Thermal Inactivation at High Temperatures and Regeneration of Green Asparagus Peroxidase

CARMEN RODRIGO,1* MIGUEL RODRIGO,2* ANDRÉS ALVARRUlZ,3 and ANA FRÍGOLA1

1Departamento de Medicina Preventiva, Salud Pública, Bromatología, Toxicología y Medicina Legal, Facultad de Farmacia, Universitat de València, C/Vicente Andres Estelles s/n, 46100 Burjassot, Valencia; 2*Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Apartado de Correspondencias 73, 46100 Burjassot, Valencia; 3Departamento de Tecnología de Alimentos, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain

(MS# 95-305: Received 29 November 1995/Accepted 13 February 1996)

ABSTRACT

A spectrophotometric method was developed for determining the peroxidase activity of green asparagus in small samples. The optimum conditions for the analysis in the cuvette were 45 mM of H2O2, 36 mM of guaiacol, and pH 7. The method can be used to determine enzyme activity at up to two decimal reductions. A study was performed of the regeneration and inactivation kinetics of the enzyme when heated between 90 and 125°C. Regenerated asparagus peroxidase reached its maximum activity after being stored 6 days at 25°C. The regenerated enzyme followed first-order inactivation kinetics, showing an Ea = 13.62 kcal/mol and k100°C = 2.07 min⁻¹.

Key words: Asparagus peroxidase, enzyme thermal inactivation, enzyme regeneration, enzyme kinetics, HTST

High-temperature short-time (HTST) processes can improve the quality of canned foods, provided that enzyme inactivation is achieved. Peroxidase (POD) is the enzyme used as an index of the adequacy of fruit and vegetable blanching to reduce quality losses in subsequent storage, because it is considered to be the most heat-resistant vegetable enzyme (8).

The method most commonly used for studying the thermal inactivation of peroxidase involves the use of glass tubes (capillary tubes or tubes of various sizes) heated in a thermostatic bath and cooled in ice water (8, 27). The use of capillary tubes reduces the heating-time lag and improves the accuracy of the estimated parameters if the data analysis is performed on the basis that the process is isothermal. Nevertheless, the small diameter and capacity of capillary tubes means that the amount of sample to be analyzed must be small (10 to 50 μl), and consequently a simple, sensitive method for determining POD activity is required to detect low levels of residual activity.

* Author for correspondence. Tel: 34 6 3 90 00 22; Fax: 34 6 3 63 63 01; Email: Mrrodrigo@iata.csic.es.
† This study constitutes partial fulfillment of the Ph.D. requirements for Carmen Rodrigo.
exposed to a particular temperature, so that HTST treatment is less effective for the irreversible inactivation of POD than a longer treatment at a lower temperature. The ability of POD to regenerate after it has been denatured by heat varies between different species of vegetables and also between isoenzymes of the same species (20). McLellan and Robinson (16) found that purified Brussels sprouts anionic isoperoxidases, unlike the crude preparations from Brussels sprouts and cabbage, did not regenerate following heat inactivation. The studies of McLellan and Robinson (16) and Moulding et al. (17) suggest that regeneration depends on some factor that is eliminated during the purification process. Therefore, when studying the thermal inactivation of these enzymes, any purification process should be avoided (28). Most authors studying asparagus POD (8, 11) did not separate the soluble POD fraction and the cell-wall-bound form. Wang and Luh (27), who separated the soluble and ionically bound fractions, observed that there were no great differences in their thermal behavior at low temperatures.

The aims of this study were to optimize a spectrophotometric method for determining the POD activity of an unpurified green asparagus extract in small fresh and and heated samples, and to study the thermal inactivation and regeneration kinetics of this enzyme at high temperatures. We first studied the stability of the POD extract at two temperatures, 4 and −40°C, and then looked for optimum conditions to determine the POD activity of an unconcentrated asparagus extract in citrate buffer and in phosphate buffer with 1 M NaCl, and of a concentrated extract in phosphate buffer with guaiacol. Finally we compared the sensitivity of the assay with guaiacol with the sensitivity obtained when using o-dianisidine, ABTS, and MBTH-DMAB as substrates. For the inactivation studies we heated the concentrated extract to temperatures ranging from 90 to 125°C for different times, stored the sample at 25°C for 6 days, and estimated the kinetics parameters of the regenerated enzyme with three different least-square methods.

MATERIALS AND METHODS

Green asparagus

Fresh green asparagus (Asparagus officinalis L. 'Mary Washington') was obtained from El Santo Cristo cooperative in Navarrés (Valencia, Spain). The vegetables were 1 to 2 cm in diameter and 20 to 25 cm in length, with closed tips. The asparagus was cut 12 cm from the tip, washed, drained, frozen with liquid N₂, wrapped in aluminum foil, and stored at −40°C for 15 days.

Extraction of POD

The extraction of POD from the asparagus spears was carried out using the procedure described by Wang and Luh (27), but without separating the soluble and the ionically bound wall fractions. The entire procedure was performed at 4°C. One hundred grams of frozen asparagus was cut into small pieces and triturated for 5 min at maximum speed in a blender with stainless steel blades (Hamilton Beach) with 150 ml 0.05 M sodium phosphate buffer pH 7 (prepared by mixing 39 ml of 0.2 M sodium phosphate monobasic dihydrate with 61 ml of 0.2 M disodium hydrogen phosphate anhydrous (6)), 1 M NaCl, and 5% polyvinylpyrrolidone (PVPP) (Sigma Chemical Co., St. Louis, MO, USA). The pulp was vacuum filtered through 6 layers of cheesecloth and the residue was centrifuged at 21,000 × g for 30 min at 4°C in polycarbonate tubes. The supernatant was stored for 9 months in 2-ml vials at −40°C and designated asparagus extract.

Concentration of the asparagus extract

The asparagus extract was concentrated by ultrafiltration to one-third of its original volume by using a concentrator developed at our Institute. The concentrator was a cylindrical polycrystalline chloride and methacrylate box with a socket in which a PM10 membrane (Diaflo Ultrafilters, Amicon Inc., Beverly MA, USA) was located. The membrane was made of polymers which allowed molecules with a molecular mass smaller than 10,000 Da to pass through (POD has a molecular mass of approximately 40,000 Da). The cylinder was connected to a nitrogen pump, and a pressure of 3.7 atm (ca. 375 kPa) was applied to obtain the desired filtration flux. The filtrate ejected was collected in a precipitate vessel at the bottom. A magnetic stirrer prevented the membrane from being compacted. The entire process was carried out at 4°C. The concentrated extract inside the box was homogenized, stored in 2-ml vials, frozen at −40°C and designated concentrated asparagus extract.

POD assays

Method with guaiacol. Basically we used the method described by Wang and Luh (27). The substrate saturation concentrations were obtained by varying the hydrogen peroxide and guaiacol concentrations while keeping the enzyme concentrations constant. The process was repeated until the enzyme was saturated with both substrates. With these concentrations, the pH was varied to obtain the level allowing the highest enzyme activity values. Fifty microliters of asparagus extract (concentrated and unconcentrated) was placed in polycarbonate tubes together with 4 ml of citrate-phosphate buffer (pH 7 with 1 M NaCl) (prepared by mixing 20 ml of 0.1 M citric acid with 77 ml of 0.2 M disodium hydrogen phosphate anhydrous, (6)) for the unconcentrated asparagus extract, and 3 ml of phosphate buffer (pH 7, 1 M NaCl) and 0.1% gelatine gold (Pancreas, Barcelona, Spain) for the concentrated asparagus extract. The solution was agitated and kept at 25°C for 7 min. The reaction began when 2.39 ml of the solution was placed in a plastic cuvette and mixed with 0.61 ml of a reaction mixture consisting of guaiacol, H₂O₂, and bidistilled water, with the absorbance being measured at 470 nm every 3 s for 5 min at 25°C. For the unconcentrated asparagus extract, the cuvette concentration when using citrate buffer as the reaction medium varied from 0.02 to 9.0 mM for hydrogen peroxide and from 0.07 to 15.8 mM for guaiacol; the pH was varied from 5 to 7.5. When phosphate buffer was used as the reaction medium, the cuvette concentration varied from 0.23 to 55 mM for hydrogen peroxide and from 2 to 36 mM for guaiacol. For the concentrated asparagus extract the cuvette guaiacol concentration was 36 mM and the hydrogen peroxide was variable between 0.23 and 88.45 mM.

Disposable plastic 3-ml cuvettes (ELKAY Lab System, Finland) were used. The spectrophotometer was a Milton Roy Spectronic 301 with a thermostatic cell, and absorbances were recorded with a Toshiba T1100 Plus computer. Enzyme activity was calculated from the slope of the linear part of the graph of absorbance versus time. One unit of POD activity was expressed as one absorbance increment (at 470 nm in the conditions in which the assay was carried out) per minute.

Method with other chromogens. For the assay with o-dianisidine we used the method of Wang and Luh (27). For ABTS and MBTH-DMAB, the methods of Arnao et al. (3) and Ngo and Lenhoff (19) respectively were used.
Regeneration of POD

We studied the regeneration of the concentrated asparagus extract POD after heating at 90°C for up to 20 s and at 110°C for up to 15 s. For each particular time-temperature combination, approximately 200 glass capillary tubes (Hirschmann Laborgeräte, Germany) with capacity 0.01 ml and length 12.5 cm, were used in groups of 15. Each capillary tube was filled with 0.01 ml of concentrated asparagus extract using a microliter syringe. The tubes were then sealed at both ends in a gas oxygen flame and heated in a stirred oil bath using a manual adaptation of the micromethod of Stern and Proctor (24). Fifteen unheated capillary tubes were used as controls. After heating, the tubes were rapidly cooled in ice water. The contents of each set of 15 capillary tubes were pooled in a vial containing 9 ml of 0.05 M phosphate buffer pH 7 (prepared by mixing 39 ml of 0.2 M sodium phosphate monobasic di-hydrate with 61 ml of 0.2 M disodium hydrogen phosphate anhydrous, (6)) with 1 M NaCl and 0.1% gelatine gold and stored at 25°C to allow maximum regeneration. Vials were stored for different periods before the residual activity was measured. The POD assay was performed using the guaiacol method described above. With 200 capillary tubes, approximately 13 points were obtained on the curve of activity versus storage (for each time-temperature combination).

Thermal inactivation of POD

POD inactivation was measured at 90, 100, 110, 120, and 125°C with exposure times ranging from 7 to 120 s. Capillary tubes in groups of five were filled with 0.01 ml each of concentrated asparagus extract, heated, and cooled by the same procedure as for the regeneration study. Heat-inactivated samples were stored at 25°C for 6 days before activities were assayed. The POD was assayed using the same procedure as for the regeneration study.

Estimation of reaction order and kinetics parameters

Kinetic deactivation of an enzyme can be described (2) by:

\[ \frac{dA}{dt} = -k_T A^n \]

where \( k_T \) = reaction rate constant (min\(^{-1}\)); \( A \) = enzyme activity at time \( t \); \( n \) = reaction order; and \( t \) = time (min).

The dependence of \( k_T \) on temperature can be described by two models:

\[ k_T = k_R \exp \left(-\frac{E_a R}{T(1/T - 1/T_{ref})}\right); \]

\[ D_T = D_R 10^{(T_{ref} - T)/\Delta T} \tag{2b} \]

Equation (2a) is the Arrhenius model and Equation (2b) is the Bigelow model (4), where \( k_R \) = reaction rate constant at reference temperature (min\(^{-1}\)); \( E_a \) = activation energy (kcal/mol); \( R \) = ideal gas constant, 1.987 cal/(mol K); \( T \) = temperature (K or °C); \( T_{ref} \) = reference temperature (K or °C); \( D_T \) = decimal reduction time at temperature \( T \) (min) (\( D_T = 2.303 k_R/T \)); \( D_R \) = decimal reduction time at temperature \( T_{ref} \) (min); \( z \) = temperature dependence of decimal reduction time (°C).

For isothermal conditions, Equation (1) can readily be integrated and Equation (2a) substituted, i.e.:

\[ A = A_0^{1-n} - (1-n)k_R \exp \left(-\frac{E_a R}{T(1/T - 1/T_{ref})}\right) t^{1/(1-n)}. \tag{3} \]

A nonlinear regression, Equation (3) was made with the whole set of data to estimate \( n \). For \( n = 1 \), integration of Equation (1) yields

\[ A = A_0 \exp (-k_T t). \]

Substituting from Equation (2a):

\[ A = A_0 \exp \left(-\frac{E_a R}{T(1/T - 1/T_{ref})} t\right). \tag{4b} \]

The same procedure using the Bigelow model yields

\[ A = A_0 10^{\left(-\frac{E_a R}{T_{ref} - T} \right) t}. \tag{5a} \]

\[ A = A_0 10^{\left(-\frac{E_a R}{T_{ref} - T} \right) t}. \tag{5b} \]

Taking logs twice in Equations (4b) and (5b) yields

\[ \log (\log (A/A_0)) - \log t = -log D_R - (T_{ref} - T)/z. \tag{6b} \]

The kinetics parameters \( E_a \) and \( k_R \) for the Arrhenius model and \( z \) and \( D_R \) for the Bigelow model were estimated using three least-square methods: two-step linear regression, one-step linear regression, and nonlinear regression.

Two-step linear regression is the most common procedure for estimating kinetics parameters; in the first regression, \( k_T \) and \( D_T \) values at each temperature were estimated by taking log and ln from Equations (4a) and (5a) respectively; in the second regression, the log and ln expressions of Equations (2a) and (2b) were used to calculate \( E_a \) and \( k_R \), and \( z \) and \( D_R \), respectively. In the one-step linear regression procedure Equations (6a) and (6b) were used to calculate kinetics parameters with the whole set of data, and in the nonlinear regression method Equations (4b) and (5b) were used for the same purpose. Calculations for nonlinear regression were performed using BMIDP 3R software (BMDP Statistical Software, Inc., Los Angeles, CA, USA).

RESULTS AND DISCUSSION

POD assays with guaiacol

It was seen that POD in asparagus extract stored at 4°C lost 67% of its activity after 1 day of storage, 83% after 2 days and 90% after 3 days; however, when stored at -40°C it was stable over 9 months. These data confirm the results obtained by Gkinis and Fennema (10), who consider that at such temperatures the enzymatic and chemical reactions are very slow or cease completely.

The optimization study of the analysis method began with citrate buffer as the reaction medium. When the hydrogen peroxide and guaiacol concentrations were varied for a fixed enzyme concentration, the enzyme followed Michaelis-Menten kinetics with both substrates. The substrate saturation concentrations were 3.25 mM of \( \text{H}_2\text{O}_2 \) and 11.26 mM of guaiacol, and for these concentrations pH 7 ± 0.5 proved to be the optimum. For different enzyme concentrations measured in optimum conditions, up to 10% of the initial enzymatic activity can be determined, i.e., one decimal reduction of the activity.

Some authors (5) use phosphate buffer as the reaction medium for the spectrophotometric analysis of POD in
various vegetables. To increase the sensitivity of the present method, a study was made of this buffer, with added NaCl so that the ionic strength of the medium did not vary (23). Hydrogen peroxide also followed Michaelis-Menten kinetics, 45 mM of H₂O₂ being the saturation concentration for the enzyme (Figure 1). Guaiacol was insoluble in water at concentrations greater than 36 mM, and therefore that concentration was taken as optimum, suitable care being taken in the analysis so that errors in the volumetric measurement of the sample would be minimal and would not affect the results. Enzyme activity for various POD concentrations was determined at pH 7. A linear relationship was observed (significant at 99%, R = 0.9971) with up to 5% of the initial enzymatic activity being detected. The detection limit has thus improved but is not sensitive enough for thermostability studies. The improvement in the detection limit might be due to the increase in ionic strength because of the NaCl.

The asparagus extract POD was concentrated to one-third of its volume, and we observed that there were no important activity losses in the concentration process. With this concentrated enzyme extract, new optimum measurement conditions were calculated, using phosphate buffer with 1 M NaCl and 0.1% gelatine gold to improve stability of the enzyme at low concentrations (29). The measurement conditions proved to be the same as for the unconcentrated extract. At these conditions, up to 1% of the initial enzymatic activity was detected (significant at 99%, R = 0.9981) (Figure 2). Therefore the method is suitable for subsequent calculation of kinetics parameters.

**POD assay with other chromogens**

To compare the sensitivity of the guaiacol method with methods using other chromogens such as o-dianisidine (27), ABTA (3), and MBTH-DMAB (19), we studied the linearity of each method for the unconcentrated asparagus extract (Figure 3). Guaiacol provided the most sensitive procedure for the highest enzyme concentrations, followed by o-dianisidine, and ABTS was best for the lowest concentrations. In contrast, in the MBTH-DMAB method changes in the enzyme concentration produced smaller changes in optical density. With o-dianisidine and ABTS, a linear relationship between enzyme concentration and activity was observed (R = 0.9976 and 0.9999 respectively, significant at 99%). The detection limit was similar to the guaiacol method: two decimal reductions of the activity could be detected. With MBTH-DMAB it was only possible to determine up to 5% of the residual activity (significant at 99%, R = 0.9959). Shindler et al. (22) studied the evolution...
of H$_2$O$_2$ and ABTS concentration in POD activity and observed that these substrates did not follow Michaelis-Menten kinetics; therefore, small changes in the concentrations of the two substrates could cause alterations in the results and also problems with reproducibility. MBTH-DMAB reacts when the enzyme is absent, giving a colored compound analogous to the product of the peroxidase reaction.

The optimized guaiacol method is the most suitable procedure, because it provides a rapid, simple method for measuring POD activity in concentrated raw asparagus extract, so that the thermal treatment can be performed with raw extracts. It is even more sensitive than the o-dianisidine procedure, where changes in the enzyme extract produced smaller changes in optical density; furthermore, it does not have the drawbacks previously mentioned in connection with the other methods.

**Asparagus POD regeneration**

The kinetics of asparagus POD regeneration was studied by treating the concentrated extract at 90°C for 20 s and at 115°C for 10 s and storing it at 25°C. At the turn of the century it was observed that if POD-containing plant extracts were heated and the precipitate thus formed separated from the solution, activity did not regenerate until the supernatant and the precipitate were recombined. Lu and Whitaker (13) deduced that the hemin in the enzyme remains in the solution and the protein portion is precipitated. To allow regeneration of the enzyme, therefore, the precipitate formed when the extract was heated was not separated from the solution.

The regeneration of enzyme activity did not follow first- or second-order kinetics (Figure 4). There was a rapid increase in activity for about the first 100 h and then the increase slowed. These results are similar to those obtained by Wang and Dimarco (26) for horseradish POD. However, the time taken for the activity to stabilize is considerably longer in our case than the time reported by Lu and Whitaker (13) for horseradish POD, by McLellan and Robinson (15) for POD from oranges, or by Varoquaux et al. (25) for POD from peas. We also observed, as did Lu and Whitaker (13), that activity regeneration depends on the intensity of the inactivation. After the most severe treatment (at 110°C for 10 s) the regeneratable $A_{max}$ is half the level possible after treatment at 90°C for 20 s.

The data for activity in relation to storage time at 25°C fit the following exponential equation:

$$\text{enzymatic activity} = a(1 - e^{-bt}) + c,$$

where $a =$ amplitude of the exponential, $b =$ rate constant (min$^{-1}$); $c =$ intercept (activity at $t = 0$) and $a + c =$ maximum of the exponential (regeneratable $A_{max}$). In our case, $a + c = 0.11$ and $0.25 \Delta A_{abs}/min$, $b = 0.015$ and $0.017$ min$^{-1}$ and $R = 0.9813$ and $0.9912$ for $90°C$, 20 s and $110°C$, 15 s respectively. We observed that in 6 days 85% of the maximum regeneratable activity in Equation (1) was achieved, and therefore we consider this period suitable for subsequently determining the activity and studying the kinetics of the inactivation prevented by regeneration.

**Heat inactivation of POD**

A nonlinear regression of Equation (3) with the whole set of experimental activity data was performed and a reaction order of 1.29 was estimated. To verify the first-order kinetics, a linear regression of ln $A$ versus $t$ was performed and the residues showed no systematic tendency as a function of time; therefore, a first-order reaction for the heat inactivation of asparagus POD was assumed to calculate the kinetics parameters. Figure 5 shows the curves for POD inactivation obtained by determining the residual activity regenerated in the samples after being heated at each
time-temperature combination and stored at 25°C for 6 days. The POD inactivation kinetics was not determined immediately after heat treatment because in many cases there was no residual activity.

\[ A_0 = \text{calculated and the data for activity (A) were expressed as absolute values and not as a percentage of the initial activity. This was because the uncertainty for } A_0 \text{ is at least of the same order of magnitude as the uncertainty for the other activities observed (A) (2, 7). The kinetics parameters were calculated by three different least-square methods: two-step linear regression, one-step linear regression, and nonlinear regression.} \]

The results obtained using the three procedures are summarized in Table 1, which shows that there is a greater similarity between the kinetics parameters estimated by two-step linear regression and by nonlinear regression than with those calculated by one-step linear regression. It can also be seen that the asymptotic standard deviation is generally greater with two-step linear regression.

The \( z \) values estimated with the three least-squares methods (45.49, 58.31, and 48.56°C) are close to those obtained by Gibriel et al. (9) for green beans (34°C) and for spinach (66°C), but somewhat higher than those generally given for POD, which vary from 13.56°C for turnip puree POD to 37°C for corn-on-the-cob POD (12). The \( E_a \) values estimated with the three regressions (14.51, 11.31, and 13.62 kcal/mol) are also similar to those obtained by Gibriel et al. (9) for PODs of carrot (16.06 kcal/mol), mango (10.58 kcal/mol), green beans (18.22 kcal/mol), and guava (19.15 kcal/mol), and to those calculated by Naveh et al. (18) for corn-on-the-cob POD (18 kcal/mol). Holdsworth (12) reported different \( E_a \) values ranging between 49.2 and 18 kcal/mol, and Ganthavorn et al. (8) calculated an \( E_a = 33.4 \) kcal/mol for unpurified asparagus POD heated at temperatures between 50 and 70°C, whereas our values were slightly lower. The differences may be because our kinetics parameters were calculated in order to avoid the regeneration of the asparagus POD, and in this case the thermal resistance may be greater than that of the natural vegetable. These results confirm the objection made by Adams (1) that a longer treatment time is needed to inactivate the POD irreversibly, especially at high temperatures.

**TABLE 1. Activation energy \( (E_a) \), reaction rate constant at reference temperature \( (k_R) \), temperature dependence of decimal reduction time \( (z) \), and decimal reduction time at temperature \( T_{ref} \) \( (D_{ref}) \) for regenerated asparagus peroxidase in the temperature range 90 to 125°C, estimated using three least-square methods with their ASD (asymptotic standard deviation).**

<table>
<thead>
<tr>
<th>Regression least-square method</th>
<th>( E_a ) (kcal/mol)</th>
<th>( k_R ) (min⁻¹)</th>
<th>( z ) (°C)</th>
<th>( D_{ref} ) (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-step linear</td>
<td>14.51 ± 2.01</td>
<td>1.52 ± 0.16</td>
<td>45.49 ± 5.75</td>
<td>1.25 ± 0.13</td>
</tr>
<tr>
<td>One-step linear</td>
<td>11.31 ± 0.95</td>
<td>4.79 ± 0.20</td>
<td>58.31 ± 4.89</td>
<td>0.49 ± 0.02</td>
</tr>
<tr>
<td>Nonlinear</td>
<td>13.62 ± 0.96</td>
<td>2.07 ± 0.19</td>
<td>48.56 ± 3.46</td>
<td>1.13 ± 0.10</td>
</tr>
</tbody>
</table>

**ACKNOWLEDGMENTS**

This work was supported by the CICYT (ALI91-1216-C02-01) and the EC (AIRL-CT92-0746) and part of it was presented at the 55th Annual Meeting of the Institute of Food Technologists, Anaheim, California, 3–7 June, 1995.

We thank the Spanish Ministry of Education for supporting Carmen Rodrigo with a grant during her work for the Ph.D. We also thank Vicente Giner for his collaboration in the establishment of the method of analysis and Vicenta Llorens, Mercedes Climent, and Teresa Laffite for their technical assistance.

**REFERENCES**