**Abstract**

Capsiate, dihydrocapsiate, and nordihydrocapsiate are part of a group of metabolites from *Capsicum annuum* which are known as capsinoids. They are structurally and functionally similar to capsaicinoids, the substances that causes pungency in hot peppers, but the capsinoids are non-pungent compounds. The only structural difference between the capsaicinoids and capsinoids is the way in which the carbon chain is bound to the aromatic ring: by an amide moiety in capsaicinoids and by an ester moiety in capsinoids. Secretory plant peroxidases may be directly related to capsaicinoid metabolism, and the oxidation of capsaicin and dihydrocapsaicin by a pepper peroxidase was reported earlier. The aim of the present work was to study the ability of pepper peroxidases to oxidize capsiate. In order to obtain an extract enriched in basic peroxidases, a crude extract from local peppers was subjected to a two-step chromatography protocol. This extract oxidized capsiate following a Michaelis-Menten kinetic. The use of specific inhibitors confirmed the main role of secretory peroxidases in this oxidation. The MS spectrum (Orbitrap) of the principal oxidation-products, revealed a molecular ion of m/z=609, according to a 5-5' dicapsiate structure. This compound is analogous to the main product previously described for the oxidation of capsaicin [1].

**1. Introduction**

Capsinoids are a group of secondary metabolites from *Capsicum annuum* which include capsiate, dihydrocapsiate, and nordihydrocapsiate. Capsinoids were recently isolated from some sweet peppers as non-pungent compounds similar to capsaicinoids in terms of structure and biological activities. The structural difference between the capsaicinoids and capsinoids is the way in which the carbon chain is bound to the aromatic ring: by an amide moiety in capsaicinoids and by an ester moiety in capsinoids. Secretory plant peroxidases (EC 1.11.1.7; hydrogen donor: H$_2$O$_2$ oxidoreductase, Prxs) may be directly related to capsaicinoid metabolism since the vanillyl moiety of capsaicin is easily oxidized by this enzyme [2]. The first report of capsaicin oxidation by a peroxidase enzyme was from Boersch *et al.* [3]. Very soon after, Bernal *et al.* [1] reported the first data of capsaicin and dihydrocapsaicin oxidation by a pepper peroxidase. The dependence of the oxidation rate on capsaicinoids and H$_2$O$_2$ concentrations shows a kinetic behavior of the Michaelis-Menten type at low substrate concentrations, with inhibition at high substrate concentrations. The aim of the present work was to study the ability of pepper peroxidases to oxidize capsiate. In order to obtain an extract enriched in basic
peroxidases, a crude extract from local peppers was subjected to a two-step chromatography protocol. The use of specific inhibitors (tropolone/ferulic acid/potassium ferrocyanide and potassium ferricyanide) allow us confirm the main role of secretory peroxidases in the oxidation of capsiate. The MS spectra obtained by the use of an Orbitrap spectrometer provide information about the chemical structure of the main compounds obtained from the oxidation of capsiate by pepper peroxidases.

2. Material and Methods

2.1. Semipurification of pepper peroxidases

To perform peroxidase purification, we followed a three-step protocol including ammonium sulphate precipitation, adsorption chromatography on phenyl sepharose and cationic chromatography on SP sepharose. After the ammonium sulphate precipitation we considered two protein fractions, the first one from 0 to 80% of ammonium sulphate and the second one, from 80 to 95% of ammonium sulphate. Each fraction was pooled into a phenyl sepharose chromatography. The peaks from the adsorption column were then loaded into a cationic exchange chromatography, and the peroxidase bound to SP sepharose matrix was eluted with a linear gradient of 0 to 1 M KCl.

2.2. Spectrophotometric determinations

The oxidation of capsiate by the *C. annuum* basic peroxidases, in the presence and in the absence of H$_2$O$_2$, was assayed spectrophotometrically at 25°C in a reaction medium containing 50 mM Phosphate buffer (pH 5.5), different capsiate concentrations (0.22 mM, 0.44 mM, 0.87 mM, 1.31 mM), and hydrogen peroxide concentration (0.5 mM) using the $\varepsilon_{230} = 0.27$ mM$^{-1}$ cm$^{-1}$. The specific inhibitors tropolone, ferulic acid, potassium ferrocyanide and potassium ferricyanide are added at a 1 mM concentration one minute before the capsiate addition.

2.3. HPLC/MS Orbitrap analysis

The reversed-phase HPLC analyses were carried out on Thermo Accela high-performance liquid chromatograph, equipped with a Thermo LTQ Orbitrap Discovery detector. The HPLC column was a C18 SunFire (5 mm particle size, 150 mm x 4.6 mm i.d.) from Waters. The oven temperature was set at 30 °C. Extracts were passed through a 0.45 mm filter (Millex-HV, Millipore) and a volume of 10 mL of solution was injected. The flow rate was 0.8 mL/min and the mobile phase consisted of 2% acetic acid as solvent A and acetonitrile as solvent B. The gradient profile was 85% A at 0 min, 20% A at 15 min, 0% A at 35 min, 0% A at 40 min and 85% A at 45 min. The mass spectrometry system was a LTQ-Orbitrap® Discovery mass spectrometer (Thermo-Fisher Scientific) equipped with an electrospray ionization (ESI) source operating in negative ionization mode. The ESI source conditions were: source voltage -3.51 kV, heated capillary temperature 350 °C, capillary voltage -35 V and sheath gas and auxiliary gas, 50 and 10 (N$_2$ arbitrary units). For full scan MS analysis, the spectra were recorded in the range of m/z 80 to 800 with a scan speed of 1 scan/s. The analysis was carried out with source fragmentation at 35V and the mass resolution was set at 30.000.
3. Results

The purification procedure used allowed us to obtain a semipurificated extract of cationic peroxidases from pepper (figure 1). Two major peroxidase isoenzyme groups can be distinguished in Capsicum by their individual isoelectric points. The first major group is composed of peroxidase isoenzymes of acidic isoelectric point named APrx, and the second group corresponds to peroxidase isoenzymes of basic isoelectric point (BPrx) [1]. Basic peroxidases are located in the cell walls and the vacuoles, where there is only the strong basic isoenzyme B6. According to the vacuole as the hypothetical place for the accumulation of capsiate, this extract obtained seems adequate to carry out the study of oxidation of the capsiate by pepper peroxidases.

![Figure 1](image)

Figure 1
Isoelectrofocusing of the semipurification steps: (1) crude extract, (2) ammonium sulphate precipitate, (3) hydrophobic chromatography, (4) cationic chromatography

The oxidation of capsiate by pepper peroxidases was monitored by the increase in absorbance in the ultraviolet region (figure 2), showing a maximum change at 230 nm. The dependence of the oxidation rate on capsaicinoids and H₂O₂ concentrations shows a kinetic behavior of the Michaelis-Menten type at low substrate concentrations, with inhibition at high substrate concentrations.
The use of different inhibitors allows us to confirm the peroxidase nature for the activity detected (table 1). The activity of peroxidase was suppressed by 1 mM ferulic acid but not by troplone. These results strongly support a role for the *C. annuum* basic peroxidases as the responsible for the oxidation of capsiate. Further evidence was obtained from the use of a peroxidase substrate, ferrocyanide (K₄Fe[CN]₆), as a competitive inhibitor of the enzyme. The advantage of using ferrocyanide is the possibility of having an analog, ferricyanide (K₃Fe[CN]₆), which is not a peroxidase substrate, and may be used for evaluating any collateral effects on cyanide/iron complexes [4]. Ferrocyanide inhibits the capsiate oxidation, this observation and the fact that ferricyanide had no noticeable effect, suggest that the capsiate oxidation is due to a Class III peroxidase activity.
Table 1
Effect of inhibitors on the activity of C. annuum peroxidases

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Capsiate oxidation (nmol s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.61 ± 0.39</td>
</tr>
<tr>
<td>Ferulic acid (1mM)</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Tropolone (1mM)</td>
<td>2.69 ± 0.68</td>
</tr>
<tr>
<td>(K₄Fe[CN]₆) (1mM)</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>(K₃Fe[CN]₆) (1mM)</td>
<td>1.55 ± 0.57</td>
</tr>
</tbody>
</table>

The analysis of the products from de oxidation of capsiate by HPLC-MS, shows several compounds. One of these compounds has a MS spectrum with a maximum m/z fragment of 609.34 (figure 3). This spectrum supports a 5-5′-dicapsiate structure for this compound. According to the molecular weight of 5-5′-dicapsiate (610.35), a negative ionization generates a fragment with a m/z of 609.35. The figure 4 shows a hypothetical reaction of capsiate and peroxidases that produces the 5-5′-dicapsiate.

![Figure 3](image3.png)
Figure 3
Spectra for the compound obtained by oxidation of capsiate A) PDA spectrum, B) MS spectrum

![Figure 4](image4.png)
Figure 4
Reaction proposed for the synthesis of 5-5′-dicapsiate throw the oxidation of capsiate by C. annuum peroxidases
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