoto K., *Bioorg. Med. Chem.*, **12**, 5349—5354 (2004).
27) Cheynier V., Moutounet M., *J. Agric. Food Chem.*, **40**, 2038—2044 (1992).
28) Erat M., Sakiroglu H., Kufrevioglu O. I., *Food Chem.*, **95**, 503—508 (2006).
29) Pedersen J. Z., Oliveira C., Incerpi S., Kumar V., Fiore A. M., De Vito P., Prasad A. K., Malhotra S. V., Parmar V. S., Saso L., *J. Pharm. Pharmacol.*, **59**, 1721—1728 (2007).
30) Lin W. L., Wang C. J., Tsai Y. Y., Liu C. L., Hwang J. M., Tseng T. H., *Arch. Toxicol.*, **74**, 467—472 (2000).
31) Iranshahi M., Shahverdi A. R., Mirjani R., Amin G., Shafiee A., *Z. Naturforsch. C*, **59**, 506—508 (2004).
32) Filippini R., Piovan A., Innocenti G., Caniato R., Cappelletti E. M., *Phytochemistry*, **49**, 2337—2340 (1998).
33) Nabiev A. A., Khasanov T. K., Malikov V. M., *Chem. Nat. Compd.*, **18**, 547—549 (1983).
34) Kiseleva V. V., Nikonov G. K., Karryev M. O., *Chem. Nat. Compd.*, **11**, 358—361 (1976).