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Bioepoxidation of isosafrol catalyzed by radish and turnip peroxidases

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Peroxidases (PODs) from radish (*Raphanus sativus* L.) and turnip (*Brassica napus* L.) were extracted and precipitated with ammonium sulfate using a simple, low cost and quick method. The activities of all steps performed by the vegetable PODs were measured via guaiacol assay. The epoxidation of isosafrol, catalyzed by radish (*R. sativus L.*) and turnip (*B. napus* L.) peroxidases was conducted in 20% (v/v) aqueous ethanol solution using 30% (v/v) H_2O_2 as the terminal oxidant. High conversion (88%) and selectivity (>98%) were obtained after 48 h. The products of the reaction were analyzed by high resolution gas chromatography (GC) and mass spectrometry.

Key words: *Raphanus sativus* L, *Brassica napus* L., peroxidase, epoxidation, isosafrol.

INTRODUCTION

Peroxidases (PODs) are heme proteins involved in the oxidation of a wide variety of organic and inorganic substrates that use H_2O_2 or organic peroxides as terminal oxidants (Hamid and Rehman, 2009; Veitch, 2004). PODs can be found in multiple isoforms in several species of fruits and vegetables, and are related to changes in flavor, texture and color, during post-harvest ageing and/or the processing of vegetables and fruits (Lopes et al., 2015). In spite of being ubiquitous in nature, horseradish (*Armoracia rusticana*) is the only commercial source of these enzymes. The commercially available horseradish peroxidase (HRP) is normally used in immunoassays, diagnostic kits (Veitch, 2004) and for development of biofuel cells (Ramanavicius et al., 2015; Ramanavicius and Ramanaviciene, 2009), but it is expensive due to its elevated purification costs. Many reactions catalyzed by HRP can be found in the literature: in addition, demethylation, epoxidation, hydroxylation,

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Abbreviations: HRP, Horseradish peroxidase; **GC,** gas chromatography; **POD,** peroxidases.

polymerization of phenolic compounds (Cheng and Harper Jr, 2012; Savic et al., 2013; Gilabert et al., 2004), electro-oxidation of phenol by heterogeneous catalysis (Carvalho et al., 2007) and the oxidation of bisphenol, which is a common industrial pollutant (Hong-Mei and Nicell, 2008). Hydroxylation and epoxide production are conducted by monooxygenases or peroxidases, which have biological functions that control the transfer of one oxygen atom from the dioxygen or H_2O_2 to an organic compound (Lin et al., 2011). Several studies reported the selective oxidation of alkenes using these biocatalysts (García-Granados et al., 2004; Hirata et al., 1998, Kim et al., 2007).

Epoxides are relevant compounds in the pharmaceutical industry, as they are important synthetic intermediates (Liang et al., 2004; Choudhary et al., 2004; Lambert et al., 2005; Piovezan et al., 2005). This fact is due to the versatility of the oxirane function, which can be converted into numerous chemicals with biological activity (Archellas et al., 1997). One important example is the oxidation of isosafrol, which products epoxides, that also can be converted to aldehyde (piperonal for example), an intermediate on the route to L-Dopa (Santos et al., 2004), used in the treatment of Parkinson's disease, and to α-methyldopa, used as an anti-hypertensive agent (Gu et al., 2012).

Chemical methods to synthesize epoxides are generally based on heavy metal catalysis and/or the use of stoichiometric reagents, such as *m*-chloroperbenzoic acid (MCPBA), which generate highly polluting chlorinated byproducts (Costas et al., 2000). Some reports in the literature describe the catalytic oxidation of isosafrol in the presence of 30% H_2O_2 or other oxidants with vanadium catalysts at reflux. These conditions promote the cleavage of the double bond (C=C) to form the corresponding aldehydes and epoxides (Alvarez et al., 2006; Alvarez et al., 2007). However, this reaction was not yet reported using plant POD catalysis. The study of olefin epoxidation mediated by peroxidases under mild conditions is of great interest for the synthesis of chiral building blocks.

In this work, peroxidases from radish (*Raphanus sativus* L.) and turnip (*Brassica napus* L.) were extracted and concentrated by precipitation with ammonium sulfate using a simple, low cost and quick method. These partially purified protein fractions were used in the epoxidation of isosafrol in 20% (v/v) aqueous ethanol solution using 30% (v/v) H_2O_2 as the terminal oxidant.

MATERIALS AND METHODS

Chemicals

Isosafrol (a mixture of isomers) was obtained from "Geroma do Brasil" (PR/Brazil) and consists of isosafrol *Z* (15%) and isosafrol *E* (majority species, 85%). All solvents were purchased from Vetec (Brazil) as PA grade. All other chemicals used for the broth media were of analytical grade and purchased from Sigma–Aldrich (USA). *m*-chloro-perbenzoic acid (MCPBA) was purified according to the methodology already established in the literature. Briefly, the peracid was dissolved in ether and washed with buffer solution (410 mL 0.1 M NaOH, 250 mL 0.2 M KH_2PO_4 made up to 1 L, pH 7.5). The ether layer was dried over Na_2SO_4 and carefully evaporated under reduced pressure.

Preparation of the raw extract

Turnip and radish were obtained at a local market, and were washed, pilled, homogenized and stored in freezer in fractions of 25 g up to the experiments. Extraction of the crude enzyme was carried out according to the procedure described in the literature (Fricks et al., 2006; Fricks et al., 2010). The vegetable (25 g) was homogenized with 50 mL of 100 mM phosphate buffer $Na_2HPO_4 \cdot 2H_2O$ (pH 6.5). The extract was filtered and centrifuged at 2300 x *g* (5000 rpm) for 60 min at 4°C. The supernatant solution, which contained the enzymes, was stored at 4°C.

Precipitation and determination of proteins

21 g of $(NH_4)_2SO_4$ was slowly added to a volume of 30 mL of the raw extract, reaching up to 85% saturation. After the dissolution of the salt, the solution was placed in the freezer at -18°C for 1 h. Next, the solution was centrifuged at 2300 x *g* (5000 rpm) for 40 min at 4°C and the supernatant was discarded. The precipitate was dissolved in around 5 mL of 100 mM phosphate buffer $Na_2HPO_4 \cdot 2H_2O$, pH 6.0 and was used as a source of peroxidases. The total concentration of proteins obtained in the solutions was determined by the Bradford method, using bovine serum albumin as standard (Bradford, 1976).

Determination of peroxidase activity

The enzymatic activity of peroxidases was determined by a colorimetric method based on the change of absorbance at 470 nm due to the formation of tetraguaicol, the product of guaiacol oxidation (Fricks et al., 2006; Fricks et al., 2010). Peroxidase assay medium was composed of 2.78 mL of 100 mM phosphate buffer (pH 6.0), 0.02 mL of enzyme (previously diluted 20 x), 0.1 mL of the 100 mM guaiacol solution and 0.1 mL of 2.0 mM H_2O_2 solution at 25°C. One unit of enzyme (U) was defined as the quantity of enzyme capable of forming 1 µmol of product in a minute at 25°C and pH 6.0, $\varepsilon_{tetraguaiacol}$= 26.6 $mM^{-1}cm^{-1}$ (Hirata et al., 1998). The reaction progress was followed with a UV-Vis UV-HP8452-Diode array spectrophotometer. Control experiments were carried out in the absence of peroxidases.

Stability test of the enzyme in organic solvents

Aqueous solutions of ethanol and acetonitrile were prepared with concentrations of 20, 40 and 60% (v/v). The enzymatic samples (0.1 mL) were incubated in 25 mL of the ethanolic solution and 0.9 mL of 0.1 M guaiacol solution. At certain time intervals, aliquots (2.9 mL) were collected and added to a 2 mM solution (0.1 mL) of H_2O_2 to start the enzymatic reaction. Thus, the residual activity of the enzyme pre-incubated in the aqueous solutions of ethanol was determined. An analogous methodology has been described in the literature (Azevedo et al., 2003).

Standard oxidation reaction

10 mL of dry solvent, 0.06 or 0.08 mmol of dry *m*-chloro-perbenzoic

Table 1. Radish and turnip peroxidases activities.

Parameter	Radish		Turnip	
	Raw extract	Precipitation $(NH_4)_2SO_4$	Raw Extract	Precipitation $(NH_4)_2SO_4$
Total protein (mg)	21.6±0.55*	11.3±0.14	44.2±0.24*	15.0±0.36
Specific activity (U/mg)	13.3±0.35	20.1±0.56	41.1±2.51	36.7±1.84
Total activity (U)	96.0±2.82	76.0±3.73	605±10.08	212±5.00
Recovery of activity (%)	100.0±5.00	78.0±2.51	100±5.44	35±2.47

*mg protein/ g tissue: radish (1.72±0.12) and turnip (3.54±0.21).

acid (MCPBA) and 0.04 mmol of isosafrol were stirred in a 20 mL flask under an inert atmosphere for 48 h at room temperature. Next, the reaction medium was washed with a $NaHCO_3$ solution to eliminate excess MCPBA. The reaction products were extracted with CH_2Cl_2 and the organic phase was treated with anhydrous Na_2SO_4 and subjected to chromatographic analysis (GC).

Biotransformation by POD *R. sativus* L. and/or POD *B. napus* L.

0.04 mmol of isosafrol, 20 µL of the enzymatic solution and 0.04 mmol of 30% (v/v) H_2O_2 were added to 10 mL of 20% (v/v) ethanol solution. The reaction medium was stirred (at 120 rpm) for 48 h at 25°C. After the medium was extracted with dichloromethane and dried with anhydrous Na_2SO_4, the reaction products were analyzed by GC and GC-MS.

Methods for identification and quantification of substrate and product

Reactions were monitored by high resolution gas chromatography. An HP5890 chromatograph with an HP WCOT (25 m x 0.32 mm ID) column was used in this study. H_2 was used as a carrier gas at a flow rate of 3 mL/min (96 cm/seg), with a pressure of 20 psi. The initial temperature was 100°C and the final temperature was 250°C, with a ramp rate of 3°C/min. The injector was held at 150°C and the detector at 240°C. The injection was operated in splitless mode for 0.2 µL of the injected solution. Retention times of authentic standards and their respective retention indices were obtained from a mixture of homologous hydrocarbons and used as identification parameters. Selectivity values for each product were calculated from GC data, using the products peak area, according to the following expression:

Selectivity (%) = (area peak of the product / total area peak of the products formed) * 100

Mass spectrometry was employed to confirm the identification of the product through the use of electronic libraries and published data. The analysis was performed in a HP5973 gas chromatograph connected to a HP5972 mass spectrometer, with ionization by electronic impact at 70 eV (1 scan/min, acquisition m/z: 40-400). H_2 was used as a carrier gas, with speed of 1.0 mL/min in accordance with the conditions already described.

RESULTS AND DISCUSSION

Activity assays of radish and turnip PODs were performed based on previous experience (Lopes et al., 2015), through the reaction of a guaiacol/H_2O_2 (100 mM) system. Guaiacol was selected as a standard substrate for peroxidase activity monitoring. In recent study Kumar and co-authors showed that a plant peroxidase (*Euphorbia cotinifolia*) has maximum activity with guaiacol as reducing substrate compared with pyrogallol, dianisidine-dihydrochloride, *o*-phenelene diamine, α-aminopterin and phloroglucinol (Kumar et al., 2011). Table 1 presents the results of extraction and pre-purification of PODs from radish and turnip. The main reason for performing the precipitation of proteins from the crude extract with ammonium sulfate at 85% saturation was allowed to concentrate the vegetable peroxidases in small volumes with an easy by easily and practical method, thus reducing the volume of peroxidase solution in the epoxidation medium. 30 ml of crude extract of each plant provided 4.5 and 6.0 ml of radish and turnip precipitate, respectively. For radish POD, 78% of the enzyme was precipitated, value indicated by the recovery of the activity. However, for turnip POD, a low recovery level was observed (35%). In terms of purification, It should be noted that the precipitation of the radish raw extract with $(NH_4)_2SO_4$ was efficient, due to the increased in the specific activity (13.3 to 20.1 U/mg, purification factor 1.51) with good recovery level (78%). However, a decrease in the specific activity (41.1 to 36.7 U/mg) was observed for turnip, which indicates that part of the POD turnip activity was lost during the process.

Biochemical systems involving aqueous/organic media and mild conditions are of extreme importance due to an increased demand for environmentally friendly processes. The possibility of using peroxidases in organic solvents enhances their application in the oxidation of hydrophobic molecules. Figure 1 shows that while both extracts retain part of their original activity in aqueous ethanolic mixtures, a decrease is observed at the beginning of the exposure time. After 5 h of treatment, the activity remains constant. Higher organic solvent concentrations lead to a decrease in enzyme activity. The partially purified protein fraction of the radish extract indicated that around 50 and 15% of its initial activity is preserved after 26 h of incubation in solutions of 20 and 40% (v/v) of ethanol/water solution, respectively. The

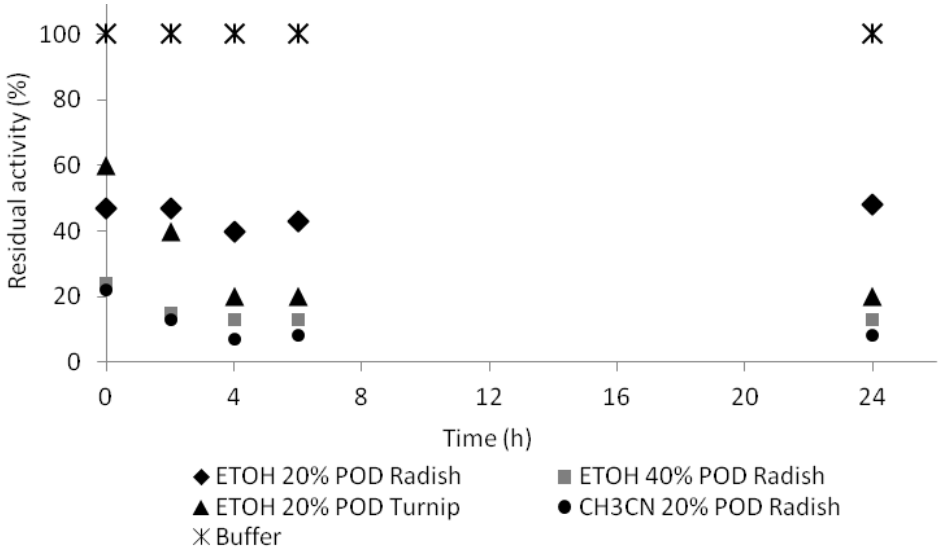

Figure 1. Residual activity of radish and turnip peroxidases in ethanol (ETOH 20 to 40% and acetonitrile (CH_3CN 20%) (v/v). In all points the deviation was less than 5%.

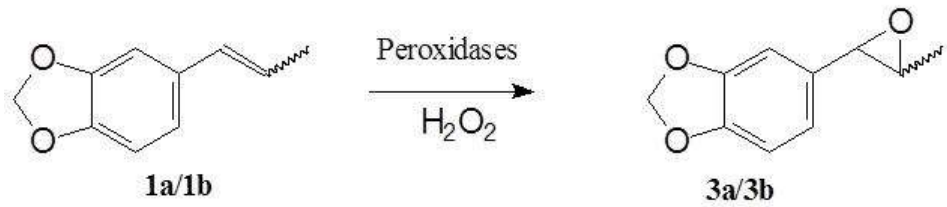

Figure 2. Microbiological oxidation of isosafrol (1a/1b) to 3a/3b.

same phenomenon was observed in the protein fraction from the turnip extract. After 26 h of incubation in 20% (v/v) ethanol/water solution, the residual activity was around 20% of the original, and virtually zero in 40% (v/v) ethanol/water (not shown in Figure 1). Radish POD extract incubated in 20% aqueous acetonitrile solution showed activities below 10% of the original activity. The results are in agreement with literature: PODs are active in organic solvents, and they have been used to catalyze the polymerization of phenolic compounds for example (Eker et al., 2009; Ryu and Dordick, 1992). In polyphenol synthesis, HRP was shown to be most stable in ethanol solutions around 20 to 40%, as higher ethanol concentrations induced a loss of activity (Ayyagari et al., 2002). Some studies in the literature indicated that HRP is more stable in polar than non polar solvents, and that sub saturated hydration levels cause a decrease in the catalytic efficiency of enzymes (Ryu and Dordick, 1992). Also, the literature shows that heme peroxidases may also have catalytic activity in non-native states (Lin and Wang, 2013).

Furthermore, large amounts of oxidant may inactivate the enzyme (Van der Velde et al., 2001). Therefore, the proportion of organic solvent, the quantity and speed of the addition of oxidant and the reaction time must be monitored to ensure enzyme activity (Azevedo et al., 2003; Santos et al., 2003; Santos et al., 2004). The epoxidation of isosafrol (1) was conducted (Figure 2) at room temperature (298 K), using the partially purified protein fractions from the extracts. Epoxidation with MCPBA was also performed to afford a direct comparison of the epoxidation with POD extracts. Blank tests showed that the substrate was not oxidized in the absence of extracts. Table 2 shows the results obtained in the experimental runs. Epoxidation with MCPBA as an oxidant gave low conversions (max. 44%) and selectivities (max. 71%) under the same experimental conditions. In addition to the epoxide, there was presence of glycol, derived from isosafrol, and piperonal, with maximum selectivities of 15 and 14%, respectively (Table 2, entry 5). According to the literature, the conventional epoxidation process utilizes acid to elicit oxygen transfer to double bonds, resulting in low yields due to side reactions such as the acid-catalyzed ring opening of oxiranes (Kim et al., 2007). In the order hand, the enzymatic epoxidation provides a mild and simple alternative, especially for the production of sensitive epoxides. The best result for the epoxidation of isosafrol

Table 2. Description of catalytic systems to oxidize Isosafrol 1 (0.04 mmol), 25°C.

#	Catalyst	Oxidant (mmol)	Solvent (10 mL)	Time (h)	Conversion (%)[a*]	Selectivity (%)			
						Epoxide	Glycol	Piperonal	By products
1	Radish -**Ia** (1,0 U)	H_2O_2 30% (0.04)	20% C_2H_5OH / H_2O	48	88	> 98	-	-	-
2	Turnip - **Ia** (1,8 U)	H_2O_2 30% (0.04)	20% C_2H_5OH / H_2O	48	7	> 98	-	-	-
3	-	MCPBA (0.06)	CH_2Cl_2	48	14	63	22	-	10
4	-	MCPBA (0.06)	CH_3CN	48	32	71	18	8	-
5	-	MCPBA (0.08)	CH_3CN	48	44	70	15	14	-

[a*]Determined by GC. Piperonal and glycol had retention times of 6.75 and 10.8 min, respectively.

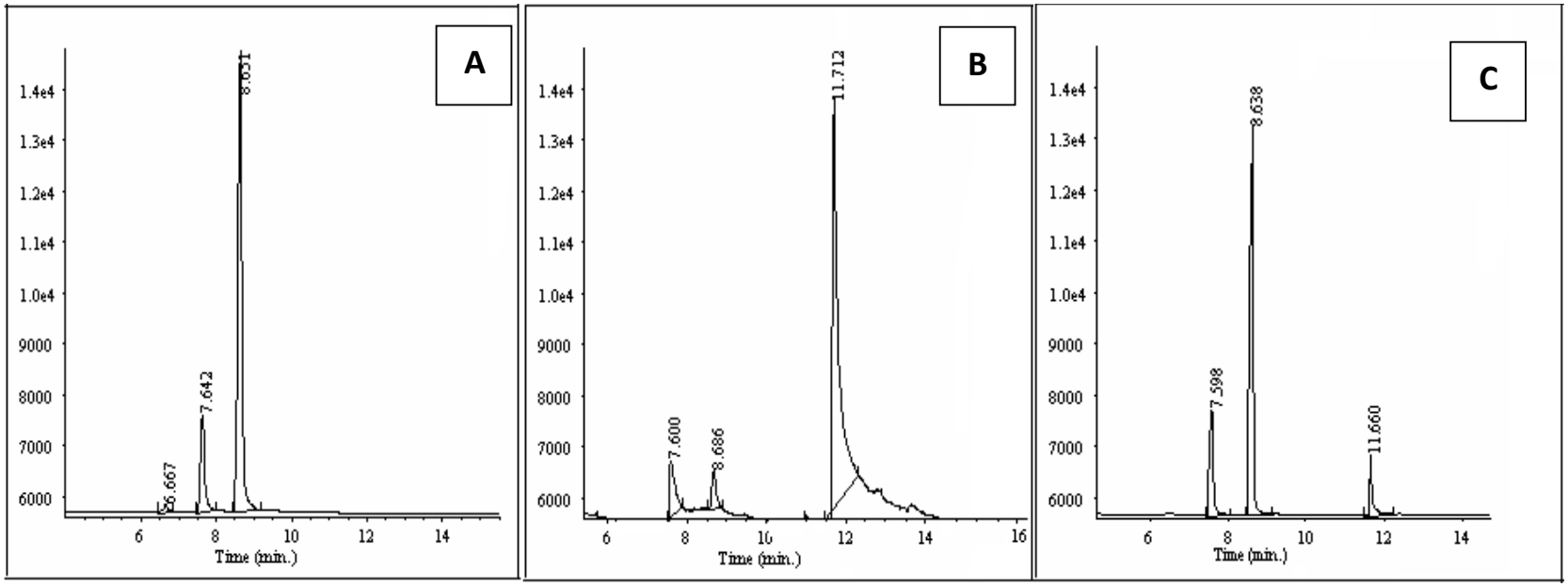

Figure 3. Chromatograms of the GC analysis of the reaction products. A) Control reaction. Retention times: Z-isosafrol (7.64 min) and E-isosafrol (8.65 min). B) Catalysis by POD *Raphanus sativus* L. (entry 1). C) Catalysis by POD *Brassica napus* (entry 2). Peak at 11.7 min is attributed to epoxide 3 (oxirane), which is validated by the mass spectrum.

was obtained with the POD extract obtained from radish as the catalyst, with the production of 3-methyl-[3´,4´-methylenedioxiphenyl]-oxirane 3 as the sole product (88% conversion and 98% selective for forming the epoxide). POD derived from turnip resulted in lower conversions of the reactant (7%), likely due to its lower stability in alcohol compared to radish POD, but with high selectivity for the epoxide (greater than 98%). Figure 3 presents the chromatograms of the GC analysis of the reaction products for the control (Figure 3A) and POD-catalyzed runs (Figure 3B,

C). Peaks derived from the isosafrol isomers are identified at 7.64 min (Z isomer) and 8.65 min (E-isomer). Control sample analysis showed only the presence of the isosafrol isomers (Figure 3A). The peak at 11.7 min is attributed to epoxide 3 (oxirane), which is validated by the mass spectrum.

The results indicate that it is possible to obtain higher conversions and selectivity with the use of plant POD as a catalyst for the epoxidation of isosafrol. In comparison, epoxidation using other plant peroxidases as catalyst show low yield. Hirata and colleagues performed the epoxidation of styrene using peroxidase from *Nicotiana tabacum*, reaching maximum yield of only 7.5% using *cis*-2-methylstyrene as substrate (Hirata et al., 1998). Our group report the oxidation of *E*- and *Z*-4-(1-propenyl)-1,2-methylenedioxybenzene (*E*- and *Z*-isosafrole) into 4-carboxaldehydro-1,2-methylene-dioxybenzene (piperonal) using different strains of *Aspergillu*s, *Cladosporiu*m, *Peacilomyces* and *Pseudomona*s. These microorganisms are able to oxidize the above compounds to piperonal, in the presence of H_2O_2, but not in its absence, indicating that this biotransformation is catalyzed by peroxidases in these microorganisms (Santos et al., 2003; Santos et al., 2004). Also, heme-monooxygenases (P-450 CIT), ω-monooxygenases and methane monooxygenases are capable of catalyzing an epoxidation reaction (Archellas and Furstoss, 1997). Some authors have also reported the oxidation of olefins using chloroperoxidase (CPO) (Allain et al., 1993). Enzymes from other sources, such as *Coprinus cinereus* peroxidase, myeloperoxidase (Tuynman et al., 2000) and chloroperoxidases (Dexter et al., 1995; Hu and Hager, 1999), are capable of catalyzing epoxidation both mildly and selectively.

Conclusion

Peroxidases from radish (*R. sativus* L.) and turnip (*B. napus* L.) were extracted and precipitated with ammonium sulfate. By this methodology only radish POD was pre-purified (purification factor 1.51). The protein fractions from the radish and turnip extracts applied in the epoxidation of isosafrol in 20% (v/v) aqueous ethanol solution using 30% (v/v) H_2O_2 as the terminal oxidant are effective catalysts to epoxidize isosafrol with high selectivity (> 98); but only with POD derived from radish, excellent chemical conversion is observed (88%).

Conflict of interests

The authors did not declare any conflict of interest.

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