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An investigation on structural aspects influencing product formation in enzymic and chemical oxidation of quercetin and related flavonols

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Abstract

Quercetin oxidation, with mushroom polyphenol oxidase (PPO), horseradish peroxidase (POD) and potassium ferricyanide, was studied by means of reversed-phase high-performance liquid chromatography (RP-HPLC). In order to establish structural requirements for product formation, two other flavonols, morin and rutin (quercetin 3-*O*-rutinoside), which possess key structural differences, were also treated. Oxidations were performed, either in aqueous acetonitrile (MeCN) or aqueous *N*,*N*-dimethylformamide (DMF), due to poor solubility of flavonols in aqueous media. The chromatographic profiles obtained from quercetin solutions, treated with PPO, POD, or potassium ferricyanide, were very similar, yielding the same set of products. In contrast, morin and rutin oxidations proceeded via different pathways. From the examinations carried out, it became clear that some products which possess significantly lower polarity compared with quercetin have, as a strict requirement, both the *o*-catechol function and the 3-hydroxyl group.

Keywords: Chemical oxidation; Enzymic oxidation; HPLC; Morin; Peroxidase; Quercetin; Rutin; Tyrosinase

1. Introduction

Improper post-harvest processing and storage of plant tissues and products may lead, in some instances, to oxidation of certain components which can affect the overall quality of foods. With the exception of some food commodities, such as black tea and coffee, these oxidations are considered undesirable. Various groups of polyphenolic compounds, such as catechins, flavonols and anthocyanins, have been found to contribute to non-enzymic and enzymic browning of foods. The relative reactions in most cases involve oxidative steps and subsequent changes in the flavonoid skeleton, simultaneously altering the chemical and biological properties of these molecules. Given the increasing interest of flavonoids in human nutrition and health, the study of flavonol oxidation, as well as the nature of the oxidation products and their properties, should be addressed as an issue of high significance.

Quercetin is a hydroxyl (•OH) radical scavenger (Husain, Cillard, & Cillard, 1987), and serves as strong antioxidant against lipid peroxidation in phospholipid bilayers (Terao, Piskula, & Yao, 1994), and in human low-density lipoprotein (Manach et al., 1995; Meyer, Heinonen, & Frankel, 1998). Previous investigations, based on alkaline (Makris & Rossiter, 2000c) and buffer solutions (Makris & Rossiter, 2000a), constituted the first approach to quercetin and rutin (quercetin 3-*O*-rutinoside) degradation, and provided evidence concerning the role of the glycosidic linkage between quercetin and rutinose in flavonol degradability and product formation. Further investigations into hydroxyl free radical-mediated oxidative decomposition of quercetin and morin permitted a deeper insight into the mechanisms underlying oxidative cleavage of flavonols (Makris & Rossiter, 2000b, 2001). In the study presented here, oxidations of quercetin, morin and rutin were accomplished by enzymic methods, in order to elucidate pathways of quercetin oxidative degradation and distinguish differences deriving from structural features. Oxidations were carried out with two major enzymes involved in flavonol degradation in plant tissues, polyphenol oxidase (PPO), and peroxidase (POD). Oxidations with potassium ferricyanide were also undertaken for comparison reasons, and to test the possibility of using chemical means for performing model oxidations.

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2. Materials and methods

2.1. Chemicals

Water used for high-performance liquid chromatography (HPLC) analyses was distilled, purified by Easy-Pure® *RT* ultrapure water system, and filtered through a 0.45-μm filter (Millipore). Acetonitrile (MeCN) was HPLC grade (BDH Chemicals Ltd., Poole, UK). 2,4-Dihydroxybenzoic acid (β-resorcylic acid), and 2,4,6-trihydroxybenzoic acid (phloroglucinol carboxylic acid) were from Aldrich Chemical Co Ltd. (Gillingham-Dorset, UK). Butylated hydroxytoluene (BHT), 3,4-dihydroxybenzoic acid (protocatechuic acid), morin, peroxidase (from horseradish, 116 U/mg), quercetin, rutin (quercetin 3-*O*-rutinoside) and tyrosinase (from mushrooms, 2140 U/mg) were from Sigma Chemical Co. (St. Louis, MO, USA). *N*,*N*-dimethylformamide (DMF), potassium ferricyanide and trichloroacetic acid (TCA) were from BDH Chemicals Ltd. (Poole, UK).

2.2. Treatment of flavonols with enzymes

For the tyrosinase-catalysed oxidations, the reaction medium (1 ml) consisted of 0.70 ml of a tyrosinase (PPO) solution (1 mg ml^{-1} in citrate–phosphate buffer, pH 6.6, 1498 U), and 0.30 ml of a flavonol solution (6 mM in DMF). For the peroxidase-catalysed oxidations, the reaction medium (1 ml) consisted of 0.60 ml of a peroxidase (POD) solution (1 μg ml^{-1} in citrate–phosphate buffer, pH 6.0, 116 mU), 0.30 ml of a flavonol solution (6 mM in DMF), and 0.10 ml H_2O_2 (0.88 mM in citrate–phosphate buffer, pH 6.0). For rutin oxidation, 100 μg ml^{-1} POD solution was used. Incubations were carried out at room temperature (20±2 °C).

2.3. Treatment of flavonols with potassium ferricyanide

For potassium ferricyanide oxidations, appropriate volumes of oxidising agent (potassium ferricyanide/sodium hydrogen carbonate) were added dropwise into flavonol solutions (2 mM in 70% aqueous MeCN), over 10 min. The final concentration of potassium ferricyanide and sodium hydrogen carbonate in the reaction mixture was 2 and 1 mM, respectively. Following steps were as earlier.

2.4. Reversed-phase high-performance liquid chromatography (RP-HPLC)

2.4.1. Sample preparation

For the potassium ferricyanide treatments, 1 ml was withdrawn from the reaction flask and treated with 0.6 ml BHT (11.5 mM in MeCN) and 0.2 ml Na-EDTA (10.8 mM). The solution was taken to dryness under vacuum, dissolved in 1 ml acetic acid:DMF (1:9), and spun down. The clear supernatant was analysed by HPLC. Samples were analysed immediately after preparation. For the enzymic treatments, 0.1 ml was withdrawn from the reaction medium and treated with 0.02 ml TCA (30% in MeCN). The mixture was spun down, and the clear supernatant was injected onto the HPLC.

2.4.2. Analytical HPLC procedure

A previously established procedure was used (Makris & Rossiter, 2001). A Waters 600E gradient pump and an Applied Biosystems 757 detector, set at 290 nm, were used. The system was interfaced by a JCL 6000 software. Chromatography was carried out on a Waters Symmetry C_{18}, 3.9×150 mm, 5 μm, column, with a Waters guard column, packed with the same material. Columns were thermostatically controlled to maintain a temperature of 40 °C. Eluents were (A) 4.34 mM aqueous orthophosphoric acid (pH 2.5) and (B) MeCN:eluent A (6:4), and the flow rate was 1 ml min^{-1}. Injection was made by a Rheodyne injection valve with 20-μl fixed loop. The elution was from 0% B to 40% B in 60 min, then 50% B in 10 min, then 20% B in 10 min, and then isocratic for another 10 min. The column was washed with 100% MeCN and re-equilibrated with 100% eluent A before the next injection. Standard solutions of known compounds were prepared in EtOH, and kept at −20 °C. Identification of degradation products was based on comparing the retention times of unknown peaks with those of standards, and on spiking reaction mixtures with standard solutions.

2.5. UV–vis spectroscopic studies

The equipment used was a Shimadzu UV–vis scanning spectrophotometer, interfaced with a UV-2101PC software. Oxidations were followed in 1 cm path length cuvette, by scanning with fast scan speed every 120 s, for up to 12 min. Scans were performed over a wavelength range varied from 700 to 230 nm.

3. Results

3.1. Examination of quercetin oxidation

The incubation of quercetin with PPO produced characteristic changes in the UV-spectrum (Fig. 1), which did not occur in mixtures without enzyme or quercetin. Two distinct isosbestic points appeared at 279 and 351 nm, while absorbance declined at 256 and 375 nm with a concomitant increase at 291.5 and 335.5 nm. Following the quercetin oxidation by POD was not feasible as the reaction was instantaneous, even when very low levels of either POD or H_2O_2 were used. However, after the incubation, two maxima were recorded at 292 and 326 nm (data not shown). No reaction

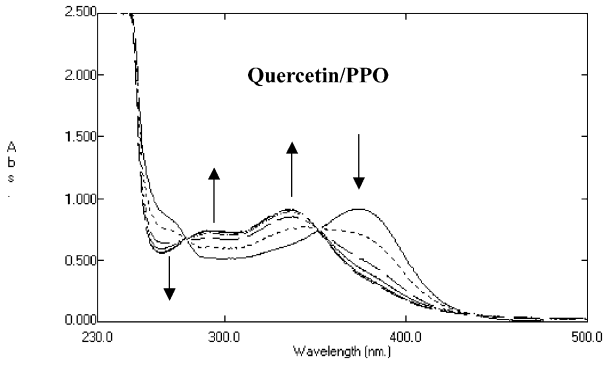

Fig. 1. Oxidation of quercetin (0.1 mM) with mushroom tyrosinase (polyphenoloxidase, PPO), as shown by UV–vis spectroscopy. Scans were performed at 120-s intervals for up to 12 min. Arrows indicate increases and decreases in absorbance.

was observed when POD or H_2O_2 were omitted from the mixtures.

The HPLC analysis of quercetin solutions treated with PPO for 20 min (Fig. 2A) revealed the formation of two major products, termed Q-DP 3 and Q-DP 4. Smaller amounts of two other products, assigned as Q-DP 1 and Q-DP 2, were also found. Q-DP 3 and Q-DP 4 were less polar than quercetin, a finding which suggests that the flavonol skeleton might not have been broken down. Quercetin treatment with POD yielded the same set of products (Fig. 2B), indicating that quercetin transformation followed the same pathway(s).

Upon addition of potassium ferricyanide, the quercetin spectrum showed profound changes, giving a 42- and a 50-nm bathochromic shift in Bands I and II, respectively. These intense modifications may indicate the formation of a complex between the flavonol skeleton and ferricyanide ion, as proposed by Pelter, Bradshaw, and Warren (1971). The oxidation of quercetin by potassium ferricyanide gave two isosbestic points at 313 and 447 nm, and maxima at 306 and 329 nm. Absorbance decrease was observed at 417 nm. The HPLC analysis revealed that oxidation of quercetin led to the formation of Q-DP 2 and Q-DP 3 as predominant products, which were accompanied by smaller amounts of Q-DP 4 (Fig. 2C). Nevertheless, in this case no Q-DP 1 was detected.

3.2. Examination of morin oxidation

Morin oxidation by PPO resulted in absorbance increases at 283 and 325 nm, and appearance of isosbestic points at 281, 359, and 450 nm (Fig. 3). POD oxidation, as in the case of quercetin, was almost instantaneous, and gave maxima at 292 and 328 nm, but one isosbestic point at 280 nm could be distinguished (data not shown). In both cases the HPLC analysis showed one compound, termed M-DP 1, to be the predominant oxidation product (Fig. 4A, B). In PPO-treated mixtures DHBA and RC, along with three other peaks, with retention times of 39, 43 and 46 min, were also detected as minor constituents. Only the peak with retention time 46 min was found in POD-treated mixtures. It is also notable that, unlike quercetin, morin did not give rise to products of lower polarity, such as Q-DP 2, 3 and 4.

The addition of potassium ferricyanide to morin solution provoked a pronounced bathochromic shift in Band I (53 nm), whereas Band II was virtually unaffected. Further examination for over than 20 min did not show appreciable changes, suggesting that oxidation occurred instantaneously. As in the case of PPO and POD treatments, the HPLC analysis showed the formation of only M-DP 1 (Fig. 4C).

3.3. Examination of rutin oxidation

Incubation of rutin with PPO did not result in detectable changes in the rutin spectrum, in spite of prolonged incubations for over 20 min, and it was assumed that rutin was not acted upon. However, rutin treatment with POD resulted in notable modifications in its spectrum, an increase at 300 nm, a decrease at 355 nm, and isosbestic points at 325 and 408 nm. Nevertheless, these spectral changes were observed only when a 10-fold higher POD concentration was used, than for the quercetin and morin treatments. This fact suggested that rutin was oxidised at a much lower rate.

Although the UV–vis examination revealed that rutin was not oxidised by PPO, the HPLC analysis of PPO-treated rutin mixtures revealed that oxidation did occur, yielding at least three major oxidation products more polar than rutin, at 20, 21 and 28 min, as illustrated in Fig. 5A. It is not why the generation of these peaks was not accompanied by spectral changes, but it is probable that these products possess very similar spectral characteristics to the parent molecule, and therefore the spectrum of the reaction mixture does not change appreciably as oxidation proceeds. The trace of POD-treated rutin solution exhibited important similarities, in that the major oxidation product was the one with a retention time of 28 min. Products at 20 and 21 min were also detected. (Fig. 5B). Characteristic quercetin oxidation products, such as Q-DP 2, 3, and 4, were not found at any time. It is not known the reason why.

Potassium ferricyanide had a clear effect on the rutin spectrum, as it provoked a 39- and a 44-nm shift to Bands I and II, respectively, showing chelation between rutin and ferricyanide ion. Considerable increases occurred at 302 and 322 (shoulder) nm, while absorbance declined at 401 nm (data not shown). The oxidation afforded more-or-less the same HPLC profile as that of PPO- and POD-treated solutions, the most abundant peak being the one with the retention time 28 min. In this instance, however, another two peaks appeared with very similar polarities, their retention times being 26 and 27 min (Fig. 5C).

4. Discussion

4.1. PPO-catalysed oxidation

Polyphenol oxidases are capable of catalysing two distinct reactions: (1) introduction of a hydroxyl group to a monophenol converting it to an *o*-diphenol and (2) oxidation of the *o*-diphenol into *o*-quinone (Matheis & Whitaker, 1984; Vámos-Vigyázó, 1981). The second reaction is considered as a fundamental step in enzymic food browning, since *o*-quinones are very reactive and condense spontaneously into brown or black polymers

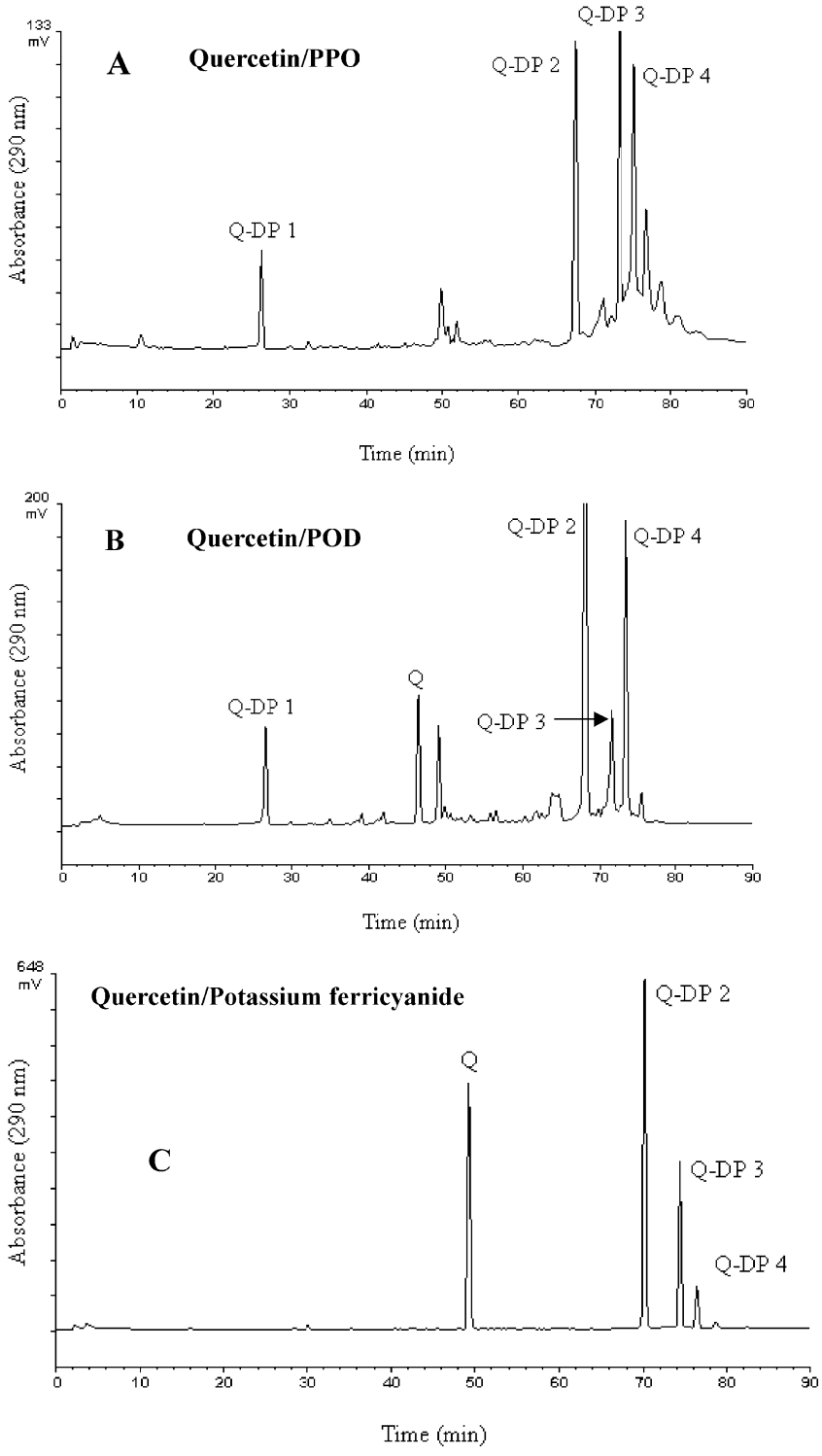

Fig. 2. HPLC profiles of quercetin solutions treated with (A) polyphenoloxidase (PPO), (B) peroxidase (POD) and (C) with potassium ferricyanide. Monitoring was performed at 290 nm. Q, Quercetin, Q-DP 1, 2, 3, and 4, Quercetin oxidation products.

(Friedman, 1996; Matheis, 1983; Pierpoint, 1966; Sapers, 1993). *o*-Quinones, however, may also form in coupled oxidation reactions, which may lead to the oxidation of compounds that cannot be directly oxidised by PPO, or are less readily oxidised. Such reactions have been observed in model solutions containing *trans*-caftaric acid *o*-quinone and 2-*S*-glutathionylcaftaric acid (Cheynier & van Hulst, 1988; Cheynier, Rigaud, & Moutounet, 1990), caftaric acid and catechin (Cheynier, Basire, & Rigaud, 1989), chlorogenic acid and catechin (Oszmianski & Lee, 1990), (−)-epicatechin and theaflavin (Opie, Clifford, & Robertson, 1993), caffeic acid *o*-quinone and cyanidin 3-glucoside (Kader, Irmouli, Zitouni, Nicolas, & Metche, 1999), and chlorogenic acid and pelargonidin 3-glucoside (Kader, Nicolas, & Metche, 1999).

The PPO-mediated oxidation of all three flavonols did not result in the formation of brown pigments, as is implied from spectral changes, which indicate that the reaction products exhibited maxima between 283 and 335 nm. Previous studies on quercetin oxidation with broad bean seed PPO showed an increase in absorbance at 291 nm and a decrease at 372 nm, but isosbestic points occurred at 272 and 342 nm (Jiménez & García-Carmona, 1999). Quercetin oxidation by POD has been reported to provoke decreases at 260 and 370 nm, and a pronounced increase at 300 nm (Schreier & Miller, 1985). The low polarity of Q-DP 2, 3, and 4, which arose from quercetin oxidation, suggested that these compounds are either considerably less polar than the parent molecule, or are larger molecules. The formation of these products appears to have, as a requirement, the *o*-diphenol group in the B-ring, because similar products were not detected in morin solutions treated with PPO. Protocatechuic and phloroglucinol carboxylic acids, which have been demonstrated to be two of the breakdown products of POD-treated quercetin (Schreier & Miller, 1985), were not detected, even when treatments were prolonged to 10 min. PPO-catalysed morin oxidation yielded a single product (M-DP 1), and rutin oxidation apparently proceeded in a very different manner, as judged by the HPLC profile. Therefore the blockage of the 3-hydroxyl group is another significant structural feature that should be considered.

Incubation of rutin with PPO resulted in oxidative phenomena which, however, were not manifested in spectral changes, since UV–vis examinations did not show detectable modifications in the rutin spectrum. Thus, previous studies, based on spectrophotometry, which failed to demonstrate PPO action on flavonol glycosides (Baruah & Swain, 1959), may not provide accurate data with respect to PPO–flavonol glycoside interactions. Oxidation of rutin was demonstrated only when PPO-treated samples were analysed by HPLC. Other HPLC investigations on model systems have also shown PPO-mediated degradation of various flavonol glycosides (Cano, De Ancos, & Monreal, 1994; Finger, 1994). Characteristic quercetin oxidation products, such as Q-DP 2, 3, and 4, were not detected. This finding constitutes clear evidence that, in addition to the catechol moiety, the availability of the 3-hydroxyl group is necessary for their formation. It is clear, therefore, that both B- and C-ring are involved in the mechanism(s). The fact that typical degradation products, such as protocatechuic and phloroglucinol carboxylic acids, were not found in rutin solutions, suggests that cleavage of the flavonol skeleton did not occur.

4.2. POD-catalysed oxidation

POD-catalysed oxidation of phenolic compounds may also give rise to either quinones, as in the case of PPO, or other polymerised products (Matheis & Whitaker, 1984; Vámos-Vigyázó, 1981). Quercetin oxidations performed with POD have been demonstrated to result in cleavage of the flavonol skeleton, giving protocatechuic and phloroglucinol carboxylic acids, as well as dimers and trimers (Schreier & Miller, 1985). Likewise, morin oxidation, with horseradish POD, was shown to yield β-resorcylic acid (Schreiber, 1974), and kaempferol *p*-hydroxybenzoic and phloroglucinol carboxylic acids (Miller & Schreier, 1985).

Nevertheless, POD action does not always result in decomposition of the flavonol skeleton. Crude preparations from *Cicer arietinum* L. have been reported to convert flavonols, such as kaempferol, quercetin and morin, into the corresponding 2,3-dihydroxyflavanones (Hösel & Barz, 1972). Horseradish POD, and POD from *Mentha piperita* plants and *Mentha arvensis* cell suspension cultures, were shown to perform the same reaction (Barz, 1977; Frey-Schröder & Barz, 1979). Transformations with preparations originating from the latter two plants were found to be H_2O_2-independent, but O_2-dependent. Also, incubation of quercetin with a crude enzyme preparation from red clover (*Trifolium*

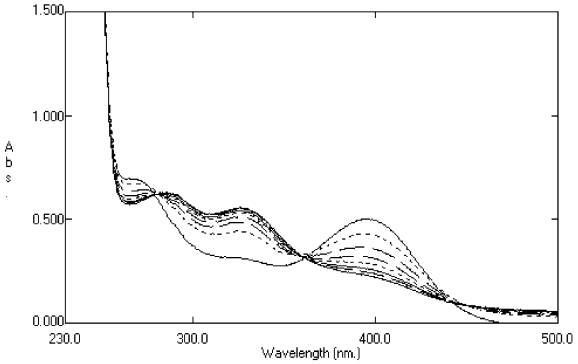

Fig. 3. Oxidation of morin (0.1 mM) with mushroom tyrosinase (polyphenoloxidase, PPO), as shown by UV–vis spectroscopy. Scans were performed at 120-s intervals for up to 12 min. Arrows indicate increases and decreases in absorbance.

pratense L.) resulted in the formation of a dihydroxy derivative (Igarashi & Akai, 1990).

Flavonol glycosides may also be attacked by POD. This has been observed for rutin and horseradish POD (Schreiber, 1974), rutin, robinin and *Vicia faba* POD (Takahama & Egashira, 1991), quercetin 3-arabinoside, quercitrin, myricitrin and grape POD (Morales, Pedreño, Muñoz, Ros Barceló, & Calderón, 1993), flavonol glycosides and horseradish POD (Finger, 1994), and quercetin 4′-glucoside and onion POD (Hirota, Shimoda, & Takahama, 1998). In fact, oxidation of rutin, leading to sufficient amounts of degradation products, was achieved using 100-fold higher enzyme activity than that used for quercetin and morin oxidation. This finding may be evidence that, although flavonol glycosides may be attacked by POD, they are not ideal POD

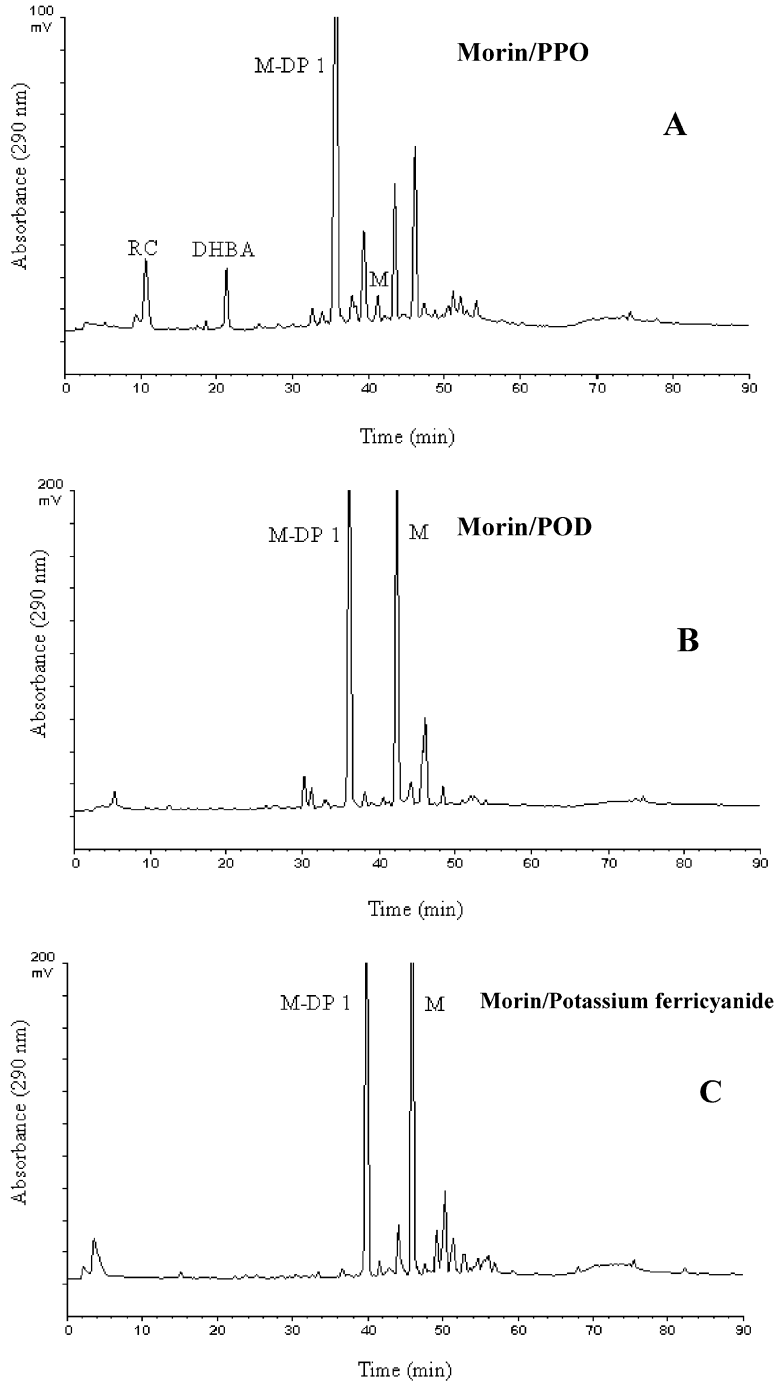

Fig. 4. HPLC profiles of morin solutions treated with (A) polyphenoloxidase (PPO), (B) peroxidase (POD) and (C) with potassium ferricyanide. Monitoring was performed at 290 nm. M, Morin, M-DP 1, Morin oxidation product.

substrates. A similar phenomenon, however, was not observed for PPO.

POD-treatment of quercetin, morin, and rutin did not give rise to characteristic benzoic acids, under the conditions employed. No protocatechuic, β-resorcylic or phloroglucinol carboxylic acids were detected in the reactions mixtures, even after incubations exceeding 10 min. The detection of relatively small amounts of Q-DP 1 may evidence that this product is formed either to a lesser extent, or represents an intermediate and does not accumulate. This hypothesis does not hold true for morin, because its oxidation has been shown to lead to a single more polar product (M-DP 1). It appears, therefore, that the *o*-diphenol structure in quercetin is a key feature with respect to the formation of Q-DP 2, 3, and 4. This finding enhances the assumption that Q-DP 1 is

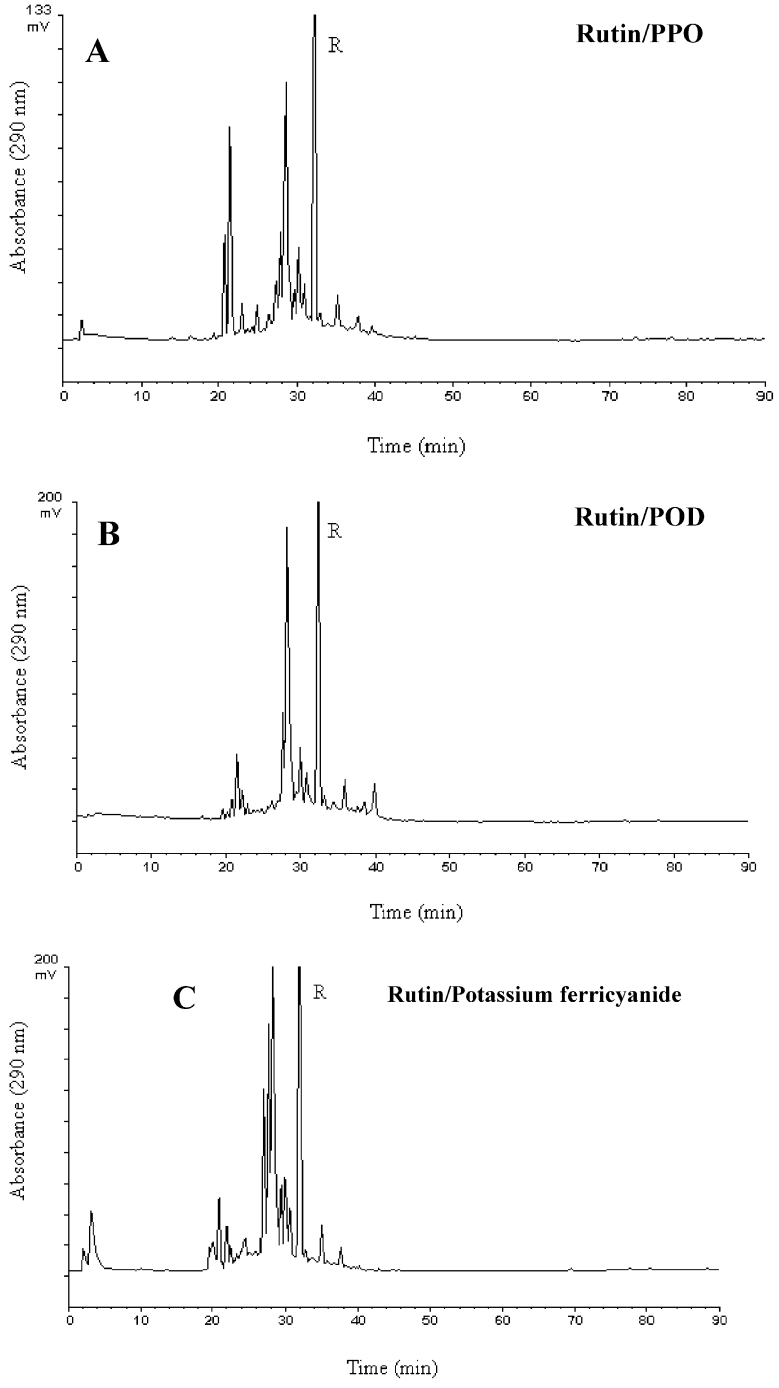

Fig. 5. HPLC profiles of rutin solutions treated with (A) polyphenoloxidase (PPO), (B) peroxidase (POD) and (C) with potassium ferricyanide. Monitoring was performed at 290 nm. R, Rutin.

rather an intermediate, and may be further transformed yielding other less polar compounds.

In all cases, POD oxidation was found to yield products which were found in PPO-treated mixtures. That is, quercetin oxidation gave rise to Q-DP 2, 3, and 4, and morin to M-DP 1, while the HPLC trace of POD-treated rutin solutions showed many similarities to the corresponding ones treated with PPO. The finding that PPO and POD treatments of quercetin yield the same oxidation products indicates that horseradish POD may act with the same mechanism(s) which, as mentioned earlier, depends on the B-ring structure and the availability of the 3-hydroxyl group.

4.3. Potassium ferricyanide oxidation

Early studies indicated that a strict requirement for oxidation of some chalcone derivatives by potassium ferricyanide is the presence of a hydroxyl group in the B-ring (Dean & Podimuang, 1965). Oxidation of various flavonoids with potassium ferricyanide (Pelter et al., 1971) further confirmed that only the B-ring is acted upon, and no reaction takes place if hydroxyl groups on the B-ring are methylated. These observations strongly suggest that modifications in the B-ring of the flavonoid skeleton are primarily involved in the oxidation mechanism.

Potassium ferricyanide has been used for model oxidations of mixtures of gallic acid and (−)-epicatechin-3-*O*-gallate (ECG) to produce a benzotropolone derivative similar to those occurring in black (fermented) tea (Bailey, Nursten, & McDowell, 1993). The structural elucidation of such a derivative, termed theaflavate A, generated from ECG oxidation by potassium ferricyanide and isolated from tea (Wan, Nursten, Cai, Davis, Wilkins, & Davies, 1997), provided sound evidence that potassium ferricyanide mimics oxidations performed during tea fermentation, which are catalysed by PPO and POD (Finger, 1994).

On the basis of the earlier considerations, it would be reasonable to presume that potassium ferricyanide-mediated oxidation of quercetin proceeds as does PPO and POD. Hence it is evident that oxidation mechanism(s) involve(s) formation of *o*-quinone in the B-ring, but the 3-hydroxyl group also appears to have a crucial contribution, as indicated from PPO and POD rutin oxidation, and the spectral changes that occurred in potassium ferricyanide/quercetin mixtures. The finding that both potassium ferricyanide and PPO/POD-catalysed reactions yielded Q-DP 2, 3, and 4 strongly supports such an assumption. Also, the fact that treatment of both morin and rutin with potassium ferricyanide generated products deriving from PPO/POD oxidation is a further corroboration that this particular oxidising agent mimics the action of mushroom PPO and horseradish POD on flavonols.

5. Conclusions

The oxidation of quercetin, morin, and rutin with horseradish POD and mushroom PPO showed that, in every case, oxidation resulted in the formation of the same products, giving very similar chromatographic profiles. The structural differences arising from the substitution pattern of the B-ring in quercetin and morin are very crucial with respect to oxidation product formation. The availability of the 3-hydroxyl group is another significant factor in this respect. Flavonol glycosides structurally related to rutin may be acted upon, but to a lesser extent than aglycones, such as quercetin and morin. Oxidations, performed using potassium ferricyanide, simulate enzymic oxidations, and this may be of importance in using chemical means for producing specific flavonol derivatives/products. Currently, work is in progress to isolate and identify some of the quercetin oxidation products. It is believed that the structural elucidation of those compounds will further aid in establishing pathways of quercetin oxidation.

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