Optimisation of onion peroxidase-catalysed formation of aureusidin using 2',4',6',3,4-pentahydroxy chalcone as substrate

ABSTRACT

Previous investigations demonstrated that crude peroxidase (POD) obtained from onion solid wastes has the ability to catalyse the formation of the aurone aureusidin (ARS), using 2',4',6',3,4-pentahydroxy chalcone (PHC) as substrate, although this reaction under physiological conditions is mediated by a polyphenol oxidase-like enzyme, called aureusidin synthase (AS). In this study, a crude onion POD preparation was used to study the effect of some critical factors affecting the reaction, including reaction time, pH and temperature. The optimal set of conditions was identified by deploying central composite factorial design and response surface methodology. The results obtained showed that the optimum values for pH and temperature were 5 and 20°C, respectively, while time was found to exert a statistically non-significant effect. These values were the same or very close to optimal conditions found for structurally different onion POD substrates. The outcome was discussed with regard to the applicability of the onion POD as a versatile tool of biocatalysis.

Key words: aurones, aureusidin, onion, peroxidase, response surface methodology
B-ring [intermediate (a)] from 2’,6’,3,4-tetrahydroxy chalcone (THC) or PHC. The activated double bond of this intermediate undergoes then nucleophilic attack by its 2’-hydroxyl group to yield the intermediate (b), which in turn undergoes further rearrangement (tautomerism) to produce the aurone.

![Proposed mechanism of aurone synthesis from PHC catalysed by aureusidin synthase (AS).](image)

**Preparation of the onion solid waste homogenate**

The protocol previously described was used (Osman et al., 2008). Briefly, onion solid wastes were ground in a domestic blender and an aliquot of 2 g of the ground tissue was suspended in 15 mL buffer solution under stirring. The suspension formed was centrifuged at 3,000×g for 20 min and then filtered through paper filter to remove cell debris. The clear supernatant obtained was treated with activated charcoal for decolourisation, and filtered through celite under vacuum. The clear filtrate was used as the crude enzyme source.

**Peroxidase activity**

A previously described methodology was used (Osman et al. 2008), to determine the POD activity of the crude preparations. An aliquot of 0.1 mL of quercetin (0.1 mM in DMF) solution was mixed with 0.1 mL crude extract and 0.8 mL H$_2$O$_2$, and the reaction was followed by measuring the decrease in absorbance at 370 nm. One enzyme unit (U) was defined as ΔA$_{370}$ s$^{-1}$. Control reactions by omitting H$_2$O$_2$ or using heat-inactivated crude extract were also carried out. In assays performed at different temperatures, all constituents of the reaction mixture were pre-incubated in a thermostated water bath. Protein content of the homogenate was determined according to Bradford (1976), using BSA as standard. For all determinations, a computer-controlled HP 8452A diode-array spectrophotometer was used.

**Aureusidin synthesis**

Stock solutions of PHC (17.3 mM) were prepared in ethanol. PHC solution was diluted 1/10 with DMF prior to reactions. The reaction mixture was composed of 0.8 mL citrate/phosphate buffer (pH 3, 5 or 7) containing 3 mM H$_2$O$_2$, 0.1 mL crude enzyme extract (6.5 mU) and 0.1 mL of the diluted PHC solution. The mixture was incubated at various temperatures for predetermined periods, as dictated by the experimental design (Table 1). The reaction was arrested by adding 0.1 mL TCA (10% w/v in ethanol) and centrifuging the mixture at 5,000×g for 10 min.

**Liquid chromatography-mass spectrometry (LC-MS)**

The LC-MS analysis was performed using an LC-DAD-MS system comprising a Finnigan MAT Spectra System P4000 pump, coupled with a UV6000LP diode array detector and a Finnigan AQA mass spectrometer. The separation was performed on a 125×2 mm Superspher 100–4, RP-18 column (Macherey-Nagel, 4 μm particle size) at a flow rate of 0.33 mL min$^{-1}$, the column being kept at 40°C. The detection was...
monitored at 278 nm. The MS-ESI(+) spectroscopy was performed at a probe temperature of 350°C, probe voltage of 4 kV and at 12 and 40 eV collision energy in the mass analyzer. The mass range was set at 121–787 amu and the scan rate was 0.8 scans s⁻¹. The following gradient programme was used: (A) acetic acid (2.5%) and (B) methanol; 100% A for 5 min, 0% A in 15 min and kept at 0% A for another 5 min. The data were processed using the Xcalibur 1.2 software.

Table 1. Experimental values and coded levels of the independent variables used for the 2³ full-factorial design.

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Code units</th>
<th>Coded variable level</th>
</tr>
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<tbody>
<tr>
<td>T (°C)</td>
<td>X₁</td>
<td>-1 0 1</td>
</tr>
<tr>
<td>pH</td>
<td>X₂</td>
<td>5 6 7</td>
</tr>
<tr>
<td>Time (h)</td>
<td>X₃</td>
<td>1 3 5</td>
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Experimental design and statistical analyses

A 2³ full-factorial, central composite experimental design was used to identify the relationship existing between the response function and process variables, as well as to determine those conditions that optimised the POD-catalysed process. The response function considered was the ARS production, estimated by the peak area at 278 nm, in the chromatograms obtained by the LC-MS system. The three independent variables or factors considered were temperature (X₁, varying between 20 and 40°C), pH (X₂, varying between 3 and 7) and time [X₃, varying between 1 and 5 h]. Each variable to be optimised was coded at three levels, -1, 0 and 1 (Table 1). The choice of value ranges for each variable was based on preliminary experimentation and literature (Moussouni et al., 2010).

The three independent variables were coded according to the following equation:

\[ x_i = \frac{X_i - X_0}{\Delta X_i}, \quad x_i = 1, 2, 3 \]

where \( x_i \) and \( X_i \) are the dimensionless and the actual value of the independent variable \( i \), \( X_0 \) the actual value of the independent variable \( i \) at the central point, and \( \Delta X_i \) the step change of \( X_i \) corresponding to a unit variation of the dimensionless value. Response at each design point was recorded (Table 2). Data from the central composite experimental design were subjected to regression analysis using least square regression methodology to obtain the parameters of the mathematical models.

The statistical significance of the regression coefficients deriving from the model was assessed by Student’s t-test. Analysis of variance (ANOVA) was used to evaluate the overall statistical significance of the model. Response surface plots were obtained using the fitted model, by keeping the independent variables simultaneous. All determinations were carried out at least in triplicate and values were averaged and given along the standard deviation (± S. D.). For all statistics, Microsoft Excel™ 2000, SigmaPlot™ 11 and JMP™ 8 were used.

Table 2. Measured and predicted ARS peak area values determined for individual design points.

<table>
<thead>
<tr>
<th>Design point</th>
<th>Independent variables</th>
<th>Response Peak area (aureusidin)</th>
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<tbody>
<tr>
<td></td>
<td>X₁</td>
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<tr>
<td>1</td>
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Results and Discussion

Tentative identification of the reaction product

In Figure 2 can be seen a chromatographic trace recorded at 278 nm. The compound eluted at 8.28 min could easily be identified as the starting material (PHC), based on the retention time of an original standard. The peak eluted at 7.75 min showed a typical aurone UV-vis spectrum, with \( \lambda_{max} \) at 398 nm and two shoulders at 268 and 322 nm (Figure 3). The mass spectrum revealed a pseudo-molecular ion at \( m/z = 287 \).
and diagnostic ions at \(m/z = 309\) (Na\(^+\) adduct) and \(m/z = 595\), which could be ascribed to a Na\(^+\) adduct of aureusidin dimer [2M+23]\(^+\). Thus this peak was assigned to ARS.

### Optimisation of reaction conditions

Values of the independent process variables (\(X_1\), \(X_2\) and \(X_3\)) considered, as well as measured and predicted values for the response (aureusidine production as peak area) are analytically given in Table 2. The experimental values of the response were analysed by multiple regression to fit the following second-order polynomial equation:

\[
\text{Peak area} = 34.54 - 21.75X_1 - 115.03X_2 + 10.4X_3 + 43.43X_1X_2 - 1.25X_2X_3 - 3.62X_3 - 30.41X_1^2 + 115.39X_2^2 - 30.86X_3^2
\]

The quality of fit was ascertained using the regression coefficients (\(R^2\)). The experimental data obtained showed a good fit with the equations (\(R^2 = 0.95\), \(p = 0.0083\)). This fact indicated a satisfactory agreement between observed and predicted responses and that the equation found can adequately predict the experimental results.

After removal of the non-significant factors, as revealed by the ANOVA analysis, the theoretical model could be simplified as follows:

\[
\text{Peak area} = 34.54 - 115.03X_2 + 43.43X_1X_2 + 115.39X_2^2
\]

The utilisation of the predictive model enabled the theoretical calculation of the optimal set of conditions, that were \(\text{pH} = 5\), \(t = 1\) h, and \(T = 20\) °C. Under these conditions, the maximum theoretically calculated peak area was 328.7±83.0. The trend concerning the co-variation of the two significant variables (T, pH) was recorded in the form of a three-dimensional plot (Figure 4).

The optimum pH value is in accordance with previous findings on onion POD for a variety of substrates, including the flavonol morin (Osman & Makris, 2011), as well as simpler molecules, such as caffeic acid (El Agha et al., 2009) and \(p\)-coumaric acid (El Agha & Makris, 2012). In addition, ARS production upon AS action on THC as substrate was found to have an optimum \(\text{pH} 5.4\) (Sato et al., 2001). Likewise, the optimum temperature is in line with that found for chlorogenic acid (Osman et al., 2012), but also close to the values reported for the flavonols fisetin (Osman & Makris, 2010) and morin (Osman & Makris, 2011) and for caffeic acid (El Agha et al., 2009). Based on this information and the data presented herein, it would appear that the crude onion POD exhibits similar biochemical characteristics with a variety of substrates. However, optimisation using response surface methodology enabled the identification of the ideal combination of variables that favoured high turnover rates for the specific reaction of PHC oxidation into ARS. The latter remains to be elucidated by kinetic studies.

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**Figure 2.** An HPLC trace recorded at 278 showing both the starting material (PHC) and the product (ARS).

**Figure 3.** UV-Vis (upper figure) and mass spectrum (lower figure) of ARS.

Peroxidases are considered versatile biocatalytic tools, mediating a range of oxidative reactions and have been used for purposes of bioremediation (Hamid & Rahman, 2009; Barakat et al., 2010), synthesis of chemicals, as well as generation of bio-based substances, including sinapic acid dimers and other oligomers (Liu et al., 2007) and ferulic acid. [http://www.jbb.uni-plovdiv.bg](http://www.jbb.uni-plovdiv.bg)
acid/resveratrol heterodimers (Yu et al., 2007). Peroxidases have also been shown to form ferulic and caffeic acid dimers (El Agha et al., 2008a; 2009) and similar products from chlorogenic and p-coumaric acids (El Agha & Makris, 2012; Osman et al. 2012).

However, quercetin oxidation by crude onion POD was demonstrated to afford oxidation products not encountered in reactions with peroxidases from other sources (Osman et al., 2008). Further to that, there has been substantial evidence that onion POD can act in the same manner with PPO, producing structurally similar compounds, as exemplified by the case of ARS formation using PHC as substrate. Recent reports also showed that potato peel PPO-catalysed hydrocaffeic acid oxidation (Demian & Makris, 2013) yielded exactly the same products as the onion POD-catalysed reaction (El Agha et al., 2008b). The fact that crude onion POD might use alternative pathways for substrate oxidation merits a profounder examination, as it may lead to novel applications in green synthesis and other biocatalytic processes.

Conclusion

A crude onion POD was used to generate ARS using PHC as substrate. Critical parameters that affect the enzymic reaction, including temperature, pH and time, were assessed simultaneously by deploying central composite factorial design and response surface methodology. The optimum values for ARS production regarding temperature and pH were 20°C and 5, respectively. Time was found to exert a statistically non-significant effect and thus the shorter interval tested (1 h) was chosen. The findings concerning pH and temperature were consistent with previous reports on other, structurally unrelated substrates, which suggested that the enzyme preparation expresses optimal activity within narrow ranges of conditions. It is proposed that kinetic studies are required to identify dependence of activity on both PHC and H₂O₂ concentrations, as well as to illuminate putative mechanism of action. This is particularly important, given the sound evidence that the oxidation mechanism(s) involved might share common features with the ones proposed for polyphenol oxidases.

References


http://www.jbb.uni-plovdiv.bg


