The influence of water activity on thermal stability of horseradish peroxidase

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Summary

The thermal stability of horseradish peroxidase in the solid state was studied as a function of water activity, from 0.11 to 0.88. At all activities the enzyme was found to be much more stable in the solid state than in solution. Inactivation temperatures were in the range of 140–160°C. Inactivation curves show a biphasic behaviour which can be described by a model assuming two fractions (heat labile and heat stable) with independent first order inactivation kinetics. The labile fraction represents approximately 30% of the total activity. The z-value for both stable and labile fractions depends on water activity (moisture content) and has a maximum at \( a_w = 0.76 \) (44.4°C and 43.8°C, respectively).

Keywords

Decimal reduction time, z-value, enzyme thermal inactivation, low moisture stability.

Introduction

Peroxidase is one of the most heat stable enzymes in vegetables and its thermal characteristics in aqueous solution are well reported (Chang et al., 1988; Ling & Lund, 1978; Vamos-Vigayzo, 1981; Lu & Whitaker, 1974). Weng et al. (1991a) found a z-value of 10°C for the heat stable fraction of immobilized horseradish peroxidase in dodecane. This result was successfully used to develop a peroxidase based Time-Temperature Integrator (Weng et al., 1991b) and for the determination of the liquid particle heat transfer coefficient (Weng et al., 1991c). However, the use of this system is limited to pasteurization temperatures, since for higher temperatures the enzyme inactivates too fast. In order to develop a similar system for higher temperatures, a more stable form of the enzyme must be found.

Dehydrated enzymes have been reported in literature as being much more thermostable than the same enzymes in solution. This profound effect of water on the thermal stability of enzymes, is due to the fact that water participates in all non-covalent interactions that maintain the catalytically active conformation of
enzymes (Klibanov, 1986). Some proteins were found to be more thermostable when dehydrated e.g. porcine pancreatic lipase (Zaks & Klibanov, 1984), phospholipase D (Weng et al., 1988), pancreatic and wheat grain ribonuclease (Multon & Guilbot, 1975), β-lactoglobulin (Rüegg et al., 1975), myoglobin (Hägerdal & Martens, 1976) and α-amylase (Meerdink & van't Riet, 1991). In the case of the first two enzymes, organic solvents were used to control the amount of water that interacts with the enzyme molecule, while in the other proteins this was done by equilibrating of the solid protein above standard salt solutions of different water activities. For α-amylase the data were obtained in drying experiments.

In order to determine the effect of moisture on the thermal stability of horseradish peroxidase, the thermal characteristics (D and z-values) of this enzyme were studied as a function of water activity. The sorption isotherm of the same enzyme was also determined to help correlate the experimentally obtained kinetic parameters and moisture content.

**Materials and methods**

Duplicate samples of horseradish peroxidase (EC 1.11.1.7., RZ = 0.98; Sigma, St. Louis, MO; 80 mg l⁻¹ in distilled water, 250 µl) were lyophilized (Secfroid, Lausanne, Switzerland) in crimp top vials (Chrompack, 0.8 ml) before equilibration (6 d at 4°C) over standard salt solutions of lithium chloride, potassium acetate, potassium carbonate, sodium chloride, and potassium chloride respectively at water activities (a_w) of 0.11, 0.23, 0.43, 0.76, and 0.88 (Greenspan, 1977). The inactivation experiments were performed on the seventh day.

**Thermal treatment**

For each a_w, prior to inactivation, the vials were hand crimped tightly as rapidly as possible to avoid atmosphere changes. The closed vials were then transferred directly from 4°C to an oil bath at the desired inactivation temperature and after predetermined time intervals, quickly transferred to an ice bath. After cooling, the vials were opened, phosphate buffer (0.1 M, pH 7.0, 625 µl) added to each and held in ice until analysis for remaining activity.

**Activity analysis**

The peroxidase activity was measured (Worthington, 1978) by adding peroxidase solution (100 µl) to substrate solution (2.9 ml) at 25°C and recording the increase in optical density at 510 nm (LKB 4053 Kinetics Spectrophotometer, Cambridge, UK) every 3 s for 1 min. The substrate solution contained 0.9 mM H_2O_2 (27%, w/w), 83.1 mM phenol (both UCB, Brussels, Belgium) and 1.19 mM 4-aminocy嗣ipryne (Janssen, Beersse Belgium) in 0.1 M phosphate buffer (pH 7.0). The data were analysed using linear regression, and the initial reaction rate was used to express activity of the enzyme (AOD min⁻¹). The substrate solution of the enzyme was always freshly prepared.

**Sorption isotherm**

Duplicate samples (100 mg) of horseradish peroxidase dried above P_2O_5 at 4°C for two weeks were equilibrated at 4°C above standard salt solutions of a_w 0.11, 0.23, 0.34, 0.43, 0.59, 0.64, 0.76, 0.82, and 0.88 (Greenspan, 1977). Moisture content was determined daily by weighing the closed vials after holding at room temperature
for 30 min in a desiccator. Each final moisture content for each $a_w$ was the average of the last four constant weights for each duplicate.

Data analysis

The inactivation kinetics of soluble horseradish peroxidase were analysed by the two-fraction model (a heat stable fraction and a heat labile fraction—Ling & Lund, 1978). In the two fraction model, each fraction of peroxidase is assumed to follow first order kinetics:

$$\frac{A}{A_0} = A_{os} \exp\left(\frac{-t \cdot ln(10)}{D_s}\right) + (1 - A_{os}) \exp\left(\frac{-t \cdot ln(10)}{D_l}\right)$$  \[1\]

where $A =$ total residual activity of the enzyme (ΔOD min$^{-1}$); $A_0 =$ total initial activity of the enzyme (ΔOD min$^{-1}$); $A_{os} =$ the fraction of the heat stable part of the enzyme; $D_s =$ decimal reduction time of the heat stable fraction of the enzyme (min); $D_l =$ decimal reduction time of heat labile fraction of the enzyme (min); $t =$ time (min).

Using non linear regression analysis (Marquardt method — Marquardt, 1963) of the SAS Software package (SAS, 1982), the unknown parameters ($D_s$, $D_l$, and $A_{os}$) were estimated from the experimental data.

It is common to present the temperature dependence of the D-value as a z-value. The z-value is the temperature increase needed to reduce the D-value by one log-unit.

$$D = D_{ref} \cdot 10^{(T - T_{ref})/z}$$  \[2\]

where $D =$ decimal reduction time at temperature $T$ (min); $D_{ref} =$ decimal reduction time at a reference temperature (min); $T =$ temperature (°C); $T_{ref} =$ a reference temperature (°C).

The z-values for both labile and stable fractions were calculated, respectively, by plotting Log($D_s$) and Log($D_l$) as a function of temperature (°C).

Results and discussion

Preliminary experiments

Weng et al. (1991a) established the optimum conditions for analysis of enzyme activity as pH 7.0, 25°C and ionic strength 0.1 M. Using the same conditions we found a linear relation between enzyme concentration and ΔOD min$^{-1}$ for ΔOD-values between 0.0 and 1.2. All samples for activity measurements were diluted to be in this range.

The lyophilization was done from water, since after lyophilization in buffer, the salts remaining from buffer could control the amount of water absorbed and alter the thermal characteristics of the enzyme (Gibriel et al., 1978). Preliminary experiments showed a yield of 95% of enzyme activity after lyophilization.

The activity of the enzyme held above standard salt solutions of $a_w$ 0.11, 0.23, 0.43, and 0.88 and 4°C, showed a slight increase after the first hours of storage and then remained constant for storage times up to 10 d.

After thermal inactivation in aqueous solution peroxidase is known to regenerate (Adams, 1978), but tests on partially inactivated peroxidase gave no indication of detectable regeneration.

The sorption isotherm at 4°C (Fig. 1) was constructed to relate moisture content
to water activity because the latter was used to control the moisture content of the enzyme preparation prior to thermal inactivation.

Inactivation

Examples of inactivation curves at $a_w$ 0.23 and 0.88 for four temperatures (Figs 2 and 3) show that inactivation temperatures are much higher (140–160°C) than those available (60–90°C) for inactivation of peroxidase in aqueous and organic media. Peroxidase clearly has a much higher thermostability in the dry state. Low moisture content was also found to have a profound effect on thermostability of other proteins (Multon & Guilbot; 1975, Rüegg et al., 1975; Hägerdal & Martens, 1976; Zaks & Klíbanov, 1984). In addition, the data clearly show that (as in aqueous solution) the inactivation cannot be described by first order reaction kinetics, but that a biphasic (two fraction model) behaviour is observed.

The estimated kinetic parameters, as contribution of the stable fraction to the total activity ($A_{on}$), and decimal reduction times, using the two fraction model are summarized in Table 1, and examples of best fitted curves are included in Figs 2 and 3. $A_{on}$ values are higher in the second batch of peroxidase (Table 1); similar variation has also been reported by Weng (1991) in heat inactivation of moist peroxidase in aqueous and organic systems.

Using linear regression on the logarithm of the decimal reduction times as a function of temperature, $z$-values for labile and stable fraction were calculated for each $a_w$-value (moisture content). Results are tabulated in Table 2. In the literature, $z$-values for the heat stable fraction of 26.3°C (Weng et al., 1991a), 27.3°C (Ling & Lund, 1978) and 38°C (Wang & Dimarco, 1972) have been reported, with lower values for the heat labile fraction of 10°C, 17.2°C and 23.3°C, respectively, whereas our $z$-values for both fractions are about the same (the biggest difference is 6°C for $a_w = 0.23$). Perhaps the comparison of results is not straightforward because in the current case the experiments were carried out with the enzyme in a solid state, and as far as we know, there are no data available on the effect of moisture on the thermo-stability of peroxidase.
Figure 2. Heat inactivation kinetics of peroxidase at $a_w = 0.23$ at 140°C (■), 145°C (◆), 152°C (●) and 165 (▲), respectively, and best fitted curve using two fraction model.

Figure 3. Heat inactivation kinetics of peroxidase at $a_w = 0.88$ at 140°C (■), 143°C (◆), 146°C (●) and 152°C (▲), respectively, and best fitted curve using two fraction model.

The $z$-values for both fractions have a maximum at $a_w = 0.76$ (water content of 16% on dry basis). A minimum activation energy (maximum $z$-value) at $a_w = 0.88$ (water content of 22% on dry basis), was also found for pancreatic ribonuclease by Mutton & Guilbot (1975). No straightforward relation can be made between the $z$-value and water activity or water content. The use of differential scanning calorimetry (DSC) showed that up to $a_w = 0.88$ all water present is 'unfreezable' water (that is, bound water — Troller & Christian, 1978).

The reason why dehydrated enzymes are more resistant to heat inactivation is not very well understood. Some authors proposed that after water removal from proteins, inter- and intramolecular electrostatic interactions and hydrogen bonds have to be established in order to satisfy the sites occupied before by water (Bull
Table 1. Kinetic parameters of peroxidase, estimated from the two fraction model and 95% confidence interval

<table>
<thead>
<tr>
<th>A_w</th>
<th>Batch*</th>
<th>Temp. (°C)</th>
<th>A_w, (%)</th>
<th>D_100 °C (min)</th>
<th>D_5 °C (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.11</td>
<td>1</td>
<td>135.0</td>
<td>32 ± 8</td>
<td>162 ± 43</td>
<td>22 ± 5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>140.0</td>
<td>34 ± 5</td>
<td>116 ± 15</td>
<td>11 ± 3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>145.5</td>
<td>27 ± 8</td>
<td>53 ± 13</td>
<td>8 ± 2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>151.0</td>
<td>24 ± 5</td>
<td>38 ± 7</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>0.23</td>
<td>1</td>
<td>140.0</td>
<td>29 ± 4</td>
<td>184 ± 19</td>
<td>23 ± 3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>145.0</td>
<td>19 ± 3</td>
<td>118 ± 17</td>
<td>10 ± 2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>152.0</td>
<td>13 ± 4</td>
<td>73 ± 19</td>
<td>8 ± 1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>165.5</td>
<td>24 ± 8</td>
<td>13 ± 3</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>0.43</td>
<td>1</td>
<td>136.5</td>
<td>34 ± 2</td>
<td>145 ± 10</td>
<td>15 ± 2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>147.0</td>
<td>26 ± 4</td>
<td>66 ± 8</td>
<td>8 ± 1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>158.0</td>
<td>20 ± 5</td>
<td>35 ± 7</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>0.76</td>
<td>2</td>
<td>131.0</td>
<td>38 ± 5</td>
<td>170 ± 93</td>
<td>21 ± 8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>140.0</td>
<td>42 ± 5</td>
<td>120 ± 12</td>
<td>17 ± 3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>146.0</td>
<td>21 ± 5</td>
<td>78 ± 12</td>
<td>13 ± 2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>155.0</td>
<td>26 ± 3</td>
<td>51 ± 5</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>0.88</td>
<td>2</td>
<td>140.0</td>
<td>38 ± 13</td>
<td>248 ± 97</td>
<td>25 ± 12</td>
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<tr>
<td></td>
<td>2</td>
<td>143.0</td>
<td>40 ± 8</td>
<td>130 ± 19</td>
<td>14 ± 6</td>
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<tr>
<td></td>
<td>2</td>
<td>146.0</td>
<td>41 ± 9</td>
<td>75 ± 10</td>
<td>15 ± 4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>152.0</td>
<td>50 ± 6</td>
<td>37 ± 3</td>
<td>5 ± 2</td>
</tr>
</tbody>
</table>

* Two batches of peroxidase were used.

° A_w = The fraction of the heat stable part of the enzyme.

Do = D_1 = Decimal reduction time of the heat stable fraction of the enzyme.

* D_1 = Decimal reduction time of heat labile fraction of the enzyme.

Table 2. z-value of peroxidase for stable (z_s) and labile (z_l) fractions for different water activities and moisture contents

<table>
<thead>
<tr>
<th>a_w</th>
<th>Moisture (% db)</th>
<th>Z_s (°C)</th>
<th>r</th>
<th>Z_l (°C)</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.11</td>
<td>1.4</td>
<td>24.0</td>
<td>0.986</td>
<td>24.2</td>
<td>0.991</td>
</tr>
<tr>
<td>0.23</td>
<td>2.6</td>
<td>22.3</td>
<td>0.992</td>
<td>28.7</td>
<td>0.982</td>
</tr>
<tr>
<td>0.43</td>
<td>5.5</td>
<td>34.8</td>
<td>0.998</td>
<td>35.4</td>
<td>0.999</td>
</tr>
<tr>
<td>0.76</td>
<td>16.2</td>
<td>44.4</td>
<td>0.994</td>
<td>43.8</td>
<td>0.958</td>
</tr>
<tr>
<td>0.88</td>
<td>25.6</td>
<td>14.9</td>
<td>0.988</td>
<td>17.7</td>
<td>0.968</td>
</tr>
</tbody>
</table>

(% db) = moisture content on dry basis (g H_2O 100 g^-1 dry solid).

r = correlation coefficient for linear regression of log(D) versus Temperature.

& Breese, 1968) and these new interactions cause the increase in thermostability. Other authors, supported by DSC-data, have the opinion that the increase in thermostability is due to the lack of a water medium to bring about thermal transitions (Hägerdal & Martens, 1976). Klibanov (1986), studying enzyme catalysis in organic media, proposed that only a small 'essential water layer' is necessary to maintain
the catalytically active conformation of the enzyme. Zaks & Klibanov (1984) found that porcine pancreatic lipase is not only more stable when dehydrated in organic solvents, but its substrate specificity is also changed. This can be attributed to the effect of water acting as if it were a 'lubricant' allowing conformational mobility of the enzyme molecule (Klibanov, 1986). This fact may be the reason for the enhanced thermostability of enzymes when dehydrated, since conformational mobility is necessary for partial unfolding, which is the first step of the thermo-inactivation process (Tanford, 1968; Lapanje, 1978). Whatever the mechanisms of enzyme thermostabilization at low water contents, the increase in thermal stability reveals that water plays an important role in the thermal inactivation of enzymes when dehydrated. This is confirmed by the results obtained in this study. In this study, both the D-values and Z-values change with water activity (moisture content). As a final remark we would like to stress that conclusions on thermostability can not be drawn from studies at a single lethal temperature. Comparing the D-values of the stable fraction for 140°C at water activities of 0.76 and 0.88 and for temperatures above 146°C at the same water activities would lead to opposite conclusions (see Table 1).

Conclusion

Horseradish peroxidase was found to be much more thermo-stable in the dry state compared with aqueous solutions of the enzyme. Inactivation temperatures are in the range of 140–160°C, and the inactivation shows a biphasic behaviour. Both decimal reduction times and z-value change with water activity, the z-value has a maximum at aw = 0.76.

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References


