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# Development of an Affinity-Based Proteomic Strategy for the Elucidation of Proanthocyanidin Biosynthesis

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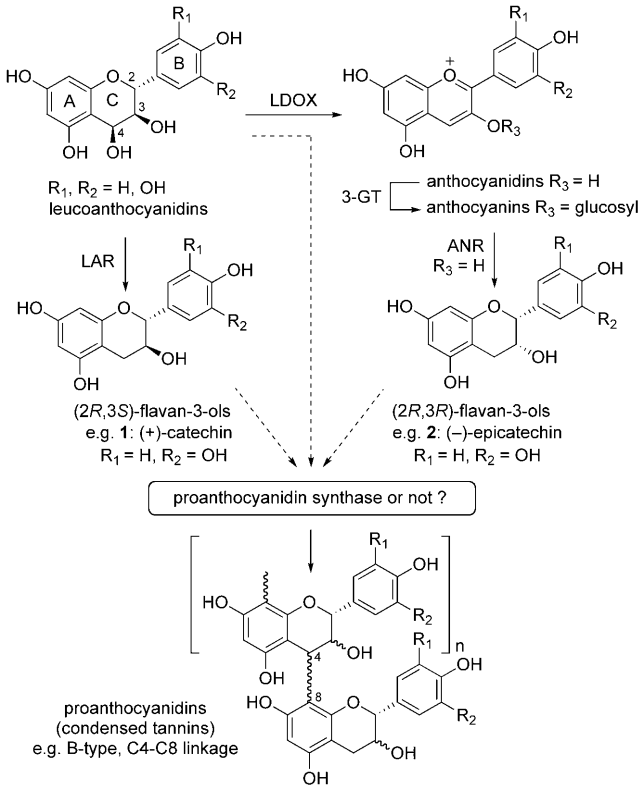
Proanthocyanidins (PAs), also known as condensed tannins, are oligo/polymeric chains of flavan-3-ols.[1] These polyphenolic flavonoid secondary metabolites are ubiquitously found in plants for which they essentially assume protective functions against microbial pathogens and herbivores.[2] Also found in grapes, PAs are an important factor in the gustative quality of wines. They are biosynthetically produced through the flavonoid pathway, one of the best-studied biochemical pathways in plants.[3,4] During the last decade, advances in genetic techniques and recombinant expression technologies have allowed isolation, from different sources, of most of the structural genes encoding the enzymes involved in flavonoid biosynthesis.[5] However, the last steps leading to the formation of PAs remain unclear and are still under debate (Scheme 1).[6–8] Leucoanthocyanidins (i.e., (2*R*,3*S*,4*S*)-flavan-2,3-*trans*-3,4-*cis*-diols) are presumed to be the common precursors of both PAs and anthocyani(di)ns. Results from in vitro studies have shown that leucoanthocyanidin dioxygenase (LDOX, also referred to as ANS—anthocyanidin synthase), a 2-oxoglutarate-dependent enzyme, is involved in the conversion of colorless leucoanthocyanidins into unstable colored anthocyanidins.[9–11] These flavylium ions are then stabilized as anthocyanins through the action of an anthocyanidin 3-glucosyltransferase (3-GT).[11,12] Two additional enzymes catalyze the production of flavan-3-ols from leucoanthocyanidins. A leucoanthocyanidin reductase (LAR) directly converts leucoanthocyanidins into (2*R*,3*S*)-*trans*-flavan-3-ols such as (+)-catechin (**1**),[13] whereas (2*R*,3*R*)-*cis*-flavan-3-ols such as (−)-epicatechin (**2**) indirectly derive from leucoanthocyanidins through the action of an anthocyanidin reductase (ANR) on anthocyanidins (Scheme 1).[14] PAs could then result from the oligomerization of flavan-3-ols such as **1** and **2**, and/or their flavan-3,4-diol precursor(s), but the exact nature of the starter and extension units, as well as whether or not a dedicated enzyme (i.e., a "proanthocyanidin synthase") is involved in the coupling process, remains unknown.[6–8]

In this context, the discovery of any novel enzyme involved in PA biosynthesis by proteomic analysis would certainly supply valuable information regarding the mechanistic nature of this oligomerization process. The recent availability of the grapevine genome sequence[15] has boosted efforts towards the proteomic analysis of tissues from various parts of the plant.[16] However, despite outstanding progress in electrophoretic and chromatographic techniques coupled to mass spectrometry-based proteomics, the quantitative resolution and identification of all proteins in a given proteome extracted from a plant tissue entail serious drawbacks. Proteins extracted from recalcitrant plant materials contain a high abundance of interfering compounds such as secondary metabolites, and thus efficient extraction is necessary in order to allow well-resolved two-dimensional polyacrylamide gel electrophoresis (2-DE).[17] Moreover, the complexity of the resulting extracted proteome (more than 700 proteins can be found just in grape skin[18]) and the wide dynamic range of protein concentrations often impede quantitative determination by 2-DE or a shotgun strategy.[19,20] Therefore, proteomic analysis of grapevine tissues have so far been essentially limited to the qualitative identification of changes in protein expression during plant development by performing comparative 2-DE analyses.[17,18]




**Scheme 1.** Putative last steps of the biosynthesis of proanthocyanidins and anthocyani(di)ns.

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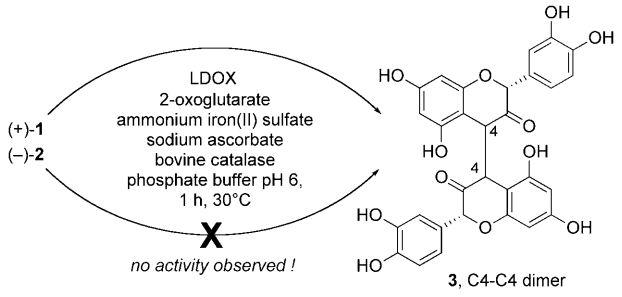
We thus surmised that analysis of the complex grapevine proteome might benefit from a preliminary protein sorting by affinity chromatography (AC). Such a strategy based on pre-fractionation of proteins prior to mass spectrometry-based proteomics has already been used with success on various proteomes,[21–25] but not from grapevine. Thus, we describe herein the development of an AC tool designed for investigating PA biosynthesis by using immobilized flavanoid probes and intended for the proteomic analysis of grapevine tissues.

The two flavanols (+)-catechin (**1**) and (−)-epicatechin (**2**), both presumably engaged in the oligomerization process leading to PAs, were selected as potential affinity-based probes. From the point of view of molecular recognition, these two flavan-3-ol probes should potentially target any unknown enzyme, as well as the three known enzymes (LDOX, ANR, and LAR) of the last steps in flavonoid biosynthesis. To evaluate the suitability of our AC tool, we selected LDOX as a model enzyme. Recent in vitro experiments carried out by Matern and co-workers[26] have shown that LDOX is capable of reacting not only with (2*R*,3*S*,4*S*)-flavan-3,4-diols (leucoanthocyanidins), but also with the flavan-3-ol (+)-**1**, which also harbors a (2*R*,3*S*)-*trans*-configured C ring, to generate the flavan-3-one C4-C4 dimer **3** (Scheme 2). However, neither its enantiomer




**Scheme 2.** LDOX assays with (+)-catechin (**1**) and (−)-epicatechin (**2**).

(−)-**1** nor its epimer (−)-**2** is processed by the enzyme.[26] Therefore, LDOX constitutes an appropriate model enzyme for this proof-of-concept study, since it should be competent only for binding with solid-supported (+)-**1**, but not with (−)-**2**.

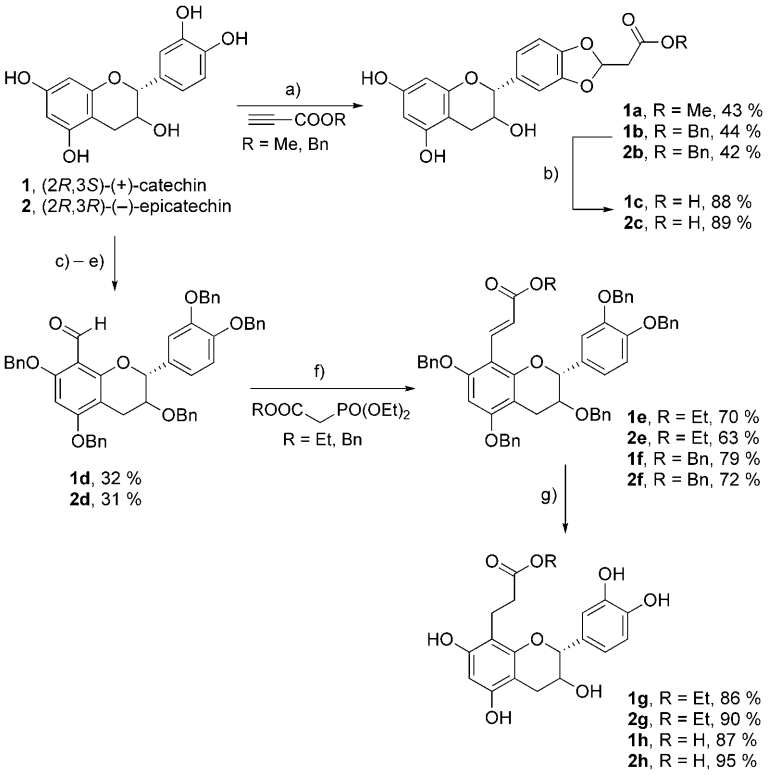
We used an N-terminal $His_6$-tagged recombinant LDOX from *Vitis vinifera* cabernet sauvignon, which was partially purified by using Ni-chelating affinity chromatography. The activity of this tagged enzyme was checked by using (+)-**1** and (−)-**2** as substrates in the presence of the requisite co-factors. LC-MS analyses of these reaction mixtures confirmed the aforementioned observations made by Matern and co-workers who used a recombinant LDOX from *Gerbera hybrida*[26] (Scheme 2 and see the Supporting Information for details).

With this active LDOX enzyme to hand, we then focused our attention on finding an adequate solid support on which to attach our two flavonoid probes by using an appropriate spacer. An ideal resin has good permeability towards the LDOX protein and is compatible with both the aqueous media used for biochemical assays and the organic solvents used for chemical grafting. The amino-PEGA resin, a copolymer of poly(ethylene glycol) and polyacrylamide with terminal amino groups,[27,28] meets these criteria, and has previously been used with success in biochemical assays.[29–31] We therefore selected the commercially available $PEGA_{1900}$ resin, which has a loading capacity of 0.2 mmol $g^{-1}$ and a molecular weight cut-off around 70 kDa, hence affording good permeability towards macromolecules and, in this case, towards the 40 kDa LDOX protein. Next, the site of attachment of the flavanoid probes to a spacer unit was chosen so as to minimize the impact of any structural modification of the native flavanols upon the molecular-recognition specificity of the enzyme. Since the primary function of LDOX appears to be a stereospecific hydroxylation at C-3 of the central pyran C ring,[32–35] we initially opted to introduce a spacer unit on the distal catecholic B ring. We settled on the use of a three-carbon spacer by taking advantage of the 2-(methoxycarbonyl)ethylidene (Mocdene) group recently developed by Vilarrasa and co-workers for the protection of 1,2-diols and catechols.[36,37] This cyclic acetal unit was chemoselectively installed onto (+)-**1** by treating it with methyl propynoate in the presence of a slight excess of DMAP (Scheme 3). Unfortunately, we failed to saponify the resulting methyl ester **1a** to its corresponding carboxylic acid **1c**, required for amidation with the amino groups of the PEGA resin. Treatment of **1a** with LiOH (1 equiv) led to partial cleavage of the Mocdene acetal. Thus, we instead used benzyl propynoate to generate under Vilarrasa's conditions the benzyl ester analogues **1b** and **2b** from (+)-**1** and (−)-**2**, respectively. Standard hydrogenolysis of these benzyl esters furnished the carboxylic acids **1c** and **2c** in good yields, ready for attachment to the resin (Scheme 3).

However, preliminary assays on the activity of the LDOX enzyme by using the B-ring-modified catechin derivatives **1a–c** in solution turned out negative. An explanation of these disappointing observations could be found in Schofield's co-crystal structure of the LDOX enzyme from *Arabidopsis thaliana* with dihydroquercetin (DHQ) as a substrate analogue,[30] in which the two DHQ B-ring hydroxyl groups are engaged in hydrogen bonding with the Tyr142 residue of the LDOX active site. Therefore, the suppression of the two catechin B-ring hydroxyl groups resulting from the installation of the spacer unit in **1a–c** might have been be detrimental to the recognition (and transformation) of such derivatives by the LDOX enzyme.

The alternative, to install a spacer unit onto the flavanol A ring, was thus implemented. Because Schofield's structure also indicates that the DHQ A-ring hydroxyl group at C-7 is hydrogen-bonded to the side-chain carboxylate group of the LDOX Glu306 residue,[32] we set out to preserve this phenolic hydroxyl group and to exploit the prominent nucleophilic character of the A-ring C-8 center to forge a functionalized C–C-linked tether at that position. This was achieved by a regioselective Vilsmeier–Haack formylation of perbenzylated (+)-**1** and (−)-**2** (Scheme 3).[38] The aldehydes **1d** and **2d** were both obtained in about 30% over three steps and were then submitted to Horner–Wadsworth–Emmons (HWE) reaction conditions[39] with triethyl or benzyl diethyl phosphonoacetate. The resulting four enoates were finally submitted to hydrogenolysis

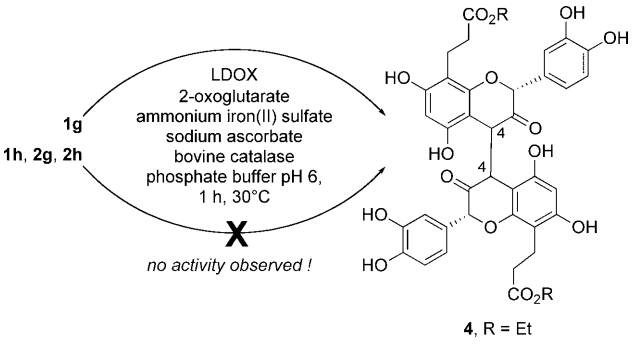



**Scheme 3.** Reagents and conditions: a) DMAP, $CH_3CN$/MeOH (R = Me) or $CH_3CN$/*t*BuOH (R = Bn), 3 h, RT; b) $H_2$, Pd/C, EtOH, 3 h, RT; c) BnBr, $K_2CO_3$, DMF, 48 h, RT; d) BnBr, NaH, 6 h, DMF, 0 °C; e) $POCl_3$, DMF, 4 h, 75 °C, then 15 h at RT; f) NaH, DMF, 15 h (R = Et) or 24 h (R = Bn), RT; g) $H_2$, Pd/C, THF/MeOH/$CH_2Cl_2$, 24 h, RT.

to mediate the cleavage of their benzyl protecting groups, as well as the hydrogenation of their olefinic bond; this furnished the two ethyl propanoate-tethered flavanols **1g** and **2g**, and their carboxylic acids **1h** and **2h** in good yields (Scheme 3).

With this new set of three-carbon-tethered flavanols to hand, we first verified that they were competent substrates for the LDOX enzyme. The ethyl propanoate-bearing (+)-catechin derivative **1g** was the only compound transformed by the enzyme. A new compound exhibiting a pseudomolecular ion peak $[M+H]^+$ at *m/z* 775 was detected by ESI-MS analysis of the enzymatic reaction mixture. By analogy with the known C4–C4 catechin dimer **3** (see Scheme 2), this compound is tentatively assigned to the C4–C4 bis-ketonic dimer **4** of **1g** (Scheme 4, see the Supporting Information for details). The fact that the (+)-catechin derivative **1h** remained intact upon exposure to LDOX could be due to its highly polar carboxylic acid function, which might interfere with the enzyme recognition process. The absence of enzymatic activity on the corresponding (−)-epicatechin derivatives **2g** and a fortiori **2h** was of course expected, since (−)-epicatechin itself is not a competent substrate for LDOX (vide supra).[26]

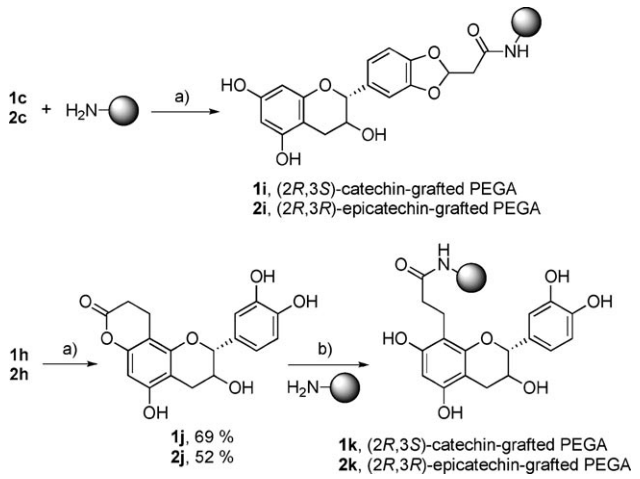
The main conclusion drawn from these preliminary LDOX activity assays was that grafting (+)-catechin (**1**) to the PEGA resin terminal amino groups through a three-carbon acyl spacer installed at the catechin A-ring C-8 locus appeared to be a viable approach for our intended AC design. (−)-Epicatechin (**2**) similarly grafted onto the resin, as well as both (+)-catechin (**1**) and **2** grafted onto the same resin but through their catecholic B rings could constitute negative controls that would allow us to discriminate nonspecific interactions with LDOX (vide infra). Hence, the B-ring-tethered carboxylic acid derivatives **1c** and **2c** (see Scheme 3) were grafted onto PEGA beads using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and 1-hydroxy-1*H*-benzotriazole (HOBt) as condensation reagents in DMF at room temperature (Scheme 5). The effectiveness of the reactions was confirmed by a 2,4,6-trinitrobenzenesulfonic acid (TNBS) test that indicated no appreciable amount of remaining free amino groups in the case of (epi)catechin-grafted resins **1i** and **2i**. However, in the case of the grafting of the A-ring-tethered carboxylic acid derivatives **1h** and **2h** under the same conditions, the coupling reactions turned out to be quasi-ineffective, mainly leading to an




**Scheme 4.** LDOX assays with tethered (+)-catechin **1g**, **1h** and (−)-epicatechin **2g** and **2h**.

intramolecular lactonization into the flavonoid derivatives **1j** and **2j** in 69 and 51 % yield, respectively. Microwave heating of the reaction mixture led to a better level of grafting, but was still incomplete, as evidenced by the TNBS test. In order to circumvent this problem, the lactones **1j** and **2j** isolated from the initial EDCI/HOBt-mediated reactions were then heated in DMF at 50 °C for 10 h under microwave assistance. Under these conditions, ring-opening of the two lactones occurred efficiently upon nucleophilic attack by the PEGA amino groups and afforded the desired (epi)catechin-grafted resins **1k** and **2k** (Scheme 5). The four grafted resins **1i/k** and **2i/k** were further analyzed by $^1H$ high resolution magic angle spinning (HR-




**Scheme 5.** Grafting of PEGA resin with flavanols through their A or B rings. Reagents and conditions: a) EDCI, HOBt, DMF, 5 h (20 h for **1h** and **2h**), RT; b) microwave 50 W, DMF, 10 h, 50 °C.

MAS) NMR spectroscopy; this confirmed the attachment of the flavanol units onto the PEGA resin (Supporting Information).[40]

Finally, we performed an SDS-PAGE analysis in order to evaluate the capacity of these grafted resins to act as affinity matrices for our recombinant LDOX enzyme (Figure 1). This partially

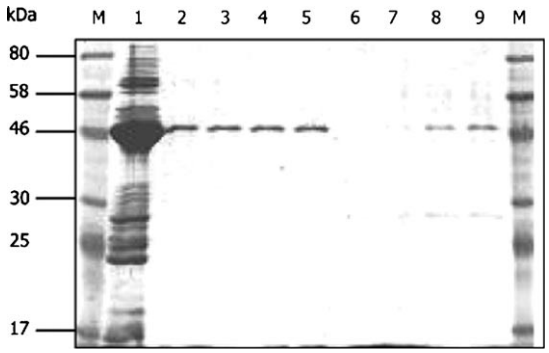



**Figure 1.** Affinity-based SDS-PAGE analysis of recombinant LDOX by using (epi)catechin-grafted resins **1i**, **1k** and **2k**. Lane M: protein markers; lane 1: partially purified LDOX; lane 2: first column wash from PEGA resin; lane 3: first column wash from resin **1i**; lane 4: first column wash from resin **2k**; lane 5: first column wash from resin **1k**; lane 6: fraction after elution with free PEGA resin; lane 7: fraction after elution with resin **1i**; lane 8: fraction after elution with resin **2k**; lane 9: fraction after elution with resin **1k**.

purified LDOX enzyme was thus added to suspensions of the resin-supported flavanol **1i**, **1k** or **2k**, or the ungrafted PEGA resin control. After 4 h of incubation with the four resins,[41] each resin was washed twice with a phosphate buffer solution (0.2 M, pH 6.0), in order to remove unbound LDOX (Figure 1, lanes 2 to 5). After the second wash, no significant amount of LDOX was detected in the column washings by SDS-PAGE analysis (not shown). Next, after elution with a denaturing Laemmli buffer solution (lanes 6 to 9), no protein was detected in fractions eluted from the resin grafted with (+)-catechin through its B ring (i.e., **1i**) or from the ungrafted resin. These negative controls confirmed the absence of any interaction between the LDOX protein and these resin materials (lanes 6 and 7). In gratifying contrast, LDOX was successfully bound to the resin grafted with (+)-catechin through its A ring (i.e., **1k**), as shown by the observation of a protein band at a molecular weight close to 40 kDa (lane 9).

This result confirmed that the (+)-catechin-derived resin-supported flavanoid probe **1k** could be used to capture LDOX in a competitive manner. The unexpected but weaker electrophoretic band observed when using the resin grafted with (−)-epicatechin through its A ring (i.e., **2k**, see lane 8) can be attributed to a specific but lower binding affinity of the epicatechin unit with LDOX. In fact, one can conceivably argue that the LDOX enzyme recognizes both catechin epimers **1** and **2**, but that the stereochemistry at the C-ring C-3 locus is the determining factor in enabling the enzymatic reaction to proceed. To complement this proof-of-concept approach to the proteomic analysis of flavonoid enzymes, the two SDS-PAGE protein bands at ~40 kDa (lanes 8 and 9) were in-gel digested. The resulting peptide fragments were then extracted from the gel and analyzed by LC-MS/MS. Peptide sequences were then identified through a proteomic database search from which we could verify a positive identification of the LDOX enzyme from *Vitis labrusca x Vitis vinifera* with a score of 28% (Supporting Information).

In summary, we have designed an affinity chromatography tool based on specific flavanol–protein interactions for the development of a proteomic approach to the identification of unknown (and known) proteins involved in the last steps of the proanthocyanidin biosynthesis. Here, two of their putative monomeric precursors, (+)-catechin and (−)-epicatechin, were covalently linked through their A rings to a PEGA resin. These grafted resins constitute suitable materials for batch affinity purifications of flavonoid enzymes. A proof of concept for this approach was established by using a recombinant LDOX protein from *Vitis vinifera* as an enzyme model system. This tool should help proteomic efforts aimed at elucidating the last steps of flavonoid biosynthesis, while facilitating the extraction and purification of specific proteins from plant tissues. We shall next exploit this coupled affinity chromatography–proteomic analysis methodology to investigate protein materials from grape tissues at different growth and maturity stages with the aim of unveiling the role of enzyme(s) in proanthocyanidin biosynthesis.

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