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Oxidation of morin (2',3,4',5,7-Pentahydroxyflavone) with a peroxidase homogenate from onion

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Abstract: This study dealt with the examination of some biocatalytic properties of a peroxidase-active homogenate, obtained from onion solid wastes, using the flavonol morin (2',3,4',5,7-pentahydroxyflavone) as substrate. Morin oxidation by the homogenate/H_2O_2 system was shown to be optimum at pH values between 4 and 6 and temperature between 20 and 40°C. High performance liquid chromatography analyses of a peroxidase-treated morin solution revealed the formation of 2,4-dihydroxybenzoic acid (*β*-resorcylic acid), and 2,4,6-trihydroxybenzoic acid (phloroglucinol carboxylic acid) as two of the major degradation products. The tentative identification of these substances indicated that oxidative cleavage of morin by onion peroxidase shares common features with quercetin (3,4′,5′,5,7-pentahydroxyflavone) degradation, and putative routes were proposed.

Keywords: Flavonols, morin, onion, oxidation, peroxidase

Introduction

Peroxidases catalyze the oxidation of a wide variety of substrates, using H_2O_2 or other peroxides (Veitch, 2004). In general terms, the majority of reactions catalysed by the classic plant peroxidases can be represented as follows, where RH and R• represent a reducing substrate and its oxidised radical product, respectively:

$$H_2O_2 + 2RH \rightarrow 2H_2O + 2R^{\bullet}$$

Reduction of peroxides at the expense of electron donating substrates, make peroxidases useful in a number of industrial and analytical applications. Because of the oxidative nature of peroxidases, there are several areas where it could replace current chemical oxidant techniques (Ragalado *et al*., 2004).

Peroxidases have been investigated not only with regard to their potential in eliminating phenolic pollutants (Davidenko *et al*., 2004; Hamid and Rahman, 2009) and synthesis of chemicals (Mazhugo *et al*., 2005), but also for their utility in the generation of novel, bio-based polyphenols with important biological activities, including sinapic acid dimers and other oligomers (Liu *et al*., 2007) and ferulic acid / resveratrol heterodimers (Yu *et al*., 2007). The ability of peroxidases to form ferulic and caffeic acid dimers has been shown using an enzyme preparation from *B. salicifolium* callus cultures (Luis *et al*., 2005), but more recently the generation of ferulic acid (El Agha *et al*., 2008a) and hydrocaffeic acid (El Agha *et al*., 2008b) dimers, as well as caffeic acid tetramers (El Agha *et al*., 2009), has been possible using a peroxidase preparation from onion solid wastes.

Of great importance is also the chemistry underlying peroxidase action on flavonoids. Particularly, the interactions of flavonols such as quercetin, with POD has been shown to generate a spectrum of other phenolics, which also exert considerable antioxidant effects (Makris and Rossiter, 2001; Gülşen *et al*., 2007a; Gülşen *et al*., 2007b).

A previous investigation on the oxidation mechanism of quercetin, a physiological substrate of onion POD, provided sound evidence that the pathway(s) followed might incorporate unusual features, as steps corresponding to dioxygenase-catalysed degradation were implicated (Osman *et al*., 2008). In the mechanism proposed, crucial role was attributed to the B-ring of the flavonol skeleton, whereas the A-ring did not appear to exert any influence.

In an effort to further elucidate this hypothesis, this study was dealt with the oxidative decomposition of a structurally similar flavonol, morin. Unlike quercetin, morin has a B-ring that contains *meta*-oriented hydroxyls (2',4'), and this characteristic makes morin an ideal substrate for the examination of the effect of the B-ring on the oxidation mechanism.

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Materials and Methods

Chemicals and reagents

All solvents used for chromatographic purposes were HPLC grade. Morin, *β*-resorcylic acid (2,4-dihydroxybenzoic acid), phloroglucinol carboxylic acid (2,4,6-trihydroxybenzoic acid) and hydrogen peroxide (H_2O_2) were from Sigma Chemical Co (St. Louis, MO, U.S.A.). For pH 2 and 8, a potassium chloride/HCl and a boric acid/NaOH buffer were used, respectively. For the pH range 3-7, a phosphate/citrate buffer was used.

Preparation of onion solid waste homogenate

The onion solid waste used in this study was obtained from a local catering facility (Chania, Crete) after processing of brown-skin onion bulbs. The waste consisted of the apical trimmings of the bulbs, as well as the outer dry and semi-dry layers (Khiari *et al*., 2008). The material was transferred to the laboratory immediately after processing, and ground in a domestic blender. An aliquot of 2 g of the ground tissue was suspended in 15 mL buffer solution under stirring, and the suspension was centrifuged at 3000 g for 20 min and filtered through paper filter to remove cell debris. The clear supernatant obtained was treated with activated charcoal for decolourisation, and filtered through celite under vacuum. The clear filtrate was used as the crude enzyme source.

Determination of peroxidase activity

An aliquot of 0.1 mL of morin solution was mixed with 0.1 mL homogenate and 0.8 mL H_2O_2, and the reaction was followed by measuring the decrease in absorbance at 390 nm (Osman *et al*., 2008). Absorbance measurements were obtained at 5 sec intervals for up to 1 min. One enzyme unit was defined as ΔA_{390} sec^{-1}. Control reactions by omitting H_2O_2 or using heat-inactivated homogenate were also carried out. In assays performed at different temperatures, all constituents of the reaction mixture were pre-incubated either in a freezer (5°C) or in a thermostated water bath (30-60°C).

Protein determination

Protein content of the homogenate was determined according to Bradford, 1976, using bovine serum albumin as standard. For all determinations, a computer-controlled HP 8452A diode-array spectrophotometer was used.

Morin oxidation

The reaction mixture (final volume 1 mL) consisted of 0.6 mL homogenate (in phosphate buffer, pH 4), 0.3 mL morin solution (6 mM in DMF) and 0.1 mL H_2O_2 (0.88 mM in phosphate buffer, pH 4). The reaction was allowed to proceed for 3 hours at 40°C, and then 0.2 mL trichloroacetic acid (10% in EtOH) was added. The solution was centrifuged at 5000 *g* for 10 min and filtered through 0.45 μm syringe filters.

HPLC-DAD

The equipment utilized was an HP 1090, series II liquid chromatograph, coupled with an HP 1090 diode array detector and controlled by Agilent ChemStation software. The column was a LiChrosphere RP18, 5 μm, 250 × 4 mm (Merck), protected by a guard volume packed with the same material. Both columns were maintained at 40°C. Eluent (A) and eluent (B) were 1% formic acid and acetonitrile, respectively. The flow rate was 1 mL min^{-1}, and the elution programme used was as follows: 0-5 min, 5% B, 5-45 min, 100% B, 45-55 min, 100% B. Monitoring of the eluate was performed at 275 and 320 nm.

Statistical analyses

All determinations were carried out at least in triplicate, the values were averaged and standard deviation (± S. D.) was calculated. For all statistics, Microsoft Excel™ 2000 and SigmaPlot 11™ were used.

Results

Biocatalytic characteristics

In Figure 1a can be seen the effect of morin concentration on the enzyme activity. Increasing concentrations of morin were shown to provoke a proportional effect on the enzyme activity, up to 0.16 mM. Thereafter, a decline in activity was observed. Increases in H_2O_2 concentration were shown to promote enzyme activity up to 2.4 mM. (Figure 1b).

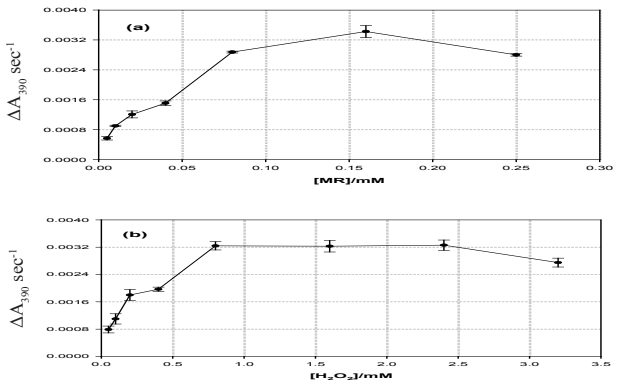

Figure 1. POD activity as a function of substrate (morin – MR) (a) and H_2O_2 (b) concentration. Reactions were perormed at pH 7 and T = 22°C, with and 13.1 μg total protein. For the substrate assay, $[H_2O_2]$ = 3 mM. For H_2O_2 assay, [MR] = 1.6 mM.

The examination of the pH effect over a range varying from 2 to 8 revealed that maximal enzyme activity was expressed at pH 5, whereas significant decline was seen when reactions were carried out at pH lower than 5 and higher than 6 (Figure 2a). In a similar fashion, temperatures ranging from 5 to 40°C were favourable, the maximal activity being found at 30°C (Figure 2b). Enzyme activity was virtually trivial at 60°C.

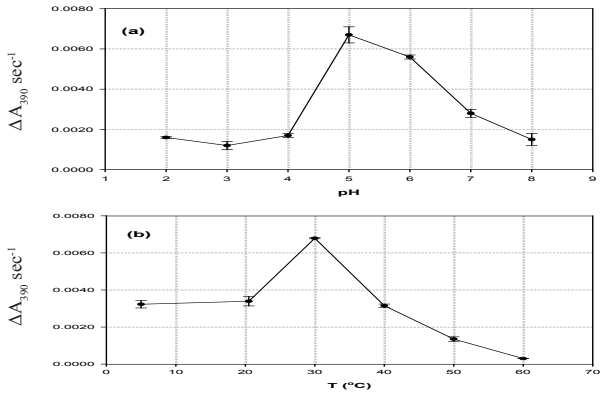

Figure 2. POD activity as a function of pH (a) and temperature (b). Reactions were carried out at T = 22°C, with [MR] = 1.6 mM, [H_2O_2] = 3 mM and 12.5 µg total protein. For the temperature assay, pH = 5.

Using optimal conditions with regard to substrate, activator, pH and temperature, different dilutions of the homogenate were assayed (Figure 3). Increasing amounts of total protein in the reaction mixture provoked proportionally higher enzyme activities in a linear manner (R^2 = 0.999), suggesting that the rate of morin oxidation is directly proportional to the total enzyme concentration.

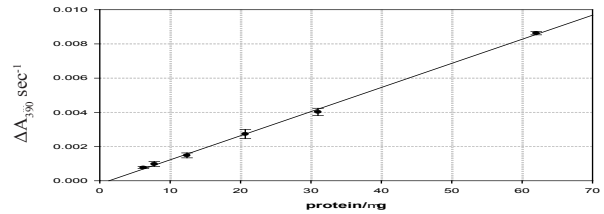

Figure 3. POD activity as a function of total protein concentration. Reactions were carried out at T = 30°C and pH 5, with [MR] = 1.6 mM and [H_2O_2] = 3 mM.

Tentative identification of morin oxidation products

A morin solution (1.8 mM) was incubated with the enzyme homogenate in the presence of 5.4 mM H_2O_2 for three hours. The mixture was then analysed by HPLC. In the trace obtained at 275 nm (Figure 4), four major peaks were detected. The peaks denoted 1 and 2 could easily be identified by comparison of their UV-vis spectra and retention times with those of original standards, and tentatively assigned to 2,4,6-trihydroxybenzoic acid (phloroglucinol carboxylic acid) and 2,4-dihydroxybenzoic acid (*β*-resorcylic acid), respectively.

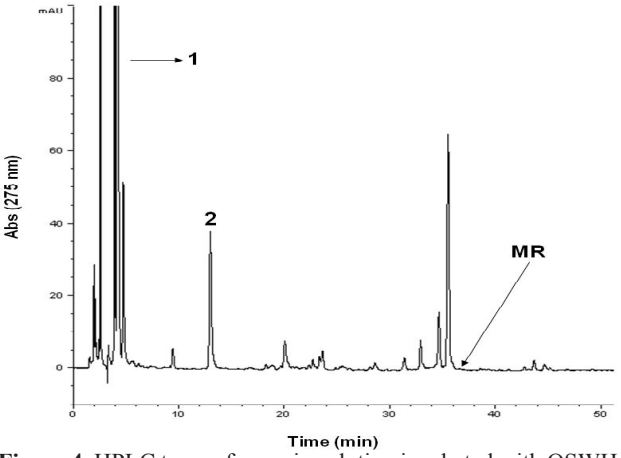

Figure 4. HPLC trace of a morin solution incubated with OSWH for 180 min. Reaction was carried out at T = 30 °C and pH 5, with [S] = 1.6 mM, [H_2O_2] = 3 mM and total protein 70.2 µg.

Discussion

Regarding onion peroxidase, the examination of some hydroxycinnamate derivatives, including hydrocaffeic acid (El Agha *et al*., 2008a) and caffeic acid (El Agha *et al*., 2009), showed that the substrate / H_2O_2 ratios were 1.33, 0.8, respectively. Using quercetin, however, which is the physiological substrate of the onion enzyme, maximum catalytic activity was observed at quercetin / H_2O_2 molar ratio of 30 (Osman *et al*., 2008).

In the case of morin, the molar ratio found was 15. This ratio is 2-fold lower than that found for quercetin, but approximately 11-18 times higher than those reported for structurally simpler phenolic acids. The evidence that oxidation of both quercetin and morin requires significantly higher amounts of H_2O_2 might indicate that the oxidation mechanism(s) implicated presumably proceed(s) in a more complicated manner, involving more than one pathway. This was demonstrated for quercetin, for the oxidation of which at least two different pathways were proposed (Osman *et al*., 2008).

The investigation of a crude onion peroxidase showed that quercetin oxidation might be favoured at pH 8 (Takahama and Hirota 2000), but this result was contrasted by more recent data indicating that quercetin oxidation rate peaks at pH 4 (Osman *et al*., 2008). In this study it was shown that morin oxidation by the POD cell-free extract had higher rate at a pH value of 5, with significant activity (> 40%) being retained between pH 5 to 7.

On the other hand, favourable temperatures were found to lie between 20 and 40°C, the optimal being at 30°C. This is somewhat lower than that observed for quercetin and hydrocaffeic acid (40°C) (Osman *et al*., 2008; El Agha *et al*., 2008), but the same with

that determined for caffeic and ferulic acids (El Agha *et al*., 2009; El Agha *et al*., 2008).

The recent investigation of the putative pathways involved in quercetin oxidative degradation by onion POD suggested that the mechanism implicated may share common features with those observed for dioxygenase-catalysed cleavage of quercetin (Osman *et al*., 2008). The predominant components detected as the result of quercetin decomposition were 3,4-dihydroxybenzoic acid (protocatechuic acid) and 2,4,6-trihydroxybenzoic acid (phloroglucinol carboxylic acid), which corresponded to the B and A-ring fragments of the flavonol skeleton. The data generated indicated that the formation of these two acids was rather attributed to hydrolysis of the previously formed depside.

Likewise, the detection of 2,4-dihydroxybenzoic acid (*β*-resorcylic acid) and and 2,4,6-trihydroxybenzoic acid (phloroglucinol carboxylic acid) in the morin solution treated with onion POD, demonstrates that the mechanism of morin cleavage follows the same pathway (Figure 5). Thus it could be postulated, in line with the findings for quercetin, that the morin radical generated upon action of peroxidase would react with dioxygen (O_2), and this binding would then produce the morin peroxide, which after nucleophilic attack on the C4 atom would generate the endoperoxide (EP).

Unlike quercetin, however, no morin heterodimer was detected under the conditions employed, an indication that its formation might not have occurred or was particularly limited. However, the fact that typical, major oxidation products were formed in accordance with the mechanism proposed for quercetin, shows that the different arrangement of hydroxyl groups on the B-ring plays no role in this regard.

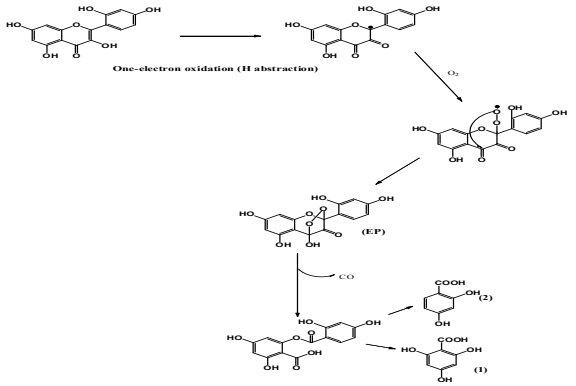

Figure 5. Putative pathway of morin oxidation by onion POD. EP, endoperoxide.

Conclusion

In the study presented herein, an attempt to clarify the role of key structural features on the oxidisability and cleavage mechanism of flavonols was sough. Using morin as substrate, it was shown that the different arrangement of hydroxyl groups on the B-ring might affect the biocatalytic properties regarding the optimal substrate-to-H_2O_2 ratio, pH and temperature. Further, efforts to elucidate the oxidative behaviour of onion POD on morin provided strong evidence that the oxidative pathway(s) implicated share common features with quercetin oxidation, which appears to be independent of the B-ring substitution pattern.

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