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Exploiting UPO versatility to transform rutin in more soluble and bioactive products

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ABSTRACT

The discovery of unspecific peroxygenases (UPOs) completely changed the paradigm of enzyme-based oxyfunctionalization reactions, as these enzymes can transform a wide variety of substrates with a relatively simple reaction mechanism. The fact that UPO can exert both peroxygenative and peroxidative activity in either aromatic or aliphatic carbons, represents a great potential in the production of high value-added products from natural antioxidants. In this work, the flavonoid rutin has been considered as possible substrate for UPO from *Agrocybe aegerita*, and its peroxygenation or its peroxidation and successive oligomerization have been studied. Different experiments were performed in order to reduce the range of process variables involved and gaining insight on the behavior of this enzyme, leading to a multivariable optimization of UPO-based rutin modification. While trying to preserve enzyme activity this optimization aimed for maximizing the production of more soluble antioxidants. Reusability of the enzyme was evaluated recovering UPO using an enzymatic membrane reactor, revealing challenges in enzyme stability due to inactivation during the filtration stages. The influence of the radical scavenger ascorbic acid on product formation was investigated, revealing its role in directing the reaction towards hydroxylated rutin derivatives, hence indicating a shift towards more soluble and bioactive products.

Introduction

Among the different natural antioxidant compounds present in nature, rutin ($C_{27}H_{30}O_{16}$) has been the target of numerous intensive researches, attracting the keen attention of pharmaceutical, cosmetic, nutraceutical and food industries in the last decades, mainly due to its interesting and varied array of pharmacological properties, from antioxidant activity to anti-SARS-CoV-2 capacity, also accounting for anti-osteoporotic, anti-diabetic, antimicrobial, anti-inflammatory, gastroprotective activities, etc [1]. Moreover, the inclusion of natural antioxidants such as rutin in the formulation of products is often more easily accepted by consumers rather than synthetic ones, given that those substances are already present in our daily diet. These facts, together with the huge potential of obtaining natural antioxidants from agroforestry and food industry wastes following biorefinery strategies based on the concept of circular economy has led to an exponential increase in the demand of these type of antioxidants in the last decades [2]. In the case of rutin, this substance has successfully been recovered from a variety of food and agroforestry-related residues: buckwheat, asparagus, apple, tomato, grapes, black tea, eucalyptus leaves, etc [1].

However, rutin, alongside with most polyphenolic natural antioxidants, displays poor physicochemical properties, namely scarce solubility in organic non-toxic, lipidic and aqueous solvents, which limits its bioavailability while also increasing difficulty in handling, low shelf life when administered, reduced stability against environmental (light, temperature, pH, etc.) and processing stresses (sterilization, drying, etc.). For these reasons, several techniques for enhancing the solubility and bioavailability of polyphenolic compounds have been extensively researched over the last decades, from the inclusion of polyphenolic antioxidants in structures or complexation with other substances [3] to their chemical or biochemical transformation into derivatives with enhanced properties [4]. Chemical hydroxyethylation of rutin deserves a special mention, since the produced mono hydroxyethyl and tri-hydroxyethyl rutin (also known as troxerutin) are already marketed under different brands (Relvene®, Paroven®, Venoruton®, Varemoid®, etc.) for the treatment of venous insufficiency [1]. The hydroxyethylation with ethylene oxide is conducted in solvents (water, methanol or ethanol) at high temperatures and under the presence of alkaline metals, ammonium water, sodium hydroxide or pyridine as catalysts, therefore implying additional downstream purification processes in

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order to prevent toxic compounds in the final composition of the commercialized product [4,5].

Enzyme-catalyzed modification of natural antioxidants presents the advantages of being more chemo-, regio-, and stereoselective than conventional chemical modifications, while catalyzing such reactions at milder conditions, which, given the reduced stability of some polyphenolic substrates, positively affects the outcome of the reaction. Indeed, the enzyme-catalyzed polymerization of rutin by laccases and peroxidases has been demonstrated as a successful process to produce rutin derivatives with highly increased aqueous solubility and interesting bioactive properties [6,7]. However, transformation products of laccase catalyzed oligomerization of rutin may show decreased biological activities and stability, particularly when using high concentration of enzyme [7]. Their low antioxidant activity was correlated with a decreased phenolic content, which could be attributed to the formation of several bonds between rutin molecules.

Fungal unspecific peroxygenases (UPOs) are considered one of the most promising biocatalysts in synthetic chemistry due to their capability of catalyzing C-H oxyfunctionalization reactions [8]. They can perform both peroxygenative reactions (insertion of one oxygen atom through a two-electron oxidation) and peroxidative reactions (formation of phenoxy radicals through one-electron oxidation). Using H_2O_2 as oxygen donor and electron acceptor, UPO can catalyze the transformation of more than 400 substrates up to date, comprising from aliphatic and aromatic hydroxylation, C-C bond cleavage, O-dealkylation, epoxidation of double bonds, etc [9]. Since the products of the peroxygenative activity of UPO on aromatic carbons can become a suitable substrate for UPO peroxidative activity, it can lead to the formation of phenoxyl radicals and their successive oxidative coupling. Aiming to prevent such unwanted oligomerization, several works have reported the use of radical scavengers such as ascorbic acid [10], which would scavenge phenoxyl radicals generated by peroxidative activity meanwhile ascorbic acid is reversibly oxidized into dehydroascorbic acid and can be further irreversibly hydrolyzed to 2,3-diketogulonic acid [11]. Concerning the modification of rutin, in contrast to laccases or peroxidases, the use of UPO as biocatalyst with both peroxygenative and peroxidative activities opens up the possibility of obtaining new products with enhanced biological and/or physicochemical properties. Rutin oligomers produced by laccases or peroxidases have higher solubility, and hydroxylated rutin oligomers produced by UPO may have also increased antioxidant activity. To the best of our knowledge, there is only one previous work exploring the use of UPO to catalyze the transformation of different natural polyphenolics into mainly monohydroxylated derivatives, preventing the further oxidative coupling reaction by supplying ascorbic acid as radical scavenger to the reaction medium [12]. However, according to this work, UPO did not oxidize rutin, and the effect of the absence or presence of ascorbic acid upon the reaction was not studied, since all experiments were performed using the radical scavenger.

Therefore, in this work, we propose to take advantage not only of the peroxygenase activity but also of the peroxidase-like activity of UPO (from *Agrocybe aegerita*, PaDa-I mutant), which could lead to the oligomerization of natural antioxidants into products with higher solubility but also with increased phenolic groups, due to the oxygen transfer activity. Optimization of UPO initial activity, H_2O_2 addition rate and the reaction time were considered in order to optimize UPO stability while allowing the transformation to more soluble products with high antioxidant activity. Under optimal conditions, reusability of UPO was evaluated by operating for the first time an enzymatic membrane reactor in several cycles. Moreover, products from the reactions in absence (expectingly leading to the formation of oligomers) and presence of ascorbic acid (expectingly leading to hydroxylated compounds) at different concentrations were synthesized and compared by MALDI-TOF to select the most promising rutin derivatives towards their use on the food, nutraceutical, pharmaceutical and/or cosmetic industries.

Materials and methods

Chemicals and enzymes

The recombinant UPO (1.11.2.1) from *A. aegerita* (*Aae*UPO) expressed in *Pichia pastoris* (PaDa-I mutant) was purchased from EvoEnzyme (UPO23). 2,2′-azino-bis(3-ethylbenzenothiazoline-6-sulfonic acid) diammonium salt (ABTS, ≥ 98 %), iron (III) chloride hexahydrate (≥99 %), 2,4,6-tri(2-pyridyl)−1,3,5-triazine (TPTZ, ≥ 99 %), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, ≥ 97 %) were purchased from Sigma. 2,6-dimethoxyphenol (2,6-DMP, 99 %) was purchased from Fluka.

UPO activity

UPO activity was measured attending to two different spectrophotometric protocols using a Shimadzu UV-1800. ABTS was first considered as substrate to measure UPO peroxidative activity by monitoring the oxidation rate of 0.3 mM ABTS in citrate-phosphate buffer (100 mM pH 4.0) to its cation radical ($ABTS^{\cdot+}$) at 418 nm (ε 418 = 36,000 $M^{-1}\cdot cm^{-1}$) in the presence of 2 mM H_2O_2 at room temperature. One unit (U) of activity was defined as the amount of enzyme forming 1 μmol·min^{-1} of $ABTS^{\cdot+}$. The blue-green $ABTS^{\cdot+}$ radical can be reduced by hydrogen-donating antioxidants. So, in the presence of transformation products, UPO activity may be underestimated due to their radical scavenging activity. Hence, activity was also determined by measuring the oxidation of 3 mM 2,6-DMP to 2,2′,6,6′,-tetramethoxydibenzo-1,1′-diquinone at 469 nm (ε 469 = 27,500 $M^{-1}\cdot cm^{-1}$) caused by the enzyme under the presence of 1 mM H_2O_2 in phosphate buffer (pH 6.0 100 mM) at room temperature. To assess the effect of transformation product on activities measured with ABTS substrate, activity values were compared using also DMP after removing the transformation products from the medium by ultrafiltration (10 kDa). The experiment was performed with low and high concentration of transformation products (37 % and 99 % conversion, respectively (Figure SM1).

Rutin conversion

Rutin concentration was monitored through high performance liquid chromatography (HPLC). The analyses were performed on a Jasco XLC HPLC (Jasco Analitica) equipped with a 3110 MD diode array detector (detection at 355 nm) and a Gemini reversed-phase column (150 ×4.6 mm, particle size: 3 μm) maintained at 45 °C (Phenomenex, supplied by Jasco Analitica). Gradient elution (flow rate of 0.7 mL·min^{-1}) started with 10 % acetonitrile (CAN, v:v) in water (2 % acetic acid), increased to 90 % ACN within 8 min, and then decreased back to the initial concentration after 2 min

Ferric reducing antioxidant power (FRAP)

FRAP of obtained products (full samples including insoluble compounds – $FRAP_F$, and supernatants with only soluble compounds– $FRAP_S$) was conducted by adding 0.1 mL of each sample to 3 mL of FRAP reagent (acetate buffer, 300 mM, pH 3.6; 10 mM of TPTZ in 40 mM HCl; 20 mM of $FeCl_3\cdot 6\ H_2O$, chemicals added with a ratio of 10:1:1). The absorbance was recorded after 6 min at 593 nm.

Effect of pH and H_2O_2 feeding strategy

Citrate-phosphate buffers 100 mM with pH 7.0 and 4.0 were chosen as reaction medium to assess the effect of pH over the enzymatic modification of 5 g·L^{-1} rutin catalyzed by 10,000 U·L^{-1} (ABTS-related units), and the addition of 500 μM·h^{-1} H_2O_2 and 1000 μM·h^{-1} AA was considered, providing these compounds in one pulse per hour. Reaction controls with no UPO, but with H_2O_2 and AA supply were performed. Due to the low solubility of rutin in aqueous medium (0.13 g·L^{-1}),[1]

the substrate was suspended in the reaction medium and agitated at 1500 rpm and 25 ºC on a Labgene Scientific MSC-100 thermoshaker incubator. Ferric reducing antioxidant potential (FRAP) was measured after completing reaction time to both full samples ($FRAP_F$) and supernatants ($FRAP_S$) obtained after centrifugation (14,000 rpm at 25 °C using an Eppendorf 5417 R microcentrifuge), which were compared to their respective controls of reaction (correspondingly full and centrifuged) to quickly assess the increase or decrease in antioxidant activity cause by enzymatic transformation.

A first screening on enzymatic activity and hydrogen peroxide addition rate was carried out using 3 $g{\cdot}L^{-1}$ rutin dispersion in phosphate buffer pH 7.0 100 mM. Rutin UPO-catalyzed transformation was performed at 25 °C for 5 h in 12 mL polypropylene test tubes with a threaded cap, however only filling 5 mL of these tubes to provide proper rotatory agitation leading to the homogeneous dispersion of the substrate in the reaction medium. This agitation was provided by operating a Multi Bio RS-24 agitator (BioSan) at 50 rpm. Catalytic activity was defined by means of ABTS-related units, and 500 and 10,000 $U{\cdot}L^{-1}$ were selected, together with H_2O_2 supply rates in the range 0.01 - 5.00 $mM{\cdot}h^{-1}$, provided in pulses, leading to different combinations of these two variables, these values being selected based on literature [10,12]. Rutin conversion (RX, %) was followed through HPLC of samples from the reaction medium (triplicates), and diluted 50 times in methanol to ensure complete solubilization.

Multivariable optimization of the reaction conditions

Experiments involving continuous supply of H_2O_2 were optimized through response surface methodology following a Box-Behnken design (BBD). Continuous feeding of hydrogen peroxide was achieved by connecting the test tubes to 1 mL Omnifix-F syringes loaded with different concentrations of H_2O_2 and placed on a Syringe TWELVE New Era syringe pump (BIOGEN) set at 52 $\mu L{\cdot}h^{-1}$ flowrate. Rotation was set at 50 rpm and programmed to spin only 90° in one direction, setting the tubes in a horizontal position in which they vibrated for 2 s, and then spinning 180° to the contrary direction and vibrating again for 2 s. This movement was repeated during the complete time of reaction to provide proper dispersion of not solubilized rutin in the reaction medium, therefore allowing correct enzyme-substrate contact.

The optimization of reaction conditions considered four dependent variables: rutin conversion (RX, %) was calculated through rutin depletion as measured by HPLC, residual relative UPO activity (rUPO, %) was spectrophotometrically monitored and FRAP of full samples ($FRAP_F$, %) and FRAP of the obtained supernatants ($FRAP_S$, %) calculated in relation to their respective controls (not containing UPO but receiving the same H_2O_2 supply). Initial UPO activities (iUPO) of 1500, 5750 and 10,000 $U{\cdot}L^{-1}$ (ABTS-related units), 0.25, 1.5 and 2.75 $mM{\cdot}h^{-1}$ H_2O_2 supply rates (H_2O_2r) and reaction time (t) of 1, 3 and 5 h, were selected as independent variables for a first approach on optimizing this operational parameters, which were combined in a three factor and three level Box-Behnken design with three replicates in the central point (15 experiments). The independent variables selected in the optimization study are summarized in Table SM1 alongside with the specific values for the fixed variables.

The relationships between reaction conditions and evaluated responses were fitted to the following second-order polynomial equation:

$$y_j = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum \sum_{i<j=1}^{3} \beta_{ij} x_i x_j + \sum_{i=1}^{3} \beta_{ii} x_i^2 \quad (1)$$

where, y is the response variable, x_i and xj are the independent variables, β_0, β_i, β_{ij}, and β_{ii} are the regression coefficients determined from the experimental data by the least-squares method. Experimental data was fitted to the proposed model equations using the regression analysis function of Microsoft Excel's Data Analysis Add-In (Microsoft 365) and the quality of the generated model was checked by the coefficient of determination (R^2), the significance of the regression coefficients and the F-test value obtained from the analysis of variance.

A multi-response optimization was performed to simultaneously maximize the studied response variables through the Derringer's desirability function using the software STATGRAPHICS Centurion XVI, (16.1.03 version). The selection criteria were established in order to retain the highest UPO activity at the end of the reaction (therefore leading to the possibility of reusing the remanent active enzyme) while maximizing the transformation of rutin into products (high rutin conversion) but focusing the attention on those products with the more enhanced antioxidant performance (determined by FRAP assay methods). To validate the model, three experiments were conducted under the optimal reaction conditions, and the dependent variables measured were compared to those predicted by the model.

Enzymatic membrane reactor

The production of oligorutin allowing the reuse of the biocatalyst in successive reaction-separation cycles was performed, considering reaction steps of 1.87 h. A 10 mL Amicon Stirred Cell coupled to an ultrafiltration membrane with 10 kDa nominal pore size was used as an enzymatic membrane reactor (EMR), since PaDa-I UPO, the evolved variant from *A. aegerita* showed a molecular weight of 51.1 kDa [13].

The standard procedure reported by the supplier of the Amicon Stirred Cell was followed. As schematized in Figure SM2, each complete cycle comprised three different steps: (i) charge of substrate, (ii) enzymatic reaction with continuous feeding of H_2O_2 and (iii) discharge of products while retaining the enzyme. In the charge stage, phosphate buffer 100 mM pH 7.0 with 15 mg rutin were loaded to reach 5 mL of reaction media and 3 $g{\cdot}L^{-1}$ rutin. First cycle included enzyme charge with an initial enzymatic activity of 7546 $U{\cdot}L^{-1}$. The enzymatic transformation step took place under magnetic stirring to provide good enzyme-substrate contact and the continuous supply of H_2O_2 at 2.75 $mM{\cdot}h^{-1}$ using a TWELVE New Era syringe pump (BIOGEN) set at 52 $\mu L{\cdot}h^{-1}$ flowrate. After completing the reaction time, the separation of the soluble products and the retention of the enzyme was performed by using N_2(g) to create overpressure (1 bar) inside the reactor, favoring the filtering of the reaction media, thus promoting the pass of the transformation products (4 mL) through the membrane while retaining a small fraction of the reaction media enriched with the enzyme (1 mL) and the unreacted solid rutin inside of the reactor. Filtration step lasted 30–35 min. The retained fraction was then re-used in a new charge stage, when rutin (15 mg) and phosphate buffer 100 mM pH 7.0 (4 mL) were provided, thus starting a new successive reaction-separation cycle catalyzed by the activity remaining from the previous reaction. Therefore, the initial rutin concentration of successive cycles would always be at least 3 $g{\cdot}L^{-1}$, reflecting higher values if complete rutin conversion were not achieved in the previous cycle. Three consecutive reaction-separation cycles were performed to evaluate the re-use of UPO, following the conversion of rutin over time and enzyme activity drops during both the reaction and filtering steps. Permeate products (soluble products) were characterized on terms of antioxidant power (FRAP assay).

UPO-based modification of rutin in presence of ascorbic acid (AA)

Experiments evaluating the effect of different concentrations of AA on the UPO transformation of rutin were performed as described previously but with the initial addition of the radical scavenger at 5.5, 1.4 and 0.7 mM. Controls lacking UPO but in the presence of all other components were also carried out.

MALDI-TOF analysis was performed on an Ultraflex III TOF/TOF mass spectrometer equipped with a Smartbeam laser (Bruker Daltonics) in reflectron operation mode and negative polarity. The lyophilized samples were dissolved (1 g·L-1) in ACN:water (30:70, v:v) with 0.1 % TFA. Sample solutions were mixed with DHB at a 1:1 ratio. Then, 1 μL

was spotted on the MALDI plate and crystallized at room temperature. The acceleration voltage was set to 25 kV and a total of 1200 laser shots per spot were automatically acquired. Data acquisition and data processing were performed by the Flex Analysis software (Bruker Daltonics). Sample controls (lacking UPO) were also analyzed. The relative intensities were calculated based on the sum of the intensities of all peaks present in the transformation products but absent in the respective controls.

Results

Selection of pH and hydrogen peroxide feeding strategy

PaDa-I UPO, with slightly improved peroxygenase activity compared to wild type UPO [14], has been widely used as a model enzyme for multiple applications [15]. The pH profile of this evolved variant from *A. aegerita* shows optimum pH values of 4 for peroxidative activity (with ABTS) and pH 7 for peroxygenative activity (with NBD, veratryl alcohol, benzyl alcohol, naphthalene, etc) [13–15]. Therefore, these two pHs were preliminary evaluated for the enzymatic transformation of rutin to more soluble products with antioxidant activity (Fig. 1A). Reaction with UPO at pH 7 led to a decrease of the antioxidant activity of the suspension (14 %) but it promoted the solubilization of antioxidants, since the supernatant antioxidant activity increased 1.77-fold. Interestingly, at pH 4 the solubilization of antioxidants was remarkable (99.8 mg TE·L^{-1} in the supernatant), and barely affected the activity of the full sample.

Ascorbic acid is one of the strongest reductants and radical scavengers and is generally used in presence of UPO to prevent or at least decrease peroxidative activity [10]. However, AA also causes the reduction of ferric to ferrous iron, so the antioxidant activity measured by FRAP assay is affected (Fig. 1B). In this sense, the presence of AA increased the antioxidant activity, compared with the controls lacking AA. When combined UPO and AA, the antioxidant activity decreased both in the full sample and in the supernatant, indicating that peroxidative activity is necessary to produce soluble antioxidant compounds.

A pH of 7 was selected because of the highest solubility of the products, observed by the increase of the antioxidant activity of the supernatant in absence of AA. Furthermore, it has been described that at pH 4 the damaging effects of H_2O_2 on *Aae*UPO were more pronounced than at the optimal pH 7 [16].

Next step was to select enzyme initial activity and H_2O_2 stepwise addition. For this, different experiments were performed evaluating initial activities in the range of 500 to 10,000 U·L^{-1} and H_2O_2 addition rates from 0.01 to 5 mM·h^{-1} (pulses each hour) (Table SM2). The main conclusion was that low rutin conversions were achieved (<40 %), probably related to the suicide-inactivation of UPO by excess of H_2O_2. UPO can catalyze H_2O_2 dismutation via a catalase-like reaction which tends to cause enzyme malfunction in presence of excess H_2O_2 concentration, leading to both higher consumption of this co-substrate and enzyme inactivation [16]. To avoid such drawbacks, different strategies for H_2O_2 gentle supply have been studied in order to guarantee the presence of this co-substrate at low concentrations, from *ex situ* methods (stepwise addition by pipette or syringe pump systems) to *in situ* strategies (H_2O_2 generation by an oxidase, generation by illuminated flavins and electrochemical H_2O_2 generation by gas diffusion electrode). In order to enhance transformation rates, H_2O_2 feeding was changed to a continuous supply. In this sense, when 10,000 U·L^{-1} were used, and 0.5 mM·h^{-1} was added in continuous mode, the conversion was increased to 52 % (instead of 35.7 % when stepwise addition was used).

Furthermore, it was observed that transformation products present at high concentrations affected the measurement of enzymatic activity by ABTS method, underestimating the value of UPO activity (Figure SM1). Hence, residual enzymatic activity values were determined using DMP as substrate.

Multivariable optimization and validation of the model

The combined RSM and BBD were used to optimize the values for the different UPO-catalyzed rutin modification conditions. Table 1 shows the ranges of values studied for each independent variable (initial UPO activity, H_2O_2 feeding rate and reaction time), alongside with the specific values for the fixed variables (rotatory speed: 50 rpm, substrate initial concentration: 3 g·L^{-1} rutin, H_2O_2 flow: 52 μL·h^{-1}, and temperature: 25 ± 2 °C). The reaction conditions were optimized attending to four dependent variables: residual UPO activity (rUPO) measured at the end of the experiment, rutin conversion (RX) in percentage, FRAP of full samples ($FRAP_F$) and FRAP of the obtained supernatants ($FRAP_S$) expressed as percentages compared to their respective controls.

Table 1 shows the Box-Behnken Design matrix of 15 experiments, indicating the experimental values obtained for the dependent variables under different combinations of the independent variables. Low rutin conversions (< 25 %) were achieved with the lowest initial activity, indicating that a minimum UPO activity is required for efficient transformation. Additionally, lowest hydrogen peroxide rates led to lowest conversions (< 20 %), independently of the initial activity and reaction time. Finally, reactions longer than 1 h are required to achieve conversions higher than 34 %. Only three experiments led to conversions higher than 70 % and were reached for the highest H_2O_2-UPO (exp 15) or highest H_2O_2-intermedium UPO (exp 11) or intermedium H_2O_2-

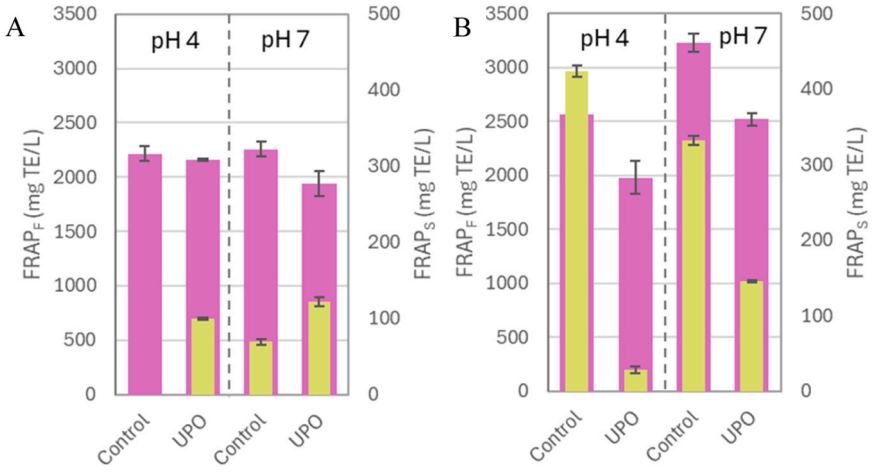

Fig. 1. Ferric reducing antioxidant potential (FRAP) of full samples ($FRAP_F$) and and supernatants ($FRAP_S$), expressed as mg TE·L^{-1}, of controls (absence of UPO) and enzymatically treated samples. A) Absence of the radical scavenger ascorbic acid, and B) in presence of ascorbic acid.

Table 1
Operational conditions assayed and experimental results obtained for dependent variables y_1 to y_4.

Exp.	Independent variables			Dependent variables			
	Initial UPO activity ($U{\cdot}L^{-1}$)	H_2O_2 rate ($mM{\cdot}h^{-1}$)	t (h)	rUPO or y_1 (%)	RX or y_2 (%)	$FRAP_F$ or y_3 (%)	$FRAP_S$ or y_4 (%)
1	1500	0.25	3	65.9 ± 5.6	11.8 ± 0.3	108.4 ± 16.7	230.8 ± 8.2
2	1500	1.5	1	89.4 ± 3.0	18.4 ± 0.5	101.7 ± 17.1	286.4 ± 23.6
3	1500	1.5	5	0.0 ± 0.0	24.5 ± 0.6	100.8 ± 2.8	276.2 ± 6.2
4	1500	2.75	3	0.0 ± 0.0	23.0 ± 1.5	121.2 ± 12.4	299.2 ± 3.2
5	5750	0.25	1	109.1 ± 6.6	11.8 ± 0.5	132.4 ± 4.2	159.2 ± 3.2
6	5750	0.25	5	65.6 ± 2.1	16.8 ± 0.5	104.2 ± 0.8	274.3 ± 14.2
7	5750	1.5	3	50.9 ± 4.2	44.9 ± 1.0	104.0 ± 3.6	565.7 ± 38.4
8	5750	1.5	3	52.6 ± 0.8	40.1 ± 1.3	102.0 ± 4.5	579.8 ± 11.7
9	5750	1.5	3	56.5 ± 5.4	46.1 ± 2.2	89.7 ± 11.4	573.4 ± 12.9
10	5750	2.75	1	91.3 ± 6.6	33.7 ± 1.0	112.0 ± 3.0	437.2 ± 4.4
11	5750	2.75	5	1.8 ± 0.2	77.1 ± 4.1	88.4 ± 12.7	648.7 ± 32.8
12	10,000	0.25	3	83.5 ± 4.2	12.3 ± 0.6	113.5 ± 15.5	233.6 ± 0.7
13	10,000	1.5	1	96.4 ± 10.0	20.2 ± 1.0	108.9 ± 18.0	313.6 ± 19.3
14	10,000	1.5	5	48.7 ± 1.1	71.4 ± 2.5	85.6 ± 0.3	730.3 ± 1.9
15	10,000	2.75	3	52.5 ± 3.2	74.7 ± 4.6	77.1 ± 10.6	776.4 ± 4.6

highest UPO and a reaction time of 5 h (exp 14).

Complete inactivation of enzyme was observed for experiments with the lowest initial activity and highest/medium H_2O_2 rate. This was probably the reason why a minimum of activity is required for efficient transformation. With the highest initial activity, it was ensured that the enzyme was active during the transformation experiments.

Antioxidant activity of the suspension either increased or decreased. The experiments where $FRAP_F$ diminished were related to those with maximum rutin conversions. On the other hand, antioxidant activity of the supernatant increased in all experiments, regardless of the rutin conversion. Interestingly, the experiments with a decreased $FRAP_F$ also produced the highest increase in the antioxidant activity measured in the supernatant (573–776 %).

A second order polynomial equation was obtained for each dependent variable through multiple regression, being their fit to experimental values assessed through the analysis of variance (ANOVA), obtaining the regression coefficients for each dependent variable, the determination coefficients (R^2, between 0.875 and 0.998) and the statistical significances (based on Student's t-test and Fisher's F test), being all this data collected in Table 2.

According to the model, residual UPO was positively affected by the initial activity, but negatively affected by the concentration of hydrogen peroxide and particularly the reaction time. Maximum values of rUPO were obtained with higher iUPO combined with shorter reaction times. The quadratic effect of reaction time was positive, whereas the quadratic effect of initial activity was negative. Regarding the conversion, it was positively affected by all the parameters, but interestingly, the effect was negative for the quadratic terms. Similar response surface was obtained for $FRAP_S$, since the mathematical operators of each coefficient were coincident with those of rutin conversion. On the contrary, the coefficients of $FRAP_F$ showed opposite signs, indicating that those

Table 2
Regression coefficients and statistical parameters measuring the correlation and significance of the model.

Coefficient	rUPO or y_1 (%)	RX or y_2 (%)	$FRAP_F$ or y_3 (%)	$FRAP_S$ or y_4 (%)
Intercept (b_0)	53.34[a]	43.71[a]	98.57[a]	558.13[a]
iUPO (b_1)	15.73[a]	11.06[a]	−5.87	117.95[a]
H_2O_2r (b_2)	−22.30[a]	21.02[a]	−7.47[c]	153.69[a]
t (b_3)	−33.75[a]	13.20[a]	−9.50[b]	86.81[a]
iUPO·H_2O_2r (b_{12})	8.75[a]	15.86[a]	−12.32[b]	118.80[a]
iUPO·t (b_{13})	10.43[a]	11.28[a]	−5.61	106.71[a]
H_2O_2r·t (b_{23})	−11.52[a]	9.60[a]	1.16	14.46
$iUPO^2$ (b_{11})	−5.61[b]	−8.76[a]	−1.76	−69.10[a]
$H_2O_2r^2$ (b_{22})	2.76	−7.57[a]	8.24	−102.96[a]
t^2 (b_{33})	10.88[a]	−1.31	2.43	−78.40[a]
R^2	0.996	0.995	0.875	0.998
F-exp	128.392	110.899	3.876	236.321
Significance level (%)	99.998	99.997	92.508	100.000

Superscripts indicates the significance of the coefficients:
[a] at the 99 %,
[b] 95 %,
[c] or 90 % confidence level

conditions leading to high rutin conversion and high antioxidant activity of the supernatant, also led to reduced values of antioxidant activity of the suspension.

Given the contrary effects of independent variables over response surfaces, a compromise solution was searched through simultaneous multivariable optimization. Hence, the desirability function was applied to perform the multivariable optimization with the goal of obtaining the highest rutin conversion implying the lowest UPO activity loss, at the same time increasing the FRAP of the supernatants while trying to preserve it in full samples. The reaction conditions that led to the optimum results attending to this objective were: 7546 $U{\cdot}L^{-1}$ iUPO activity, 2.75 $mM{\cdot}h^{-1}$ H_2O_2r and 1.87 h t, while the individual optimization of each dependent variable reported different values (Table 3).

The validation of the model was assessed by carrying out three experiments on the optimum reaction conditions. Table 4 presents the predicted and experimental values for all dependent variables under study. Errors $\leq$ 15 % show that there is a good relation between predicted and experimental values. The antioxidant activity of the full sample led to the highest error (15 %), and as observed previously, the higher the reduction of $FRAP_F$, the higher the increase of $FRAP_S$.

Potential of reusing UPO in multiple cycles

Despite usually implying mild process conditions, the use of enzymes currently represent one of the highest costs associated to biocatalyzed processes. Immobilization techniques allow enzyme reuse and, in some cases, enhance their relative catalytic activity [17]. However, reuse of immobilized UPO is not straightforward and needs further research. As for instance, no reuse of UPO immobilized in activated carriers was possible as consequence of its inactivation after two cycles of reaction [18]. On the other hand, repeated fed-batch operation was possible for 5 or 6 cycles, but partially maintaining the activity [19] [20]. Hence, the use of an ultrafiltration enzymatic membrane reactor (UF-EMR) with a molecular weight cut-off (MWCO) ensuring the complete retention of

Table 3
Values obtained for the individual optimization of dependent variables and the multivariable optimization.

Optimized variable	iUPO ($U{\cdot}L^{-1}$)	H_2O_2r ($mM{\cdot}h^{-1}$)	t (h)
rUPO	4442	0.25	1.00
RX	10,000	2.75	4.93
$FRAP_F$	9814	0.25	1.00
$FRAP_S$	10,000	2.75	5.00
All variables	7546	2.75	1.87

Table 4
Predicted and experimental values under the optimum conditions based on the simultaneous optimization of all response variables evaluated.

	rUPO or y1 (%)	RX or y2 (%)	$FRAP_F$ or y3 (%)	$FRAP_S$ or y4 (%)
Predicted value	69.64	50.14	98.15	593.37
Experimental value*	70.6 ± 3.6	50.2 ± 3.2	85.4 ± 2	627.7 ± 6.2
% error	*1.36*	*0.19*	*15.00*	*5.47*

* Mean and standard deviation of triplicate experiments at the optimal conditions

freely dispersed enzyme, would allow the reuse of free enzyme in successive synthesis cycles without compromising enzyme activity. This technology has already being demonstrated an interesting alternative to perform the enzyme-catalyzed oligomerization of rutin using laccase from *Trametes versicolor*, obtaining good substrate conversion and enzyme reusability results [6]. To our knowledge, the recovery of UPO by ultrafiltration and its reuse in several cycles has not been addressed before.

The viability of reusing UPO in several cycles was tested in a stirred cell coupled to an UF membrane. After the reaction time was completed, solubilized products were removed from the reaction medium using the 10 kDa UF membrane, while maintaining UPO and non-reacted rutin in the cell. Rutin conversion in the first cycle reached 48.8 ± 3.9 % and residual UPO was 72.5 ± 3.3 (Fig. 2, Table 5), similar values to those obtained in the validation experiment, despite the different reactor configuration used. However, since the initial UPO activity in cycle 2 was lower, the conversion was also lower, which was even more evident in the last cycle. Hence, this strategy seems not to be feasible for UPO reuse in multiple cycles. The poor stability of the enzyme, combined with the loss of activity during filtration stages (50–80 U/L of UPO activity measured in the filtrate), led to a poor conversion in the third reuse of the enzyme (13.1 %). Interestingly, the properties of the products did not reflect this low conversion, since the antioxidant activity was similar to that of the products obtained in the first cycle (Table 5). A lower cut-off membrane (1 or 5 kDa) could be evaluated to ensure that UPO is completely retained in the reactor while the transformation products are recovered in the filtrate.

Effect of ascorbic acid in the conversion of rutin and characteristics of the products

Full optimization was carried out in absence of ascorbic acid, since it

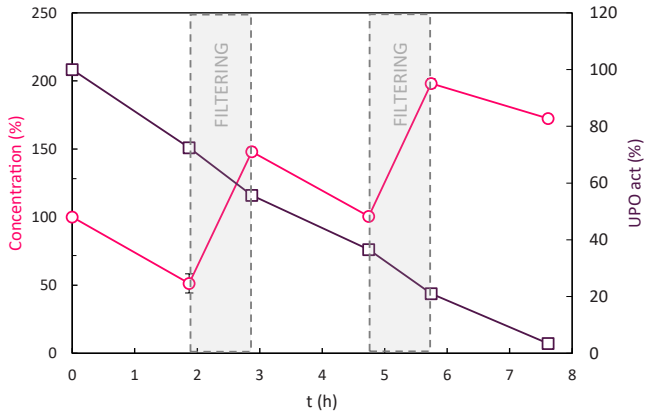

Fig. 2. Transformation of rutin by UPO in three sequential cycles. Initial activity was 7546 $U{\cdot}L^{-1}$, H_2O_2 was continuously fed at 2.75 $mM{\cdot}h^{-1}$ and reaction was stopped after 1.87 h. Once finished the reaction, transformation products were removed by ultrafiltration (10 kDa) for ~1 h, new rutin was added and reaction was initiated again with H_2O_2 addition.

Table 5
Results of rutin transformation in the sequential EMR.

Cycles	Residual UPO (%)	Rutin conversion (%)	Rutin converted (g L^{-1})	FRAP products (%)
1	72.5 ± 3.3	48.8 ± 3.9	1.02 ± 0.17	473.27 ± 8.2
2	65.7 ± 0.6	32.3 ± 2.2	0.99 ± 0.20	557.77 ± 1.6
3	16.3 ± 1.4	13.1 ± 1.1	0.28 ± 0.24	430.36 ± 1.6

was observed that at a concentration of 5 mM overshadows the antioxidant activity of the medium and did not improve the properties of the products. However, added at adequate concentrations, it could modulate peroxidative and peroxygenative activities, hence leading to different transformation products. Concentrations ranging from 5.5 to 0.7 mM were assayed and results were compared with those of the experiment lacking the radical scavenger (Fig. 3). The lowest concentration exceeds rutin aqueous solubility (0.21 mM), while the highest concentration exceeds that of the initial rutin concentration in the dispersion (4.9 mM).

Ascorbic acid did affect the residual activity of the enzyme, and the higher the concentration of AA, the higher inactivation. It also affected the conversion; in fact, 5.5 mM of AA led to complete inactivation and almost no conversion of rutin. This could be the reason why previous authors did not observe rutin transformation by *Aae*UPO, since they used a concentration of 4 mM AA. They related the lack of activity to steric hindrance by the bulky sugar substituents, suggesting that the peroxygenative active site of the *Aae*UPO cannot accommodate larger flavonoid glycosides with more than one sugar residue [12]. This has been described as an important constraint of UPO performance, since oxygenation requires the direct contact of at least part of the substrate with the ferryl oxygen of the UPO activated heme [21].

On the other hand, when using 0.7 mM of AA, although the conversion was half of that in absence of AA, the increase of the antioxidant activity of the supernatant (relative to its control containing also AA) was quite similar. Hence, it seems that the presence of ascorbic acid not only limited peroxidative transformation of rutin (the conversion decreased as the concentration of AA increased), but also affected the properties of the products.

In order to gain a deeper knowledge of the chemical composition of the transformation products using ascorbic acid, and to evaluate the potential presence of hydroxylated products, MALDI-TOF spectrometry was performed. Mass spectra revealed the presence of oligomers of rutin and hydroxylated derivatives in all samples, indicating that UPO showed both peroxidative and peroxygenative activities independently of the AA concentration used (Fig. 4). However, monohydroxylated rutin (*m/z* 625.0) was no detected in the absence of AA, and the highest relative intensity was observed with the highest AA concentration (Table SM3). In presence of AA, the highest degree of polymerization (DP) detected was 4 (observed for 5.5 and 0.7 mM AA), whereas up to 6 units of rutin were detected in absence of the radical scavenger (Table SM3).

Conclusions

This work shows, for the first time, that UPO can transform rutin to more soluble products with enhanced antioxidant activity. Different scale experiments were assayed, leading to a first optimization of initial UPO activity, H_2O_2 supply rate and reaction time in order to maximize rutin conversion into soluble antioxidant compounds, while preventing UPO from inactivation. Moreover, the effect of the highly active radical scavenger ascorbic acid upon the production of structurally and actively different products was confirmed by the analysis of antioxidant potential and MALDI-TOF spectra of the products. The prevalence of rutin oligomerization (up to DP of 6) was confirmed in the absence of ascorbic acid, while its presence likely led to the hydroxylated rutin and monohydroxylated rutin dimer. In this sense, a different profile of products with interesting bioactive properties can be obtained in the presence of a

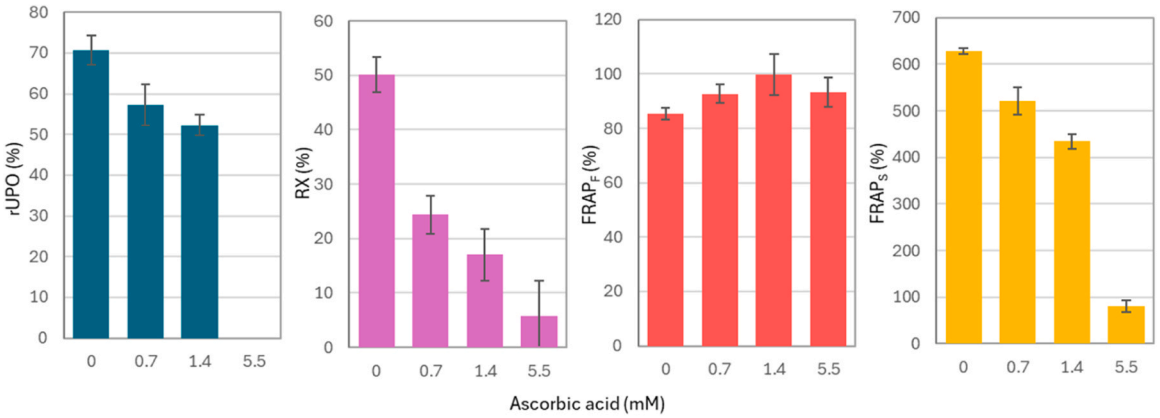

Fig. 3. Effect of different concentrations of ascorbic acid on rutin transformation by UPO.

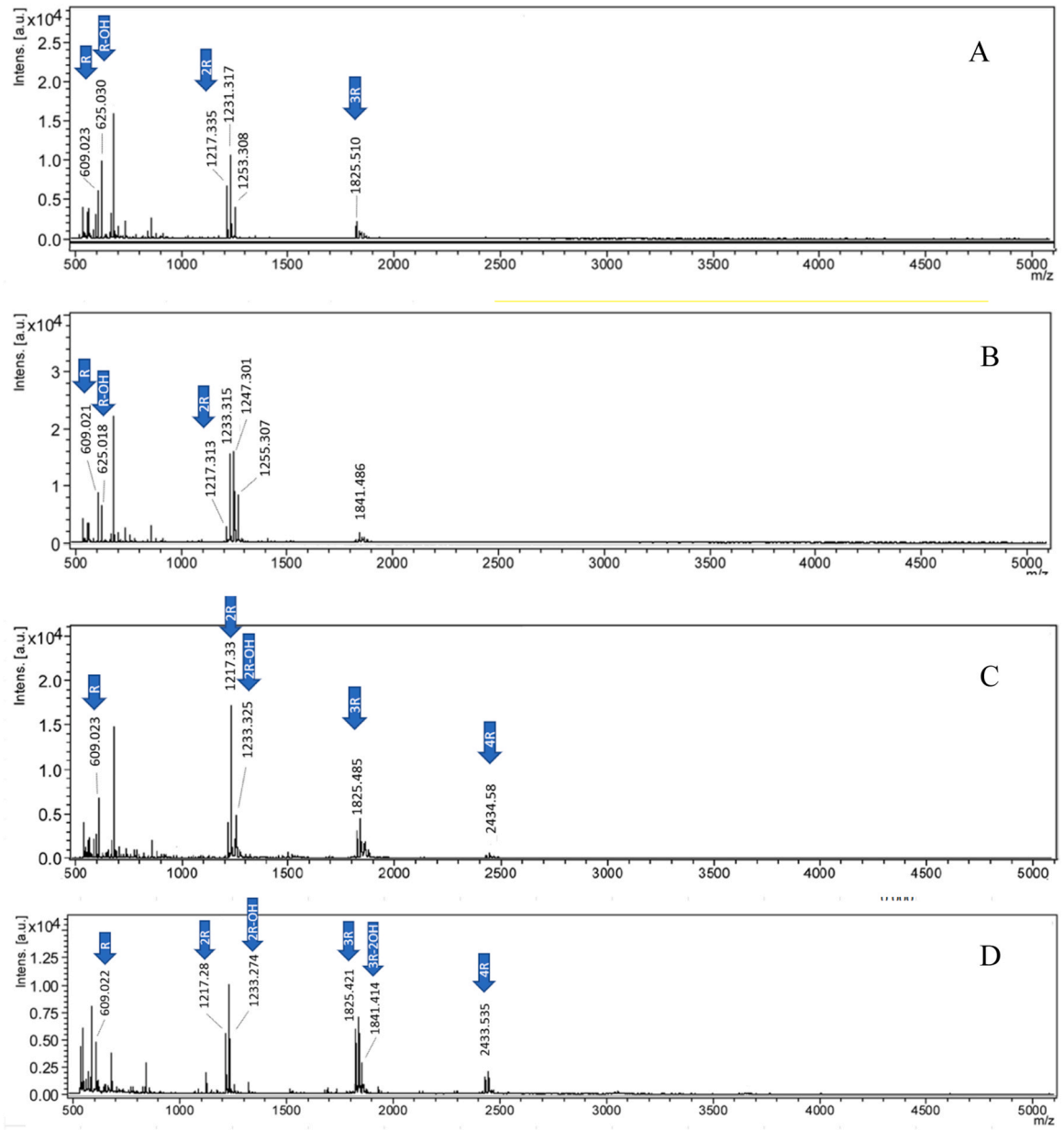

Fig. 4. MALDI-TOF analysis of UPO transformation products of rutin, in presence of ascorbic acid at A) 5.5 mM, B) 1.4 mM, C) 0.7 mM and D) in absence of ascorbic acid.

radical scavenger at appropriate concentration, hence regulating UPO peroxygenative and peroxidative activity. The increased solubility of rutin derivatives obtained was also indirectly confirmed by the comparison of FRAP antioxidant activity detected in supernatants from reaction medium, remarkably higher than those reported by reaction controls. Moreover, the re-use of UPO using EMR technology was assessed as not feasible due to the poor stability of the enzyme, combined with the loss of activity during filtration stages.

CRediT authorship contribution statement

Gemma Eibes: Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Beatriz Gullon:** Supervision, Investigation. **Abel Muñiz-Mouro:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

none.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2024.08.504.

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