Versatile peroxidase as a valuable tool for generating new biomolecules by homogeneous and heterogeneous cross-linking

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Abstract

The modification and generation of new biomolecules intended to give higher molecular-mass species for biotechnological purposes, can be achieved by enzymatic cross-linking. The versatile peroxidase (VP) from *Pleurotus eryngii* is a high redox-potential enzyme with oxidative activity on a wide variety of substrates. In this study, VP was successfully used to catalyse the polymerization of low molecular mass compounds, such as lignans and peptides, as well as larger macromolecules, such as protein and complex polysaccharides. Different analytical, spectroscopic, and rheological techniques were used to determine structural changes and/or variations of the physicochemical properties of the reaction products. The lignans secoisolariciresinol and hydroxymatairesinol were condensed by VP forming up to 8 units in the presence of organic co-solvents and Mn^{2+}. Moreover, 11 units of the peptides YIGSR and VYV were homogeneously cross-linked. The heterogeneous cross-linking of one unit of the peptide YIGSR and several lignan units was also achieved. VP could also induce gelation of feruloylated arabinoxylan and the polymerization of β-casein. These results demonstrate the efficacy of VP to catalyze homo- and hetero-condensation reactions, and reveal its potential exploitation for polymerizing different types of compounds.

Keywords

Enzymatic polymerization, organic co-solvent, lignan, peptide, β-casein, feruloylated arabinoxylan.

1. Introduction

Biotransformation is a useful way for modifying or producing novel structures and materials, which can then be exploited in a broad range of applications. The enzymatic polymerization and hetero-conjugation of various substrates is a method of primary interest to reach that goal. Biocatalysis is advantageous over chemical procedures, since: (i) it is an environmentally friendly alternative that uses milder and less contaminant reaction conditions [1] and (ii) it can produce more specific cross-links, as many enzymes have high chemo-, regio-, and enantio-selectivity [2]. The use of oxidoreductases, as radical-forming enzyme systems, to render homo or hetero-polymers of very diverse molecules is an attractive example of this, and the enzymatically synthesized polymers may exhibit new or improved properties in comparison with their respective precursors [3].

Versatile peroxidases (VP) are an interesting group of oxidoreductases (EC 1.11.1.16; described as a Reactive Black 5:hydrogen peroxide oxidoreductase) whose activity in these polymerization reactions has not been previously explored at the molecular level. These enzymes are secreted by fungi and included in the class II of the superfamily of plant-fungal-bacterial heme-peroxidases, together with manganese peroxidases (MnP), lignin peroxidases (LiP), generic peroxidases (GP) as *Coprinopsis cinerea* (synonym *Coprinus cinereus*) peroxidase (CiP) [4]. To date, these enzymes and their encoding genes have been found and characterized only in white rot, wood-decaying Basidiomycota belonging to the class Agaricomycetes, as described in the comparative genomic research recently published by Floudas et al. [5]. VPs constitute an example of enzyme multifunctionality, combining the catalytic properties of MnP, LiP, and low redox-potential peroxidases. Therefore, VP displays a wide oxidative activity on substrates having different chemical structures and redox-potentials, including compounds that cannot be oxidized by low redox-potential peroxidases, such as fungal GP, horseradish peroxidase (HRP) and other plant peroxidases [6-9]. Recent

research efforts of VPs have focused on the understanding of their kinetic reaction mechanisms and structure-function relationships [10,11], in the search of adequate systems for the expression of VP [12], and in enzyme improvement through directed evolution [13]. Nevertheless, the potential applications of VPs have not yet been fully exploited, in spite of being a very promising group of enzymes from a biotechnological point of view [14].

Different substrates previously used in enzymatic polymerization reactions [15-18], representing low-molecular mass, oligomeric, and polymeric substrates, were chosen to evaluate the polymerizing ability of VP. Lignans are diphenolic compounds found in the cell wall of higher plants, formed by β-β coupling of two cinnamyl precursors [19], and their chemical structure depends especially on the plant species from which they are isolated. These compounds can appear in side-streams from industrial processing of lignocellulosic material, *e.g.* mechanical pulping and paper processing, and should be removed to avoid undesirable effects, such as interferences with process chemicals [20]. Polymerization of lignans into larger molecules is one way to eliminate these unwanted effects. Lignans may also serve as precursors for the enzymatic production of value-added polymers or materials with improved functional properties [21]. These synthetic reactions are challenging, since most of them may only be performed in the presence of organic solvents, jeopardizing the stability of the enzyme catalyst. On the other hand, the enzymatic polymerization of bioactive peptides, proteins as β-casein, or feruloylated arabinoxylans (FAX), which have well-known functional properties [19,22-24], could lead to tailored products with improved/modified organoleptic or functional properties such as reduced fat content, texture, solubility, mouth feel, better digestibility, emulsification, viscosity, gelling, or resistance to heat or proteolytic attack during enzyme digestion [25,26].

Therefore, the aim of the present study was to determine if the VP from *Pleurotus eryngii* is able to catalyze the covalent homogeneous and/or heterogeneous cross-linking of

selected small and large molecules, in the presence and absence of organic solvents, thus producing novel biocompounds. The extent of the condensation reactions was also evaluated.

2. Materials and methods

2.1. Substrates

The lignans used in this study (**Fig. 1**), namely secoisolariciresinol (SECO), hydroxymatairesinol (HMR), matairesinol (MR), cyclolariciresinol (CYCLO), and 7-hydroxy-secoisolariciresinol (7-HSECO) were prepared as described earlier [27-29]**.** The bioactive peptides EPPGGSKVILF, RKRSRKE, VEPIPY, YIGSR, and VYV were obtained from Sigma (St Louis, MO, USA). YST was bought from Biokemis (Saint Petersburg, Russia). GLY was obtained from Fluka (Buchs, Switzerland). The bovine β-casein protein (24 kDa, 85% purity) was purchased from Sigma-Aldrich (Taufkirchen, Germany). FAX from maize bran, containing an alkali-extractable ferulic acid content of 6.2 mg g^{-1}, was kindly given to CBF by Cambridge Biopolymers Ltd. (Cambridge, UK).

2.2. Enzyme activity

VP was isolated and purified from *P. eryngii* cultures as previously described [30]. Its Mn^{2+}-oxidizing activity was determined spectrophotometrically at 238 nm through the formation of the Mn^{3+}·tartrate complex (ε_{238}= 6500 $M^{-1}cm^{-1}$) in a reaction mixture containing 0.1 mM $MnSO_4$ (Mn^{2+}) in 25 mM sodium tartrate buffer (pH 5.0), with the addition of 0.1 mM H_2O_2 to start the reaction. The effect of two organic solvents on VP activity was also checked through the evaluation of Mn^{2+}-independent activities following the oxidation of 1 mM 2,6-dimethoxyphenol (DMP) to dimeric coerulignone (ε_{469}=55000 $M^{-1}cm^{-1}$) and 1 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) to $ABTS^{+}$· (ε_{436}=29300 $M^{-1}cm^{-1}$) in 25 mM sodium tartrate buffer (pH 5.0). Solutions containing from 0% to 50% (v/v) of either ethanol or 1,2-propanediol in 25 mM sodium tartrate buffer (pH 5.0), in the presence or

absence of 0.1 mM Mn^{2+} and 0.1 mM H_2O_2, were prepared as solvents for the substrates. The VP stability during 24 h-reactions was also checked in 25 mM sodium tartrate buffer (pH 5.0), 20%, and 50% ethanol (dissolved in the same buffer at equal final concentration), with and without Mn^{2+}. The percentage of residual activity was calculated at 0, 0.5, 2, and 24 h using Mn^{2+} /H_2O_2 as substrates and taking the initial activity in buffer as 100%. Measurements were carried out in triplicate at room temperature. One unit of activity (1 U) is defined as the amount of enzyme releasing 1 µmol of product per minute under the defined reaction conditions.

2.3. Substrate solutions

The lignan SECO (3 mM), all peptides (3 mM), β-casein (1 mg mL^{-1}), and FAX (30 mg mL^{-1}) were prepared in 25 mM sodium tartrate buffer (pH 5.0). The remaining lignans were dissolved in 20% ethanol in the same buffer at a 3 mM final concentration, except 7-HSECO, which was dissolved in 50% ethanol buffer. All solutions were left standing for at least 30 min to be stabilized before commencing the enzymatic treatments.

2.4. Cross-linking assays

The enzyme reactions detailed below were initiated by addition of 0.1 mM H_2O_2, supplementing with a second dosage after 1 h of incubation and briefly agitated after each H_2O_2 supplementation. As an exception, the FAX assays were performed with a single dosage of 0.2 mM H_2O_2 at the beginning of the treatment. Unless otherwise stated, these treatments were performed in the presence of Mn^{2+} (0.1 mM), in duplicate, at room temperature. Negative controls consisted of reactions lacking VP or H_2O_2 incubated under the same conditions than the test reactions (with and without Mn^{2+}).

2.4.1. Homogeneous cross-linking of lignans and peptides

The polymerization of different substrates was performed in 1.5 mL Eppendorf tubes with a 1.5 U mL^{-1} VP in a final volume of 200 µL. Aliquots (3 µL) of each reaction mixture were

removed after 0.5 and 2 h of incubation for subsequent analysis by matrix-assisted laser desorption/ionisation-time of flight-mass spectroscopy (MALDI-TOF MS). Lignan treatments were also performed during 24 h, in the absence of Mn^{2+}. To stop these reactions 0.05% (w/v) NaN_3 was added and samples from lignan treatments were lyophilized for further size exclusion chromatography (SEC) analysis. The effect on the polymerization efficiency of a lower enzymatic dose (0.15 U mL^{-1}) and a higher H_2O_2 concentration (0.5 mM), during 0.5, 2 and 24 h, was studied and separately assayed using HMR lignan as substrate.

2.4.2. Heterogeneous cross-linking of lignans with peptides

Reactions containing equal volumes (85 µL) of the 3 mM solutions of the SECO or HMR lignans and of the tyrosine-containing peptides were mixed with 1.5 U mL^{-1} VP, in a final volume of 200 µL. Aliquots (3 µL) were withdrawn after 0.5 and 2 h reaction and analyzed by MALDI-TOF MS.

2.4.3. Homogeneous cross-linking of β-casein

Three VP doses (0.015, 0.15, and 1.5 U mL^{-1}) were assayed in 1 mL reactions for β-casein polymerization, incubating separately for 2, 6, and 24 h with continuous stirring at 300 rpm. Aliquots (30 µL) from each treatment were separated and immediately mixed with loading buffer (10 µL), boiled for 10 min and analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The remaining enzymatic reactions were stopped with NaN_3 (0.05%, w/v) and samples were lyophilized for further transmission electron microscopy (TEM) analysis.

2.4.4. Homogeneous cross-linking of FAX

The ability of VP to cross-link FAX, inducing gel formation, was investigated in reactions containing VP doses of 0.015, 0.15, 1.5 U mL^{-1} in a total volume of 1.5 mL. Mixtures were briefly vortexed and 1.3 mL was immediately removed and placed on the rheometer plate. Rheological analysis started 3 min after H_2O_2 activation. For swelling experiments, reactions

were prepared with the same enzyme doses, in 2 mL syringes (diameter 1.5 cm), in a final volume of 1 mL. Reactions were allowed to gel for 15 h before analyzing the swelling degree.

2.5. MALDI-TOF MS analyses

MALDI-TOF MS spectra of VP-treated lignans and peptides were recorded on a Bruker Autoflex II instrument equipped with a N_2-laser (337 nm, 100 µJ) and previously calibrated with peptide and protein standard solutions from same distributor (Bremen, Germany). For the analyses, 3 µL of the reaction solution were mixed 1:1 (v:v) with saturated α-cyano-4-hydroxycinnamic acid matrix from Sigma-Aldrich (St Louis, MO, USA) dissolved in 0.1% trifluoroacetic acid from Fluka (Buchs, Switzerland) containing 50% acetonitrile. 1 µL of the sample-matrix solution was spotted onto the stainless steel target plate and allowed to dry at room temperature. Positive ion mass spectra were recorded in reflector mode (m/z range 500-3500) and linear mode (m/z range 3500-10000). FlexAnalysis (version 2.4) was used for data analysis (Bruker, Bremen, Germany). Lignans were detected as their sodium adducts.

2.6. Size exclusion chromatography (SEC)

Samples from lignan treatments were dissolved in 0.1 M NaOH and analyzed by high performance SEC eluting with the same solvent (0.5 mL min^{-1} flow rate) at 25 °C, in MCX 1000 and 100000 Å columns connected in tandem and coupled to a precolumn (all from PSS Mainz, Germany). The elution profiles were followed at 280 nm with a Waters UV detector. The molar mass distributions (MMD), and weight average molar masses (M_w) were calculated against polystyrene sulphonate (Na-PSS) standards, using the Waters Empower 2 software.

2.7. SDS-PAGE analysis

SDS-PAGE was used to analyze the formation of β-casein polymers smaller than 250 kDa. Samples were loaded onto Criterion TGX Stain-Free™ precast gels (4-20%) and visualized on the Criterion Stain Free™ Imager System. Precision Plus Protein™ Standards (10-250 kDa)

were used for molecular weight estimations. All instruments and reagents were purchased to Bio-Rad (Hercules, CA, USA).

2.8. Microscopy analysis

Lyophilized β-casein samples, subjected or not to a 24 h VP treatment, were dissolved in distilled water or 6 M urea to observe non-enzymatic aggregates and enzymatic cross-links, respectively. Glow-discharged carbon-coated formvar grids were placed face-down over a droplet of sample. After 1 min, the grid was removed, blotted briefly with filter paper and negatively stained with 2 % uranyl acetate for 40 s, blotted quickly and air-dried. Samples were observed by TEM in a JEOL 1230 instrument (Tokyo, Japan) operated at 100 kV.

2.9. Rheological measurements and gel swelling analysis

The gelation of the cross-linked FAX was monitored by using an AR-G2 rheometer (TA Instruments, Crawley, UK) in oscillatory mode at a constant temperature of 22ºC. A plate-plate geometry with a diameter of 40 mm and a gap of 1 mm was used for the measurements, with a solvent trap to prevent sample drying during analysis. Gel formation was followed during 4 h, by monitoring the storage modulus (G´), the loss modulus (G´´) and the phase angle at a constant frequency of 0.1 Hz and a strain of 0.01 %.

To evaluate the gel swelling properties, cross-linked FAXs were allowed to swell in 10 mL of a 0.02 % (w/v) NaN_3 solution. After 32 h, samples were blotted, weighted, and subsequently added to new NaN_3 solutions at room temperature. The equilibrium swelling was reached when the weight of the samples did not change more than 3%. The swelling ratio (q) is calculated according to the equation: $q= (W_s – W_i)/ W_i$, where W_s is the weight of the swollen gel at each measured time and W_i is the weight of the gel before swelling.

3. Results and discussion

3.1. Influence of organic co-solvents and Mn^{2+} on VP activity and stability

The lignans included in the present study were selected for their different structures and the degree of solubility in aqueous solutions or organic co-solvents. Enzymes can be severely affected by the presence of organic solvents, which generally cause a sharp activity drop due to modification of the protein conformation [31]. For this reason, VP activity was first established in the presence of different concentrations of ethanol and 1,2-propanediol, two solvents chosen for their ability to solubilize softwood and hardwood lignins and for being completely water-miscible. Initial activities in aqueous buffers were determined to be 15 U mL^{-1} for Mn^{2+}, 1 U mL^{-1} with ABTS, and 0.4 U mL^{-1} for DMP. VP-activity against ABTS and DMP increased in the presence of Mn^{2+}, reaching 1.6 U mL^{-1} and 3.6 U mL^{-1}, respectively. This enhanced activity on phenols and dyes in Mn^{2+}-containing reactions has been previously reported [30], and it is related to the catalytic versatility of VP, acting as both Mn^{2+}-independent and Mn^{2+}-dependent peroxidase (**Fig. 2**).

Fig. 3 shows the effect of the two solvents on Mn^{2+}, DMP, and ABTS oxidation. As 1,2-propanediol concentration increased (**Fig. 3a)**, a concurrent decrease in VP residual activity was observed. The decrease was significantly lower in reactions with Mn^{2+}, especially at high solvent concentrations, although some activity was retained in all reactions even with 50% of this organic solvent**.** On the other hand, the effect of ethanol on VP activity (**Fig. 3b)** was diverse. With Mn^{2+} as the substrate, VP was quite stable in up to 40% ethanol, with only a 30% decrease in activity. The oxidation of DMP in the presence of Mn^{2+} appeared not to be affected by the use of ethanol concentrations ≤ 20%. The activity of a *B. adusta* VP at low ethanol concentrations has also been described [32] although, according to our data, the VP from *P. eryngii* seems to be more resistant. However, a high activity loss was observed in DMP reactions without Mn^{2+} that could be explained by reduced accessibility of ethanol

231 through the narrow Mn-oxidation channel, compared with the main heme access-channel
232 where DMP is oxidized [33]. From the above results, and taking into account that most of the
233 lignans used in this study are soluble at low ethanol concentrations (20%), this solvent was
234 selected for further enzyme stability assays. The enzyme in buffer, as well as in the presence
235 or absence of Mn^{2+}, retained the initial activity during the 24 h of incubation. With 20%
236 ethanol in the absence of Mn^{2+}, the residual activity was 84 and 43% at 0.5 and 24 h,
237 respectively, while in the presence of the ion was 87 and 82% (with 50% ethanol the enzyme
238 lost its activity quickly, retaining 2.1% and 3.4% activity after 0.5 h with and without Mn^{+2},
239 respectively). It can thus be concluded that the presence of Mn^{2+} exerts a stimulating and
240 stabilizing effect on the oxidation reactions and VP respectively. The former effect is due to
241 Mn^{3+}-mediated oxidation of DMP and ABTS, while the stabilizing effect indicates lower
242 inhibition when the VP Mn^{2+}-oxidation site is occupied by the ion during incubation with
243 ethanol.

244 *3.2. Small molecules cross-linking by VP*

245 *3.2.1. Lignan cross-linking analysis*

246 Several parameters, such as the degree of polymerization (DP), the molecular mass (MM),
247 and molar mass distribution (MMD) of the polymers enzymatically synthesized, should be
248 analysed to compare the efficiency of VP in lignans polymerization with that reported for
249 other oxidative enzymes [34]. The polymerization of the substrates (DP and MM) was
250 followed by MALDI-TOF MS. Control treatments, in the presence and absence of Mn^{2+},
251 containing lignans and H_2O_2 but lacking enzyme were conducted to check for substrates self-
252 polymerization, revealing the presence of dimers and trimers of lignans (**Table 1**). These non-
253 enzymatic cross-links could arise from oxidation reactions, involving O_2 or H_2O_2, mediated
254 by traces of metal ions [35,36]. Despite of these unspecific links, the VP/H_2O_2 system was
255 capable of synthesizing molecules of much higher DP compared to the controls. **Table 1**

shows that most cross-linking occurred during the first 30 min of reaction and that the presence of Mn^{2+} enhanced the polymerization efficiency, raising both the reaction rate and the maximum DP of the products over the reaction time at all solvent concentrations. **Table 2** depicts the predicted and experimental MM (accuracy $\leq \pm$ 1 Da) of the longest lignan homopolymers synthesized by VP, and the spectra of the reaction products are represented in **Fig. 4**. The assembly of lignan monomers is produced through an ether or carbon-carbon linkage that causes the elimination of two hydrogen atoms per cross-link [17]. The theoretical mass of the products can be calculated according to the equation [[nMM-(n-1)2H] + Na^{+}], where n is the number of monomers and MM is the molecular mass of lignan. The water-soluble SECO was the most efficiently polymerized substrate, forming nonamers and octamers in the presence and absence of Mn^{2+}, respectively, after 24 h incubation. This value was similar or slightly higher than those reported for the cross-linking of SECO catalyzed by fungal [21] or bacterial laccases [37]. Over the first 2 h of reaction, polymerization of both SECO and HMR progressed at similar rates, but after 24 h the maximum DP of the later decreased in one unit while the SECO polymer reached its highest length (**Table 1**). Buchert et al. [20], reported the synthesis of HMR oligomers after 2 h incubation with a fungal laccase, but the products were 3-4 units smaller than those found in the present study at the same reaction time. Regarding 7-HSECO, the lignan dissolved in 50% ethanol, it is remarkable that the addition of Mn^{+2} produced two-fold larger oligomers as compared to the reaction without the cation, which could be due to an increased VP catalytic efficiency and stability, as described in the previous section.

SEC analysis of the VP-untreated and treated lignans, in the presence of Mn^{2+}, showed the reduction of the low MM peak from the substrates along the incubation time, and a parallel increase of the M_w of the products. **Fig. 5** illustrates the MMD profiles of SECO and HMR, the two lignans that reached the highest DP. A single peak, corresponding to

281 substrates, was observed after SEC of control samples without peroxidase. The appearance of
282 oligomers along the reaction time is detected as new shoulders or peaks at the right of the
283 substrates peak. The small increase in the low MM fraction from 24 h HMR reactions could
284 be explained by the coexistence of polymerization and degradation activities in the reaction
285 mixture, as previously reported for soluble-lignin samples treated with VP [38] and MnP
286 [39]. This finding is in accordance with the fading of the signal from HMR octamers detected
287 by MALDI-TOF analysis of this sample (**Table 1**). However, the global M_w of the reaction
288 components raised approximately 100 Da from 2 h to 24 h, suggesting that polymerization
289 predominated over depolymerization during this period.
290 As in the case for all peroxidases, H_2O_2 is essential for VP activity and its eventual
291 depletion stops the reaction. The effect of different VP/H_2O_2 ratios on HMR cross-linking was
292 assayed for 24 h treatments, either increasing 5-fold the peroxide concentration or decreasing
293 10-fold VP dosage (from 1.5 to 0.15 U mL^{-1}), and samples were analyzed at different reaction
294 times. Enzyme-free controls demonstrated that HMR treatment with 0.5 mM H_2O_2 did not
295 induce any additional polymerization. In contrast, HMR polymers only one DP larger in
296 molecular size than those detected in low-peroxide reactions were detected in the VP dosed
297 with 5-fold more H_2O_2 system. The M_w values increased around 200 and 300 Da in 2 h and 24
298 h-reactions, compared to the equivalent products obtained with 0.1 mM H_2O_2. SEC profiles
299 (**Fig. 5b**) showed that the low MM peak decreased around 50% in height, while a high-MM
300 fraction of almost the same intensity appeared. Thus, higher doses of peroxide did not cause
301 inhibition of VP, but rather improved its polymerization capacity. On the other hand, a
302 reduced VP dosage resulted in the production of HMR oligomers of 5, 6 and 7 monomers
303 after 0.5, 2, and 24 h, respectively, demonstrating that the amount of enzyme affected only the
304 initial reaction rate, reaching the same DP values obtained at higher VP doses in 24 h (**Table**
305 **1**).

306 *3.2.2. Peptide cross-linking analysis*

307 The positive effect of Mn^{2+} in VP-catalyzed polymerization reactions has already been
308 deduced from the previous experiments. The VP treatment of peptides, of different length and
309 amino acid sequence, was performed in the presence of this cation. No peptide cross-linking
310 occurred in the absence of VP or H_2O_2, as well as in peptides lacking tyrosine residues (**Table**
311 **2**) such as RKRSRKE and EPPGGSKVILF. In contrast, the VP/H_2O_2 system was highly
312 efficient polymerizing tyrosine-containing peptides, being as previously described for other
313 oxido-reductases [15,40,41]. Polypeptides were formed through the loss of two hydrogen
314 atoms, probably due to the formation of dityrosine or isodityrosine bonds, resulting in
315 monoisotopic masses whose theoretical value can be calculated according to the equation:
316 [nMM-(n-1)2H], where n is the number of monomers and MM is the molecular mass of each
317 peptide. As an exception, the polymers from VEPIPY were detected as their sodium adduct.
318 **Table 2** shows the experimental and predicted MM from the highest DP molecules
319 synthesized upon VP-treatment, and **Fig. 4** depicts the spectra of the reaction products.

320 The peptide length and the position of tyrosine in the sequence had no effect on the DP
321 of the products formed by the action of VP. This finding contrasts with the results obtained in
322 similar reactions performed with HRP [41] or the peroxidase from *C. cinerea* [42], in which a
323 strong effect of these two parameters on cross-linking has been reported. VP has several
324 catalytic sites, one for (low efficiency) oxidation of phenols and dyes at the main heme access
325 channel, a second one for Mn^{2+} oxidation at a (small) second heme access channel, and the
326 third site for (high efficiency) oxidation of phenolic and nonphenolic aromatic substrates,
327 located at the protein surface [11]. These features, which are not observed in other
328 peroxidases (such as HRP) that only present the "classical" oxidation site at the main heme
329 channel, can facilitate substrate oxidation even if the reactive moiety, such as the tyrosine, is
330 placed in the middle of the sequence. Therefore, the cross-linking of tyrosine-containing

peptides by HRP may be very restricted by steric hindrances and enzyme inhibition [41]. In contrast, VP can also oxidize its substrates at a second exposed catalytic site and via Mn^{2+} diffusion (**Fig. 2**), bypassing these problems.

In addition, VP-induced polymerization of peptides was very fast, reaching the maximum DP after 30 min in all cases. Regardless the reaction time, the predominant signals in most spectra corresponded to dimers, although tetramers and trimers were the main peaks detected after 2 h of reaction using GLY or VYV as the substrates, respectively (**Fig. 4**). The MMD of the products, derived from MALDI-TOF MS spectra, changed while the reaction proceeded due to the production of larger species. The production of pentamers of GLY, using a laccase from *Trametes hirsuta* [40] and hexamers of VYV with the *C. cinerea* peroxidase [15] in 24 h, are poor values when compared to the nonamers of GLY and the undecamers of VYV obtained in VP-reactions of 30 min (**Table 2**). The results suggest that beyond a certain DP range, peptides polymerization is not favored under the reaction conditions used in this study or by other steric factors. Despite this limitation, our results show that, even at short reaction times, the polymerization degree was higher using VP, even though this enzyme has a more restricted activity compared to laccases, especially due to the low H_2O_2 concentration tested in this study.

3.2.3. Heterogeneous cross-linking of lignans with peptides

VP reactions containing SECO or HMR and each of the tyrosine-containing peptides were incubated for 30 min and 2 h and analyzed to test the ability of the enzyme to catalyze the synthesis of heteropolymers. The MALDI-TOF spectra of the reaction products for each peptide-containing reaction showed that in most cases only lignan homopolymers were formed, detecting up to nonamers of SECO and octamers of HMR. Steffensen and co-workers [43] suggested that monolignols can have antioxidant effects, avoiding tyrosine oxidation in peptides when they are added to the reaction at the same concentration. Moreover, although

the molar concentration of both substrates in the reaction mixture was the same and the MM of the tripeptides (GLY, VYV, YST) and the lignans is also similar, the later contain two-fold more reactive sites than the peptides (two phenols per one tyrosine, respectively). The probability for heterocomplex formation appears to increase with the number of reactive sites in the biopolymer. This proposition contrasts with the results obtained with VEPIPY. In this last case, and taking into account the gravimetric amount of the compound in the final reaction mixture, polymerization was not observed even though this peptide and the lignans contained a similar number of reactive sites.

Hetero-oligomers were only formed in reactions containing the peptide YIGSR with both lignans (**Fig. 6**), although lignan homo-oligomers were also detected. These heterogeneous cross-linking reactions took place through the elimination of two hydrogen atoms according to the equation: $[nMM(YIGSR)+[nMM(lignan)-(n-1)2H^{+}]-2H^{+}]$. Peaks corresponding to peptide homo-oligomers were not found, contrasting with the high DP of the YIGSR units produced when incubated alone with VP (**Table 2**). YIGSR is a peptide derived from laminin and it is considered as an adhesive ligand, what can possibly facilitate its attachment and linking to other molecules [44]. These results indicate that, in the presence of both substrates, VP has a preference for lignans and if a hetero-crosslinking occurs, only the lignan chain is further elongated.

3.3. Large molecules cross-linking by VP

3.3.1. β-Casein cross-linking

As VP was shown to efficiently cross-link small tyrosine-containing peptides, the milk protein β-casein, consisting of 209 amino acids, four of which are tyrosines [45], was selected to determine if VP could cross-link larger proteins. The products were analyzed under dissociating and reducing conditions by SDS-PAGE, confirming that the complexes observed were due to the formation of covalent bonds and not by molecular aggregation. Large-size

molecular species (approximately 150 kDa) appeared even at the lowest enzyme dosage and the band corresponding to the β-casein monomer slightly decreased over the reaction time, suggesting that a low percentage of protein was modified (**Fig. 7, lane 6**). In contrast, medium and high doses of VP resulted in an almost total fading of the β-casein monomer after 24 h (**Fig. 7, lanes 7 and 10**). Moreover, bands higher than 250 kDa were observed at the top of the running gel, corresponding to polymers of at least 10-11 β-casein monomers. The polymerization of casein has been previously reported using HRP [18] and HRP plus ferulic acid [46] with similar results to those reported in the present study. Bands with a lower mass than β-casein appeared after long incubation times and in reactions with high VP doses. As previously discussed, the peroxidase can simultaneously catalyze polymerization and degradation at extended reaction times (**Fig. 7, lanes 7 to 10**). After 24 h of incubation, a slight aggregation was detected in all samples, which could be produced by the intra- and intermolecular transference of radicals formed from proteins during the reaction [47,48]. TEM images (**Fig. 7**) from water solutions of VP-treated casein allowed the observation of the protein as fibers, representing a typical product structure upon molecular aggregation [49]. When VP-treated β-casein was dissolved in urea to disrupt aggregates, polymers were observed as irregular and compact structures with a broad range of sizes.

3.3.2. FAX cross-linking

Small deformation oscillatory rheology was used to follow gel formation caused by the oxidative cross-linking of feruloylated arabinoxylan. Control treatments lacking VP or H_2O_2 did not produce gels during 4 h reactions, and the same result was obtained with the lowest VP dosage (0.015 U mL^{-1}). Neither the use of 1.5 U mL^{-1} of VP was useful for this monitoring since it produced an immediate gelation after H_2O_2 addition. A dosage of 0.15 U mL^{-1} VP was selected for further experiments. The profile obtained with 3% FAX fitted the typical kinetic behavior displayed in enzymatic systems containing laccases [50] or other

406 peroxidases [16]. **Fig. 8** shows an initial increase of both G´ and G´´ followed by a plateau
407 region. That plateau reached G´ values of around 90 Pa and G´´ values of 0.5 Pa in 10 and 7
408 min respectively, indicating that the sample had gel-like properties. The final phase angle was
409 very low (below 0.5 degrees), highlighting the high elasticity of the gel. The gelation point,
410 calculated from the crossover point of G' and G'', took place in about 2 min. Apart from the
411 diferulic covalent bonds, non-covalent links between arabinoxylans might also occur [22,51]
412 and therefore the measured rheological properties depend on the arabinoxylan structural
413 characteristics and the ferulic acid concentration.
414 Gels were generated only in reactions with 0.15 and 1.5 U mL^{-1} VP, reaching the
415 swelling equilibrium between 4 and 6 h, with swelling ratio values (q) of 53.4 ± 0.4 and 65.4
416 ± 1.1 g water/g FAX, respectively. These values were higher than those reported in other
417 enzyme-catalysed FAX gelation studies [52]. The fast reaction rate achieved with VP could
418 cause the trapping of uncrosslinked FAX molecules inside the gel structure. These molecules
419 would expand quickly in contact with water, leading to its increased intake in the resultant
420 FAX networks [16].
421
422 **4. Conclusions**
423 The modification by enzymatic cross-linking of biomolecules using the VP from *P. eryngii*
424 has been achieved. The reaction conditions during VP treatments had a great influence in the
425 reaction yields. In general, Mn^{2+} seemed to improve the VP stability and/or its catalytic
426 efficiency even in the presence of organic co-solvents, which are essential in most reactions
427 involving lignans. Only peptides containing tyrosine residues, regardless of their position in
428 the sequence, are capable of forming a covalent bond through this kind of reactions, and
429 heteropolymerization of lignans with a peptide resulted to be feasible. Moreover, VP-
430 catalyzed cross-linking produced high mass macromolecules from β-casein and FAX. In view

of these results, the application of VP for efficient polymerization of oxidizable compounds is suggested. Further screening of other potential substrates for VP and studies on the optimization of the polymerization reaction will be designed in the future. The *P. eryngii* VP used in this work is currently being subjected to structural-functional studies enabling rational design, as well as to directed molecular evolution, to improve its resistance to pH and H_2O_2, two important challenges for its application in biotechnological processes.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure captions

Fig. 1. Chemical structures of phenolic model compounds: (a) SECO, (b) HMR, (c) MR, (d) CYCLO, (e) and 7-HSECO lignans, (f) tyrosine, and (g) trans-ferulic acid.

Fig. 2. Scheme of the versatile peroxidase (VP) catalytic cycle adapted from Ruiz-Dueñas et al. [10]. As shown, VP can oxidize, among others: (i) phenolic substrates (PhOH), such as lignans and tyrosine, to the corresponding phenoxy radicals (PhO·) which are able to polymerize; and (ii) Mn^{2+} to Mn^{3+}, the latter acting as a diffusible oxidizer of different compounds including phenols.

Fig. 3. Effect of (a) 1,2-propanediol and (b) ethanol on VP activity against three different substrates (Mn^{2+}, ABTS and DMP). ABTS and DMP reactions were followed in the presence and absence of 0.1 mM Mn^{2+}. All reactions were initiated with 0.1 mM H_2O_2. 100% corresponded to the enzymatic activity in the absence of those organic solvents, and values are expressed as the percentage of residual activity. Standard deviations from triplicates were less than 5% of the average value in all cases.

Fig. 4. MALDI-TOF mass spectra (reflector mode) from VP-treated lignans incubated during 24 h with 0.1 mM Mn^{2+} and two dosages of 0.1 mM H_2O_2 at 0 and 1 h (excluding HMR whose incubation was carried out during 2 h with 0.1 mM Mn^{2+} and two dosages of 0.5 mM H_2O_2 at 0 and 1 h): (a) HMR, (b) SECO, (c) MR, (d) CYCLO, and (e) 7-HSECO. Peptides were incubated for 2 h with 0.1 mM Mn^{2+} and two dosages of 0.1 mM H_2O_2 at 0 and 1 h: (f) GLY, (g) YST, (h) VYV, (i) YIGSR, and (j) VEPIPY. The number of monomers (n) of each lignan or peptide is shown above the corresponding peak. An enlargement of m/z 2200-3400 region of all substrates is framed excluding VEPIPY, VYV, and YIGSR whose enlargements correspond to the m/z 3500-7000 Da (lineal mode) since they form oligomers larger than 3500 Da. All the analyses were performed on duplicate (two independent reactions), showing identical spectra.

626 **Fig. 5.** Molar mass increase after VP treatment in the presence of 0.1 mM Mn^{2+} of: a) SECO
627 and b) HMR. Controls correspond to VP-untreated SECO or HMR with 0.1mM H_2O_2 at 24 h
628 of incubation. Controls corresponding to VP-untreated HMR with 0.5 mM H_2O_2 were similar
629 to the previous control and are not represented in Fig. 5b.
630 **Fig. 6.** MALDI-TOF mass spectra from VP-heteropolymerization of YIGSR peptide with (a)
631 SECO lignan and (b) HMR lignan during 2 hours in the presence of 0.1 mM Mn^{2+} and H_2O_2.
632 A second dosage of 0.1 mM peroxide was added after 1h incubation. The units' number of the
633 homo- and the hetero-polymers is shown above the corresponding peak. Analyses were
634 performed on duplicates of two independent reactions, showing identical spectra.
635 **Fig. 7.** Cross-linking of β-casein analysed by SDS-PAGE and TEM. a) SDS-PAGE of β-
636 casein (Lane 1); β-casein plus H_2O_2 (Lane 2); β-casein plus VP (Lane 3); VP/H_2O_2-treated β-
637 casein with 0.015 U mL^{-1} of VP (Lanes 4-6); VP/H_2O_2-treated β-casein with 0.15 U mL^{-1}
638 (Lanes 7-9); VP/H_2O_2-treated β-casein with 1.5 U mL^{-1} (Lanes 10-12). Arrows are signaling
639 the VP band (~43 kDa). The β-casein monomer is framed along the gel. b) TEM
640 photomicrographs of untreated β-casein (1), and VP-treated β-casein during 24 h of
641 incubation with an enzyme dosage of 1.5 U mL^{-1} dissolved in water (2) or in urea 6 M (3).
642 Arrows are signaling β-casein monomers.
643 **Fig. 8.** The effects of VP-treatment on the rheological properties of FAX gels. Samples were
644 analyzed in duplicate, showing coefficients of variation lower than 5%.

Tables

Table 1. Effect of 0.1 mM Mn^{2+} and incubation time on the VP-catalyzed polymerization of five different lignans. The control (CTL) was a VP-untreated sample incubated during 24 h, adding two dosages of 0.1 mM H_2O_2 at the beginning of the reaction and after 1 h, similarly to the VP-treatments. Analyses were performed on duplicates of two independent reactions, showing identical spectra.

Lignan	Solvent	Maximal degree of polymerization*							
		- Mn^{2+}				+ Mn^{2+}			
		CTL	30 min	2 h	24h	CTL	30 min	2 h	24h
SECO	Buffer	2	5	7	8	2	8	8	9
HMR	20% Ethanol	3	5	7	6	3	8	8	7
MR	20% Ethanol	2	4	5	5	2	5	6	7
CYCLO	20% Ethanol	2	5	6	6	2	6	7	7
7-HSECO	50% Ethanol	2	3	3	3	2	6	7	7

* Values represent the maximum number of lignan units cross-linked as detected by MALDI-TOF MS.

Table 2. Assignment of masses (m/z) of the longest lignan and peptide polymers detected by MALDI-TOF MS in VP-catalysed reactions. Homopolymerization and heteropolymerization experiments were performed in the presence of 0.1 mM Mn^{2+}, adding two dosages of 0.1 mM H_2O_2 at the beginning of the reaction and after 1 h. All reactions were incubated during 2 h excluding the homopolymerization of lignans which was carried out for 24 h. HMR polymerization was accomplished with 0.5 mM H_2O_2 during 2 h.

Substrate	**Monomer (Da)**	**DP***	**Predicted m/z (Da)**	**Experimental ** m/z (Da)**
Homopolymers				
Lignans				
SECO	362.1	9	3264.9	3264.1 (-0.8)
HMR	374.1	9	3372.9	3372.8 (-0.1)
MR	358.1	7	2516.7	2517.6 (0.9)
CYCLO	360.1	7	2530.7	2531.5 (0.8)
7-HSECO	378.1	7	2656.7	2656.4 (-0.3)
Peptides				
RKRSRKE	959.1	1	959.1	959.6 (0.5)
EPPGGSKVILF	1125.3	1	1125.3	1125.5 (0.2)
GLY	351.4	8	2797.2	2796.9 (-0.3)
YST	369.2	8	2939.0	2939.6 (0.6)
VYV	379.5	11	4154.5	4155.0 (0.5)
YIGSR	594.7	11	6521.7	6522.6 (0.9)
VEPIPY	716.8	7	5027.6	5028.2 (0.6)
Heteropolymers				
YIGSR/SECO	594.7/ 362.1	1/5	2394.2	2393.7 (-0.5)
YIGSR/HMR	594.7/374.1	1/4	2082.1	2082.6 (0.5)

* DP= maximal degree of polymerization.

** The m/z differences between the theoretical and the detected masses, shown in parentheses, are within the experimental error of the technique.

Figure 1

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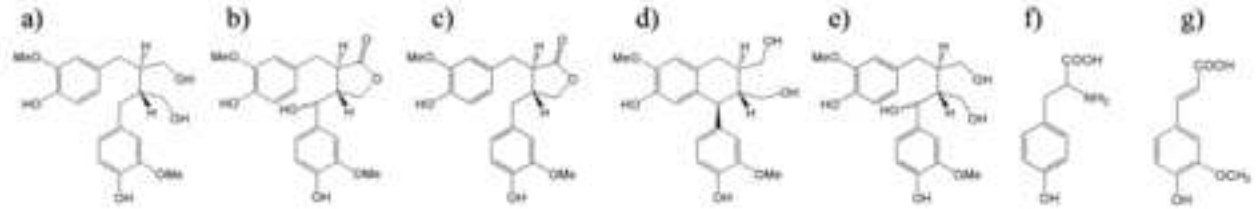

Figure 2

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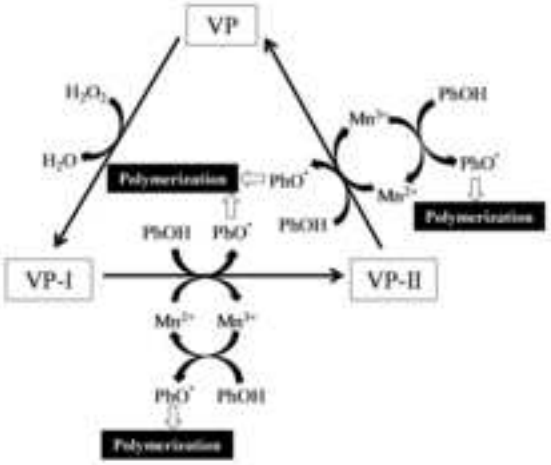

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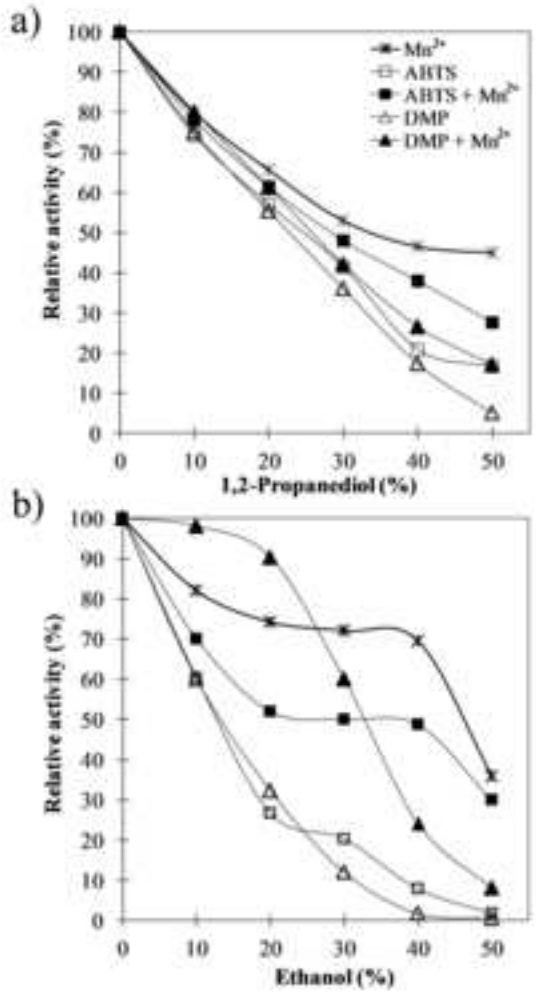

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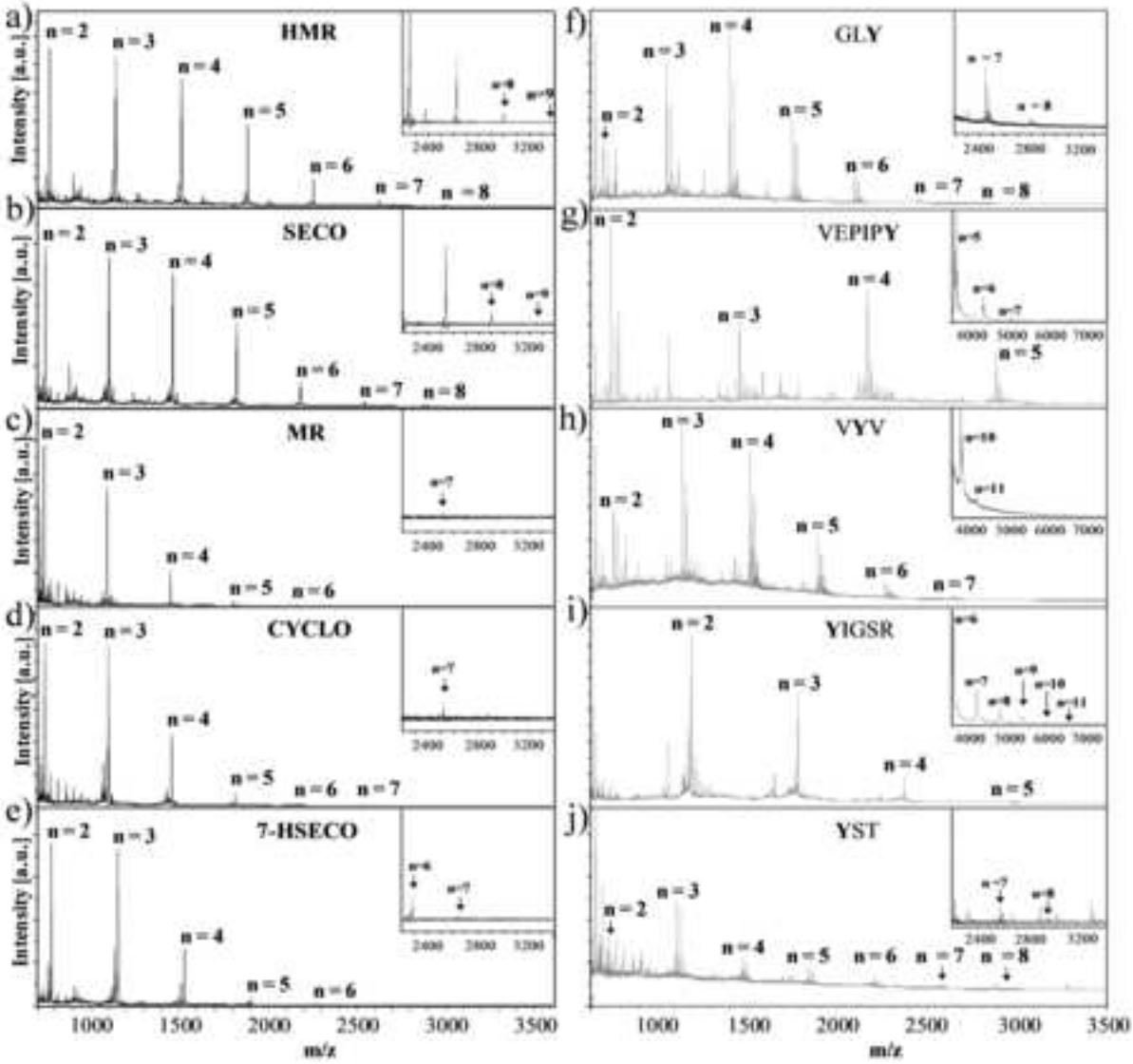

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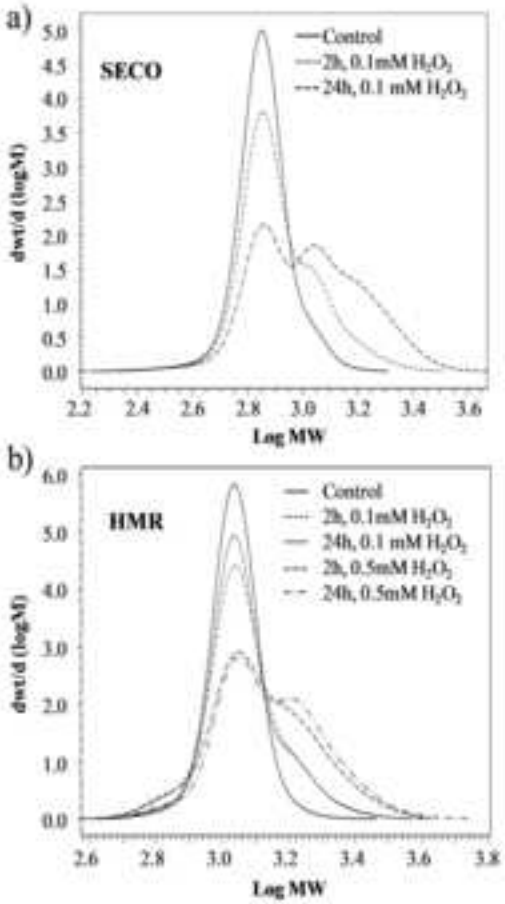

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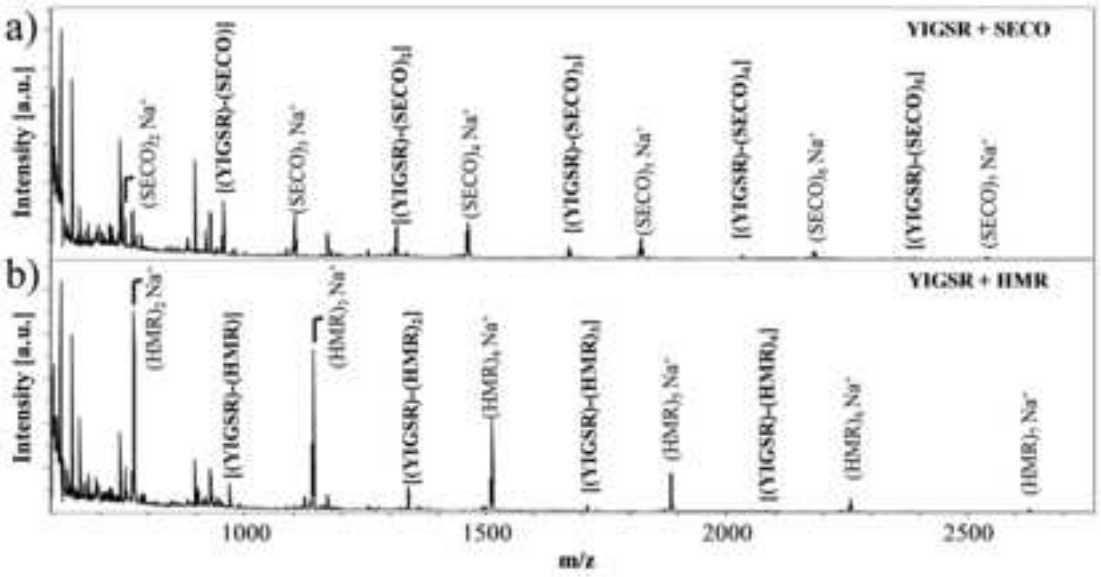

Figure 7

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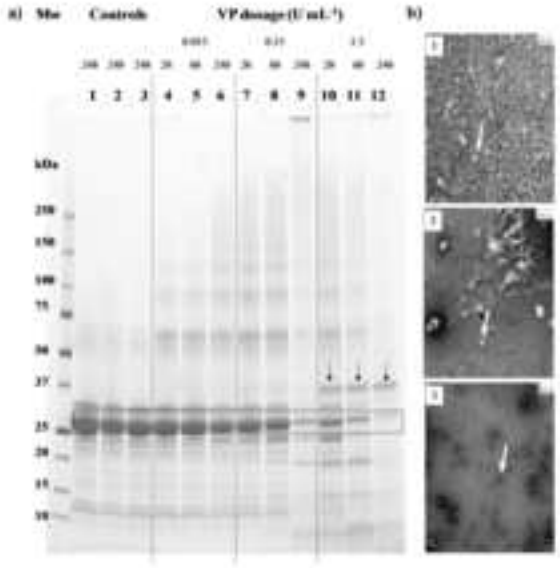

Figure 8

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