Peroxidase-Catalyzed Coupling of Solid-Supported *ortho*-Methoxyphenols

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Abstract: Enzyme-catalyzed C–C bond formation has been performed on solid-supported phenols, thereby expanding the repertoire of enzymatic catalysis on resin-bound substrates. In the presence of hydroquinone bound to various commercial resins, soybean peroxidase catalyzed the coupling of apocynin (acetovanillone) to give dimers, trimers, and oligomers with structures similar to what is obtained in solution-phase reactions. In addition to phenolic coupling, peroxidase catalyzed a Fries rearrangement presumably due to rearrangement of the acyl phenolic radicals generated on the solid phase through peroxidase catalysis. This represents the first enzymatic Fries rearrangement reported to date. Together with the solid-phase biocatalytic phenolic coupling, these results provide a route to the further expansion of enzymatic catalysis in combinatorial library synthesis.

Keywords: carbon-carbon bond formation; enzymes; peroxidase catalysis; solid-phase synthesis

Introduction

The emergence of combinatorial chemistry in new compound discovery is a direct result of advances made in transferring solution-phase synthetic chemistry to solid-phase supports. These supports, which are primarily comprised of cross-linked polymers,[1–3] provide a solvent-like environment that facilitates solution-phase chemistries on solid-phase resins. As a result, a wide range of chemical classes has been synthesized on solid supports, including substituted piperazinediones,[4] tetrahydrofurans,[5] γ-butyrolactones,[6] thiazolidones,[7] pyrrolidones,[8] isoxazoles and pyrazoles,[9] mercaptoketones,[10] and dihydropyrimidines,[11] among others.

While chemical synthesis on solid supports has progressed rapidly, enzymatic catalysis on solid-phase substrates has lagged. In particular, enzymatic reactions on solid-supported substrates have been almost exclusively limited to hydrolases; e.g., glycoside synthesis *via* glycosidase and glycosyltransferase catalysis,[12] peptide synthesis and enantioselective ester hydrolysis using proteases[13,14] and esterases/lipases,[15] respectively, and chain extension of oligonucleotides through RNA ligase catalysis.[16] To date, no carbon-carbon coupling reactions have been performed using enzymes on solid-supported substrates. Nonetheless, such reactions would represent a diverse chemistry that is difficult to achieve selectively using chemical catalysis, particularly in the case of aryl substrates.

We present here the first enzymatic carbon-carbon coupling reaction on solid-supported substrates. Specifically, we use peroxidase to catalyze the *ortho-ortho* coupling of phenols. Peroxidases catalyze the one-electron oxidation of phenols, which then undergo radical transfer and coupling.[17] The most significant natural function of plant peroxidases is in the synthesis of lignin. Because of the extremely water-insoluble nature of higher molecular weight lignins, peroxidative reactions have been proposed to occur with the growing lignin polymer in a nearly solid state.[18] Thus, peroxidases may have the innate ability to function on solid-supported phenolic substrates. Although lignins are of little interest to biological and medicinal chemists, the lignan structural motif, which consists of an *ortho*-methoxyphenol, does have significant bioactive properties. For example, apocynin (4-hydroxy-3-methoxyacetophenone) is a pro-inhibitor of vascular NADPH oxidase, and upon oxidation to oligomeric species by peroxidases (e.g., myeloperoxidases) reduces the formation of undesirable reactive oxygen species and thus may attenuate the inflammatory response.[19–21]

We have, therefore, examined the feasibility of peroxidase-catalyzed oligomerization of *ortho*-methoxyphenols with resin-bound substrates and identified the key parameters that influence soybeam peroxidase (SBP) catalysis on the solid phase. The optimal systems for SBP-catalyzed oligomerization of phenols were then used for the synthesis of apocynin oxidation products

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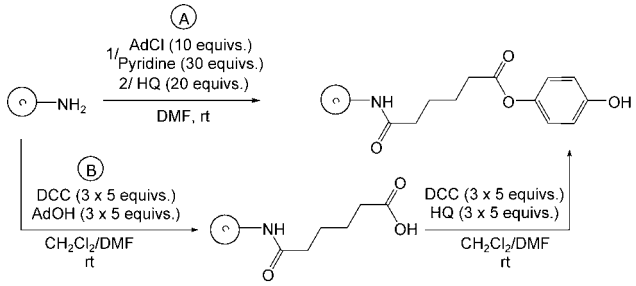
that are consistent with those obtained in solution, which show biological activity.

Results and Discussion

Attachment of Hydroquinone to Various Resins

Our strategy was to attach a "seed" phenolic onto a solid-phase support and use SBP to catalyze the C–C coupling of solution-phase phenols to the seed. Hydroquinone (HQ) represents an ideal seed, due to the presence of two phenolic groups. One group can be attached chemically to a support through a cleavable ester linkage while leaving the other phenolic group available for SBP catalysis. A number of commercially available resins were examined for HQ attachment (Table 1), and were amino-terminated to facilitate the stable attachment of an adipate linker. Two methods were used to attach HQ to the resins (Scheme 1). The first method (Method A) consisted of the one-pot reaction of the amino-support with adipoyl chloride in DMF in the presence of pyridine, followed by the addition of HQ. The second method (Method B) involved adipic acid and the coupling agent dicyclohexylcarbodiimide (DCC) in DMF in an initial step, followed by the addition of DCC and then HQ in the second step. In both cases, HQ was a poor nucleophile[22] with yields of HQ attachment ranging from 2 to 25%, based on the initial amino density of the resins. The single-pot reaction was generally more effective and, therefore, was used throughout this study. In all cases except PEG, which is a soluble support, and PL-PEGA, the HQ loading density is consistent primarily with surface functionalization. Nonetheless, the HQ loadings were sufficient to demonstrate SBP catalysis on the solid phase.



Ad = C(O)-$(CH_2)_4$-C(O); HQ = hydroquinone; DCC = dicyclohexylcarbodiimide

Scheme 1. HQ loading onto amino-functionalized resins with an adipate linker *via* two methods.

To confirm and quantify HQ attachment, the resins were treated with aqueous H_2SO_4 (1 M) to cleave the bound HQ, which was then quantified by HPLC. For the soluble PEG conjugate, ^{1}H NMR was also used to quantify the HQ loading to the polymer prior to cleavage. Subsequent hydrolysis of the HQ-adipate ester linkage confirmed the loading values obtained by NMR. Hence, acid-catalyzed cleavage was effective. Cleavage was rapid; in the case of Tentagel S essentially 100% of the loaded HQ was released after 4 h.

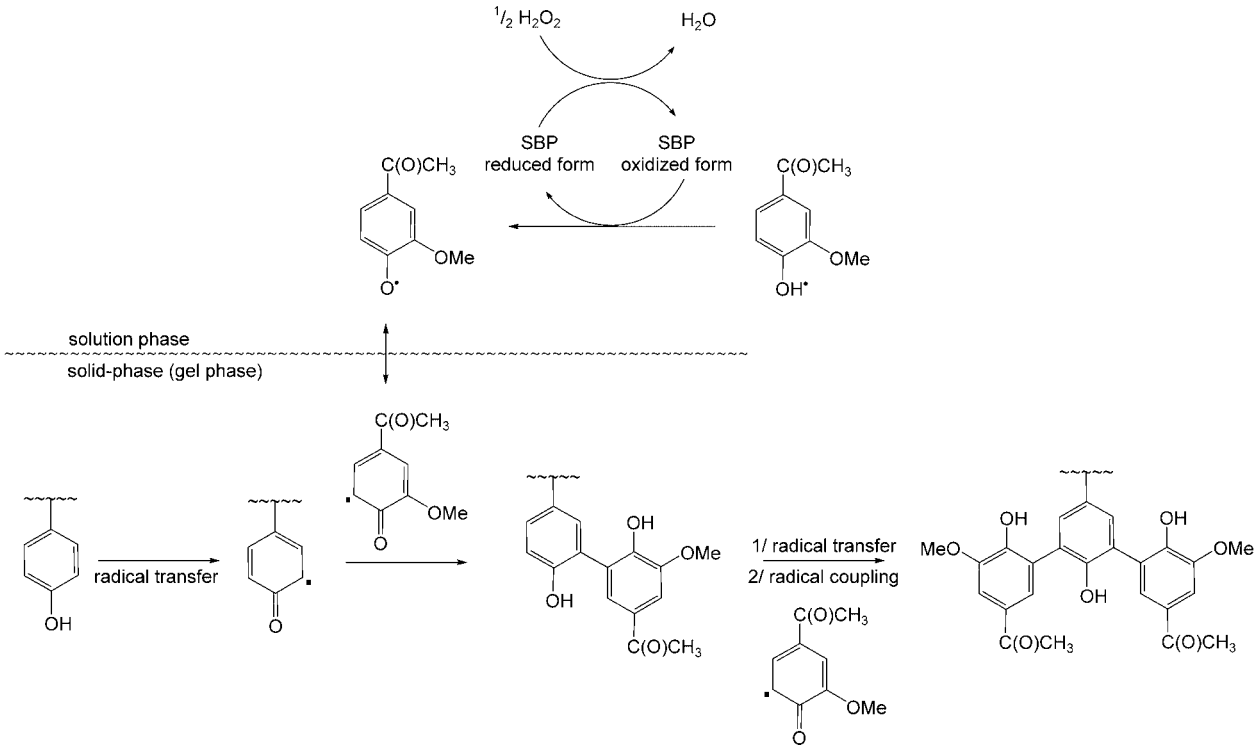
SBP Catalysis on Solid-Supported Hydroquinone

Having established that HQ could be attached and removed from several supports, we proceeded to examine SBP-catalyzed phenolic coupling, using apocynin as our soluble phenolic substrate (Scheme 2) in aqueous buffer containing 30% (v/v) DMF (added to enhance the solubility of apocynin). We reasoned that SBP would catalyze the initial oxidation of apocynin in the solution phase. The oxidized apocynin radical would then undergo radical transfer with the bound HQ to yield a carbon-centered radical on the seed phenol. Additional SBP-catalyzed oxidation of apocynin monomers would result in radical coupling and the formation of diphenolics and ultimately higher order oligomers on the support. Reactions were performed with 10–50 mg of loaded support in 2-mL reaction volumes in tinted glass vials, using an excess of reagents. DMF (600 μL) was added prior to the reaction and the bead mixture was incubated for 30 min to promote resin swelling.

Table 1. Hydroquinone loading on solid-phase and soluble polymer supports used in this work.

Name	Particle size	Core	Initial NH_2 loading	Final loading DCC method	Final loading adipoyl method	Expected surface loading[a]
PL-AMS	250–300 μm	Polystyrene	2.00 mmol/g	0.010 mmol/g	0.020 mmol/g	0.044 mmol/g
JandaJel	100–200 μm	Polystyrene/PTHF	1.00 mmol/g	0.040 mmol/g	0.040 mmol/g	0.040 mmol/g
PEG	–	PEG	1.24 mmol/g	–	0.120 mmol/g	–
Tentagel S	130 μm	Polystyrene-PEG	0.26 mmol/g	–	0.013 mmol/g	0.012 mmol/g
Tentagel HL	110 μm	Polystyrene-PEG	0.44 mmol/g	0.031 mmol/g	0.030 mmol/g	0.024 mmol/g
Tentagel Macrobeads	140–170 μm	Polystyrene-PEG	0.51 mmol/g	0.008 mmol/g	0.015 mmol/g	0.014 mmol/g

[a] Estimated as the ratio surface/volume for each particle size ($=3/r$, r being the radius of the sphere), by the initial NH_2 loading.



Scheme 2. SBP-catalyzed oligomerization of apocynin with resin-bound HQ. See text for reaction conditions.

Time course experiments were initially performed with HQ loaded onto Tentagel S with a series of identical reaction vials, five of which were removed at each time point and quenched by addition of 200 μL of catalase solution (0.1 mg/mL) to decompose residual H_2O_2, and then removal of the supernatant and washing of the support (See Experimental Section). In the absence of enzyme or in the absence of H_2O_2, no conversion of HQ was observed (Figure 1). With light agitation (100 rpm), very low conversion of HQ was achieved; however, increasing the agitation to 300 rpm resulted in nearly 80% conversion of HQ within 10 min.

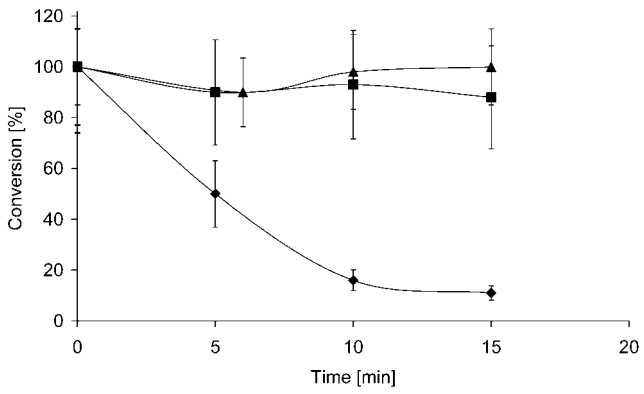


Figure 1. Enzymatic reactivity of Tentagel S-bound hydroquinone as a function of agitation speed: 100 rpm (■) and 300 rpm (with enzyme: ◆, no enzyme: ▲). See text for reaction conditions.

The striking dependence of reactivity on agitation was unexpected, as even 100 rpm should provide sufficient agitation to eliminate potential external diffusional barriers. This assumption was confirmed by calculating the observable Damköhler number [$\bar{D}\bar{a}$ in Eq. (1)] under a worst-case scenario of a stagnant boundary layer around the Tentagel particles, where the mass transfer coefficient, k_s, is approximated by Eq. (2) (D is the diffusivity of the apocynin radical, *ca.* 10^{-6} cm^2 s^{-1}, and d is the diameter of a Tentagel bead). This situation, which would result if there were no agitation, yields a $\bar{D}\bar{a}=0.12$, which is well within the reaction-limited regime. Hence, increased agitation to overcome diffusional barriers would not appear to explain the observed agitation dependence.

$$\overline{Da} = \frac{\nu}{k_S S_O} \quad (1)$$

$$k_s = \frac{2D}{d} \quad (2)$$

The aforementioned analysis, however, does not take into account the unique mechanism of SBP, where the concentration of the diffusible "substrate" is not the concentration of apocynin. Rather, SBP catalyzes the synthesis of apocynin radicals (Scheme 2), and these

radicals must diffuse to surface-bound HQ in order for the radical transfer to occur and initiate the eventual coupling with apocynin to give the dimeric product. The concentration of these radicals will be substantially lower than that of the free apocynin, and likely similar to the concentration of enzyme in the reaction mixture (~0.25 mM). Moreover, these radicals will either diffuse to the HQ bound to the support or interact with apocynin (or apocynin radicals) in the solution phase. As a result, a small fraction of apocynin radicals may ultimately diffuse to the surface of the beads and initiate radical transfer. Thus, the effective substrate concentration will be far lower than S_o in Eq. (1) and the resulting $\bar{D}\bar{a} > 1$ indicating significant diffusional limitations. Another possible explanation of the observed agitation effect is that some of the HQ is oxidized by SBP directly. In this case, the value of S_o would be the concentration of *enzyme*, not substrate, and the effective diffusivity can be obtained from the Stokes–Einstein relationship [Eq. (3)]. The resulting value for D_E is *ca.* 4.4×10^{-7} cm^2 s^{-1}, yielding a $\bar{D}\bar{a} = 5.3$ and moderate diffusional limitations in the absence of agitation. Thus, with either alternative explanation, one would expect the observed dependence of reactivity on agitation rate.

$$D_E = \frac{k_B T}{6\pi\mu r} \quad (3)$$

Influence of Resin Type on Apocynin Coupling

The influence of resin type was investigated using a series of Tentagels that differ in their available loading density along with other available supports and soluble PEG-based polymer. As depicted in Table 2, Tentagel S was the most effective support for peroxidase-catalyzed phenolic coupling, while Tentagel HL also supported relatively high activity. However, when corrected for HQ loading, the rates achieved using all supports, with the exception of PL-AMS, were similar (within four-fold of one another). This is not surprising, as HQ loading on these supports is primarily at the surface, which is exposed to the solvent in all cases. Hence, the surface environment is not likely to differ significantly among the different supports. The low reactivity of PL-AMS was not unexpected, as it is highly hydrophobic and swells poorly in aqueous solution. The unexpectedly poor conversion obtained with soluble PEG may be due to the formation of secondary structural features of the PEG chains in aqueous media[23] that could restrict accessibility to the terminal moiety containing the substrate. Nevertheless, despite the low reactivity for the PL-AMS and PEG, respectable phenolic coupling conversions were achieved.

Synthesis of Apocynin Oxidation Products on the Solid Phase

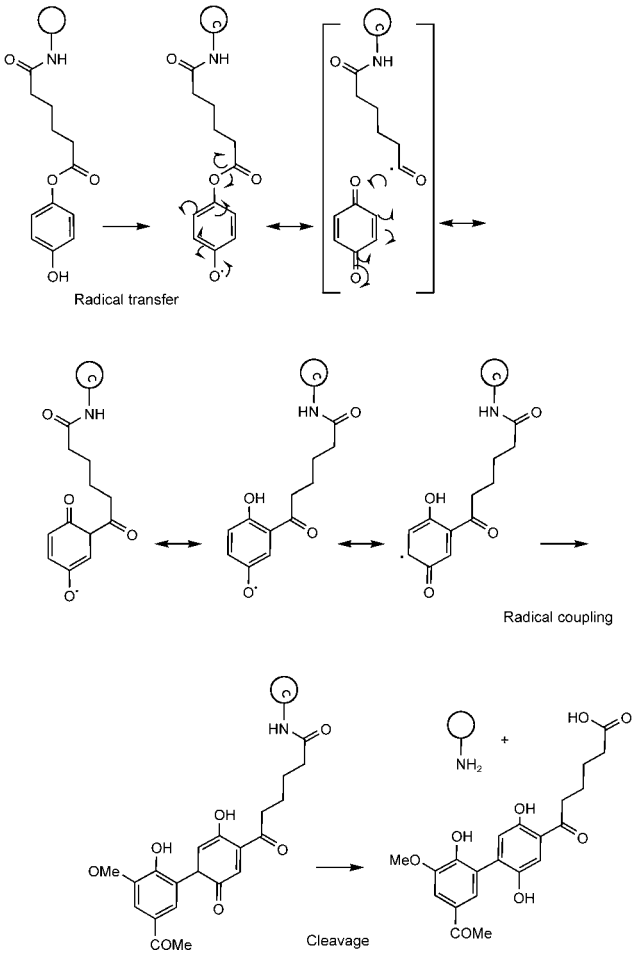
Tentagel resins were selected to investigate further the potential of SBP-catalyzed reactions to generate oligophenols on the solid phase. As depicted in Scheme 2, SBP-catalyzed oxidation of apocynin in the presence of HQ-loaded Tentagel would be expected to generate dimers, trimers, and potentially higher-order oligomers. To confirm this synthetic scheme, we performed several reactions with various amounts of bound HQ (2–15 μmol), using single or repeated addition of 0.5 mM H_2O_2 and 2.0 mM apocynin. After acid-cleavage of the oligomeric products from the adipate linker, the products were analyzed by MS and ^{1}H-NMR,[24] and compared with previous results obtained during a similar reaction with apocynin in the solution phase.[25] When a 1 h reaction was performed with a single addition of H_2O_2 and apocynin, incomplete conversion of HQ was observed and an apocynin-hydroquinone dimer (HQ-apocynin) was generated (Scheme 2). When the reaction conditions were modified by the introduction of five cycles of H_2O_2 and apocynin addition over 6 h, the sole product was a hydroxylated trimer (HQ-apocynin-apocynin)-OH, similar to what was obtained in the solution phase in the absence of HQ.[25] Thus, the presence of the seed phenol does not influence the product spectrum obtained due to SBP catalysis.

Interestingly, an additional product was observed, albeit in yields $< 10\%$, that corresponded to the migration of the adipate linker's ester group to the *ortho*-position of HQ (Scheme 3). This reaction is effectively an enzyme-catalyzed Fries rearrangement,[26] which is con-

Table 2. Initial rate and final conversion for various resins submitted to the SBP enzymatic reaction.

	Initial rate (nmol/min)	Normalized rate (min^{-1})[a]	conversion (%, 20 min)
Tentagel S	50.0	0.094	92
Tentagel Macrobeads	20.7	0.037	73
Tentagel HL	11.0	0.114	89
PEG	4.8	0.115	31
PL-AMS	1.0	0.010	41
JandaJel	1.2	0.038	22

[a] Corrected for different HQ loadings (initial rate ÷ HQ loading).



Scheme 3. Proposed Fries rearrangement of HQ-apocynin coupling catalyzed by SBP on HQ-bound to Tentagel S.

ventionally performed in the presence of strong Lewis acids or *via* irradiation to generate radical rearrangement.[27] Our results indicate that the required radical formation, leading to rearrangement, can also be generated through SBP catalysis *via* the one-electron oxidation of HQ.[28] To our knowledge, this is the first example of an enzyme-catalyzed Fries rearrangement.

Conclusion

We have demonstrated the first enzymatic C–C coupling reaction on solid-supported substrates and showed that several commercially available resins were effective in promoting the reaction. The highest conversions took place on Tentagel resins, where SBP catalysis was observed to occur primarily on surface-bound HQ. In the presence of apocynin, SBP catalyzed the formation of a dimer [HQ-(apocynin-apocynin)] and a trimer [HQ-(apocynin-apocynin-apocynin)-OH], consistent with solution-phase chemistries. This strategy expands the repertoire of biocatalytic reactions on solid-phase resins, which may lead to broader inclusion of biocatalytic transformations in combinatorial synthetic strategies to generate a broad range of small molecules.

Experimental Section

Amino-functionalized Tentagel® S, HL, and Macrobeads were purchased from Rapp Polymere (Tübingen, Germany). PEG_{1500} bis-(3-aminopropyl) and amino-functionalized Jandajel® were purchased from Aldrich (St. Louis, MO). Amino-functionalized PL-AMS was purchased from Polymer Laboratories (Amherst, MA). SBP and catalase were obtained from Sigma Chemical Co. Chemicals and solvents were of the highest grade commercially available and were purchased from Aldrich and Fisher (Hampton, NH).

Loading of Hydroquinone onto the Resins (Method A: Adipoyl Chloride)

A desired amount of resin (125–250 mg, 10–20 μmol NH_2) was swollen in a 20-mL vial under nitrogen and 10 mL of anhydrous DMF were added. Under a nitrogen flush, 20 equivalents of pyridine in 2 mL of DMF and 10 equivalents of adipoyl chloride in 2 mL of DMF were added. The mixture was then shaken at 250 rpm for 16 h followed by the addition of 20 equivalents of HQ in 2 mL of DMF. The mixture was then shaken for an additional 12 h. After filtration over a fritted disk (porosity 40–60 μm or 10–15 μm, depending of the particle size) and washing sequentially with deionized water (200 mL), acetone (200 mL), and CH_2Cl_2 (50 mL), the resin was dried overnight at 20 °C under vacuum, and then stored at 5 °C. The procedure for the loading of PEG_{1500} bis-(3-aminopropyl) was identical except that CH_2Cl_2 was used instead of DMF. For organic solvent-soluble PEG-based compounds, the work-up procedure consisted of washing the reaction mixture with deionized water (4×100 mL), decanting, and drying the organic phase over $MgSO_4$ and removing CH_2Cl_2 by rotary evaporation. The resulting white solid was washed with cold diethyl ether (200 mL) to remove unreacted organic molecules and gave purified PEG-loaded HQ. PEG loading was determined by ^1H NMR in $CDCl_3$.

Data for PEG-bis-[$(CH_2)_3$-NHCO-$(CH_2)_4$-COOC_6H_4OH]: ^1H NMR (300 MHz, $CDCl_3$, 20 °C): $\delta = 6.8$ (m, 8H aromatic, symmetrical); 3.6 (s, ethylene glycol chain); 3.4, 2.4–1.4 (m, various methylene groups).

Loading of Hydroquinone onto the Resins (Method B: DCC)

The resin (1 g) was first swollen in CH_2Cl_2 for 30 min. A solution of DCC (5 equivalents with respect to resin amino functions) in DMF (5 mL) was added to a solution of adipic acid (5 equivalents) in DMF (5 mL). The reagent solution was then added to the swollen resin in a solid-phase reaction vessel and shaken horizontally at 100–300 rpm on an orbital shaker at 25 °C. After 3 h, the reaction solution was removed and the resin washed with CH_2Cl_2 (50 mL). The coupling procedure was then repeated twice. Final washing involved DMF (50 mL), MeOH/water 1:1 (50 mL), MeOH (50 mL), and

CH_2Cl_2 (50 mL), respectively. An identical procedure was repeated with HQ. In this case, DCC solution was added to the resin after swelling, and the HQ solution (5 equivalents) in DMF (5 mL) was added to the mixture.

SBP-Catalyzed Oxidation of Apocynin with Resin-Bound HQ

Resins (10–40 mg) were loaded into 4-mL vials containing 2 mL of reaction medium. The latter consisted of 10 μg/mL SBP, 2.0 mM apocynin, and 0.5 mM H_2O_2 in phosphate buffer (20 mM, pH 8.0) containing 30% (v/v) DMF. Vials were shaken horizontally at 100–300 rpm on an orbital shaker at 25 °C. Peroxidase reactions were quenched upon addition of 200 μL of 0.1 mg/mL catalase solution. After sedimentation of the solid support, the reaction solution was removed and the support washed three times with water (4 mL) and three times with acetone (4 mL). Cleavage was carried out with 1 mL of 1 M H_2SO_4 (25 °C, 6 h). Cleavage products were recovered after addition of 1 mL of 1 M NaOH and extraction with 1 mL diethyl ether.

Samples were analyzed with a Shimadzu LC-10A liquid chromatograph equipped with an SPD-M10A diode array detector. An Alltech (Deerfield, IL, USA) Alltima C_{18} column was employed with a gradient elution profile consisting of water:CH_3CN, 95:5 to 60:40 (30 min), then to 10:90 (10 min), then back to 95:5 (10 min), and finally maintaining for 5 min at a flow rate of 0.8 mL/min. Quantification of HQ was performed using an authentic external standard with detection at 254 nm. To overcome the standard error associated with resin-based reactions on small amounts of material, each time course experiment was performed with five replicates. A Dixon test (confidence 95–99%) was then carried out for each data series to reject outliers and maintain a relative standard error of 5–20%.

Acknowledgements

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