American Journal of Food Technology

ISSN 1557-4571

Academic Journals Inc.

www.academicjournals.com

American Journal of Food Technology 1 (2): 158-165, 2006
ISSN 1557-4571

Rhyzopus oryzae Endopolygalacturonase and *Borago officinalis* Polyphenol Oxidase Thermostability, Isothermal and Thermal Gradient Methods

Jorge Mir and Ana Ferrer and Pascual López Buesa
Department of Food Science, University of Zaragoza,
Miguel Servet, 177. 50013-Zaragoza, Spain

Abstract: Heat stability of two major enzymes in vegetable processing, such as endopolygalacturonase from *Rhyzopus oryzae* and polyphenoloxidase from *Borago officinalis*, has been calculated. Isothermal and nonisothermal methods for the calculation of z values have been assayed. No significant differences between the kinetic parameters obtained by both isothermal and nonisothermal methods were found. The calculation of heat resistance of the endopolygalacturonase showed the absence of a bimodal inactivation profile. Furthermore, the present study shows the adaptation of the linearly increasing temperature method to determine the kinetic parameters of heat inactivation of those enzymes. It should be feasible to use this methodology to estimate the effects of heat treatments on the inactivation of critical enzymes

Key words: *Borago officinalis*, polyphenol oxidase, endopolygalacturonase, heat inactivation

Introduction

Food stabilisation requires not only microbial destruction but also efficient enzyme inactivation or inhibition, being heating the most common method to achieve the desired stability. As microbiological destruction requires more severe treatments than enzymatic inactivation, the thermoresistance of microorganisms has been more deeply studied. Nevertheless some enzymes from fruits and vegetables exert a negative effect on the quality of some thermal treated vegetable products. In the field of processed fruits and vegetables, such as canning, juice processing or freezing a special attention is required on the inactivation of certain enzymes. This is true even for minimally processed products, which are solely submitted to heat shocks. Furthermore, in recent years, heat treatments have been even used effectively to extend the shelf-life of fresh fruits (Zhou *et al*., 2002). In all cases, thermal treatments have problems derived from the high thermoresistance of some pectic enzymes, such us endopolygalacturonase (Ludikhuyze *et al*., 2003; Pilnik *et al*., 1991) or oxidation enzymes, such us polyphenol oxidase (Lopez *et al*., 1994). Those enzymes may have endogenous or microbiological origin.

Endopolygalacturonase (endoPG; E.C. 3.2.1.15) is a pectolytic enzyme produced by fungi and higher plants which catalyses the hydrolysis of α-1-4 glicosidic linkages among the galacturonic acid residues, randomly hydrolyzing the pectin polymer. Endogenous endopolygalacturonase plays a role in softening during the ripening of the fruits, since pectins are, with cellulose and hemicelluloses, the major components of the vegetable cell wall (Huber, 1983). The extracellular enzymes produced by fungi have been implicated in postharvest rot of fruits and in the disintegration of preserved fruit following bottling or canning (Yates *et al*., 1967; Harper *et al*., 1973). Several reports exist in the literature concerning thermotolerant endopolygalacturonase from a range of fungi. This fact has been explained attending to the existence of high thermoresistant enzymes or to the two-phased kinetic thermoresistance of endoPG (Harris and Dennis, 1980, 1982).

Corresponding Author: Pascual López Buesa, Department of Food Science, University of Zaragoza, Miguel Servet, 177. 50013-Zaragoza, Spain Tel: 34-976-762533 Fax: 34-976-761590

Another major enzymatic concern when processing vegetables is polyphenol oxidase activity, because of its role in enzymatic browning and phenolic degradation. Polyphenol oxidase (PPO; EC 1.10.3.1) is a copper-containing enzyme that acts as a monooxygenase in the O-hydroxylation of monophenols and as a two-electron oxidase in the oxidation of o-diphenols ot o-quinones which undergo further modifications to give brown pigments (Mayer and Harel., 1991). It seems important to know the parameters of PPO inactivation in the different products, as PPO inactivation is one of the major methods to increase phenolic-related quality (Tomás-Barberán and Espin, 2001).

Both enzymes must be specially taken into account on those products that only suffer mild heat treatments, as the minimally processed stems of borage (*Borago officinalis*). Borages are vegetables with high consumption rates in our region, which are usually commercialized in fresh. Lately some alternatives to the fresh product have been searched to extend the area of business. After preliminary operations to facilitate its consumption, including washing, peeling and cutting, product is packed and pasteurized by applying only 'mild' thermal treatment (60-90°C) for a few minutes. After processing, chilling is mandatory to ensure an appropriate shelf-life. In order to optimize the thermal treatment and to evaluate the product shelf-life it is essential to have at our disposal kinetic data for the thermal inactivation of endogenous and exogenous enzymes. Those data with some other from models of heat and mass transfer must be associated to evaluate the safety and quality of foods during heating processes (Wang and Sun, 2003).

The procedure of heating and the method applied for analyzing the experimental data must be considered when determining the thermostability of a food component during heat treatment. Studies have been conventionally carried out in isothermal conditions, however during practical heat treatment the components are subjected to conditions that often substantially differ from those of isothermal experimental conditions. An alternative is to apply noninsothermal heating methods which, in addition, provide more information with a single experiment.

The purpose of this work is the study of the thermoresistance of *Rhyzopus oryzae* endoPG and *Borago officinalis* PPO comparing a classical isothermal method with a thermal gradient method in order to obtain kinetic data of thermostability of enzymes. The configuration of the of warming-up system and the mathematical treatment of the results are also considered to facilitate their future application.

Materials and Methods

Materials

Endopolygalacturonase from *Rhyzopus oryza*e used in this investigation was supplied by SERVA (Heidelbeg, Germany) under the name of Pectinase Borozym M5. The galacturonic acid (sodium salt) was supplied by Sigma (St. Louis, MO, USA). The common reagents used were of analytical grade.

Polyphenol oxidase of borage was extracted in a 0.2 M sodium phosphate buffer (pH 7.0) containing 10 g L^{-1} of polivinilpirrolidone and 5 g L^{-1} of Triton X-100 (Cano *et al*., 1990).

Heat Treatments

Two types of treatments were assayed: isothermal and thermal gradient assays. Heat treatments at constant temperature were performed in a thermostatized bath by immersing capillary tubes containing the enzyme samples. The reaction was performed at temperatures ranging between 52°C and 60°C in endoPG studies and between 70°C and 80°C in PPO studies to compare the data with earlier studies.

Linearly increasing temperature profiles were obtained in a thermostated metallic glass by water circulation from a water bath equipped with a programmable temperature controller (Almemo ZA 9020-FS NiCr).

At different time intervals, aliquots were removed and rapidly cooled in iced water. Enzyme activity was immediately measured. Each assay was perfomed in duplicate.

Enzyme Activity Assay

Endopolygalacturonase was assayed by measuring the increase in reducing groups of the polygalacturonic acid substrate (López *et al*., 1994a).

Polyphenol oxidase (PPO) activity was spectrophotometrically determined by monitoring the rate of dopachrome formation from DL-dopa at 475 nm (Lopez *et al*., 1994).

Parameters of Thermal Inactivation

Because of its advantages for heat treatment calculations, it is common when working on thermostability of food enzymes to express enzyme inactivation in terms of the pertinent parameters (D and z values) of the inactivation plots (Versteeg *et al*., 1980; Christen and Marshall, 1985; Richardson and Hyslop, 1985; Diermayr *et al*., 1987). This was the approach followed in the present study.

The D value, or decimal reduction time, is defined as:

$$D_T = \frac{x}{\log(N_0 / N_x)}$$

where, N is the number of cells before and after a x minutes of thermal treatment at T temperature. The z value expresses the temperature dependence of thermal inactivation and is defined as:

$$z = \frac{\Delta T}{\log(D_{T1} / D_{T2})}$$

In those experiments applying thermal gradient profiles, the z values can be calculated using the following equation:

$$\log A = \log A_0 - (z/\alpha DT_{ref} \ln 10)\, 10^{(-Tref/z)} (10^{T/z} - 10^{To/z})$$

as previously described (López *et al*., 1994b)

Results and Discussion

Adaptation of the Warming-up System

As the proposed method requires linear temperature profiles, we designed a device to reach the assay conditions. The implemented system allowed us to obtain high linearity ($R^2 = 0.9997$), despite its simple design and construction. Nevertheless, a precise design of the size of the containers and heater plates is mandatory, in order to get an appropriate control of the warming-up gradient.

Endopolygalacturonase Thermostability

The isothermal heat treatments were carried out between 52°C and 60°C. Figure 1 shows the values of residual activity as a function of time in those experiments carried out at 52, 54, 56, 59.3 and 60°C. These experiments allow us to obtain the z value of the enzyme in this range of temperature (Fig. 2). The average z value thus calculated is 5.1°C ($\sigma = 0.141$). In this range of temperature, endoPG inactivation follows a first-order kinetic and the D value temperature dependence fits well to the Arrhenius equation ($R^2 = 0.9968$).

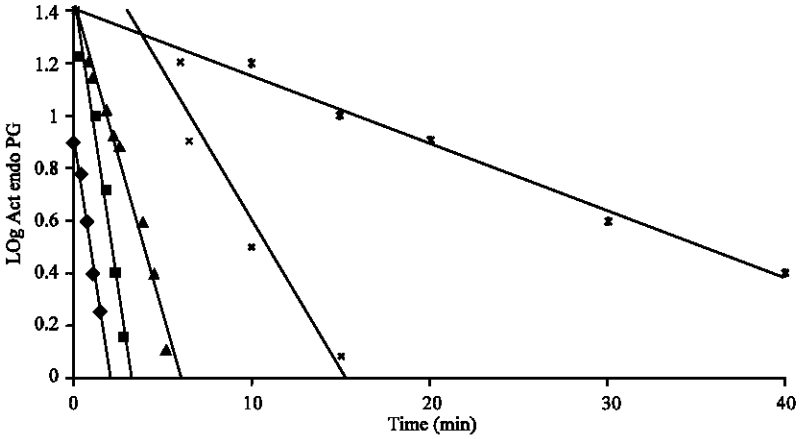

Fig. 1: Heat inactivation of *Rhyzopus oryzae* endopolygalacturonase under isothermal conditions at 52°C (◆); 54°C (■); 56°C (▲); 59°C (✖); 60°C (✳)

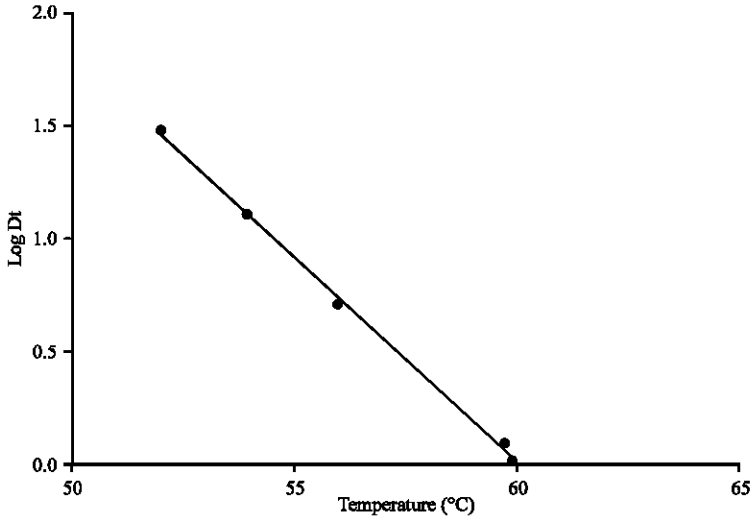

Fig. 2: *Rhyzopus oryzae* endopolygalacturonase z values (log Dt, vs. Temperature)

Harris and Dennis (1980) reported that *R. oryzae* endoPG show the same D value at 80°C than at 48°C in 50 mM citrate-phosphate buffer (pH 5.0). From those results it can be concluded that the D value at 80°C should be approximately of 150 to 200 min. Nonetheless we have treated one sample of endoPG at 80°C in isothermal conditions and no activity has been detected, even in the first aliquot taken only after 5 sec. We do not either notice any biphasic behavior in the inactivation of *R. oryzae* endoPG. One reason for the discrepancy between our findings and the previous results may be ascribed to the low specificity of the method assayed by Harris and Dennis (1980). Another reason could be the monomer-dimer transition which increases the enzyme thermostability. Tomato endoPG isoenzyme has been described as being composed by a catalytic subunit and another subunit which provide heat resistance to endoPG isoenzyme. It is possible that *R. oryzae* endoPG also contains a type of subunit and that was not present in the enzyme preparation we used. Another reason could be that the association between subunits was not possible under our experimental conditions.

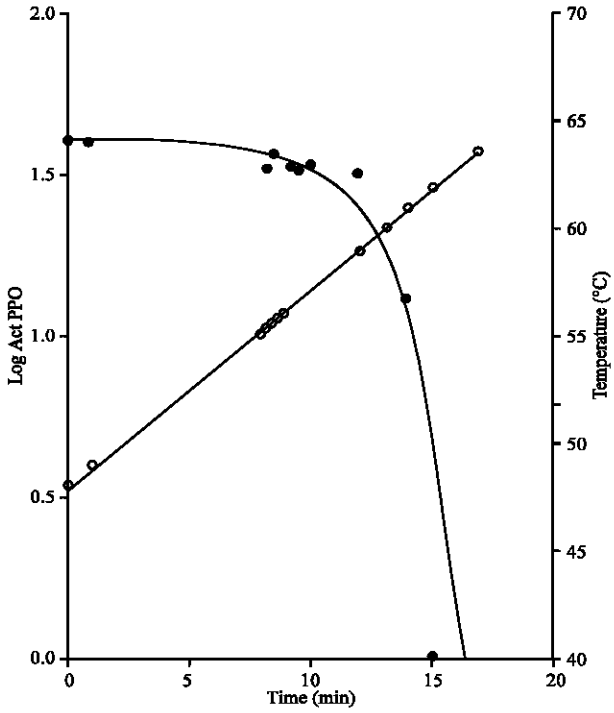

Fig. 3: Heat inactivation of *Rhyzopus oryzae* endopolygalacturonase in linearly increasing temperature sweeps. Enzyme activity (○); temperature (●)

The heat treatment of enzymes with linearly increasing temperature profiles permits to obtain z values in a single experiment, avoiding the usage of different samples of enzyme. By the application of this method, the variability within the results is minimized and time and labor cost are saved. In the present study the calculation of z values was carried out following the equation previously described (López *et al*., 1994b) and the nonlinear regression program Enzfitter (Leatherbarrow, 1987). An example for the result of this experiment is shown in Fig. 3, where residual endoPG activity is plotted against temperature.

The medium z value thus obtained (5.5°C) does not significantly differ from the obtained in the experiments at different constant temperatures. This result confirms the low thermal resistance of the endoPG of *Rhyzopus oryzae* and demonstrates the adaptation of the method for the calculation of thermoresistance of endopolygalacturonases. The existence of endoPG isoenzymes with different thermostability and monomer-dimer transition, affects enzyme termoestability (Tucker *et al*., 1981) and makes difficult the interpretation of studies of enzymatic thermostability (Moore and Bennett, 1994). The application of the methods assayed in this study can be an invaluable aid to study the stability of endoPG of fungi origin and from other sources, especially in the tomato.

PPO thermostability

In this case the isothermal heat treatments were carried out between 70 and 80°C. The Fig. 4 samples the values of residual activity as a function of time in experiments carried out to 70, 74, 76, 78 and 80°C.

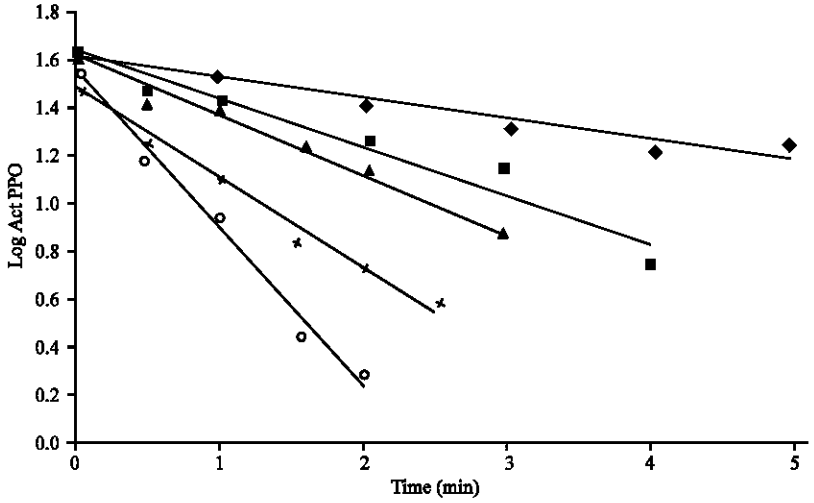

Fig. 4: Heat treatment of Borago officinalis polyphenol oxidase under isothermal conditions at 70°C (○); 74°C (✖); 76°C (▲); 78°C (■); 80°C (◆)

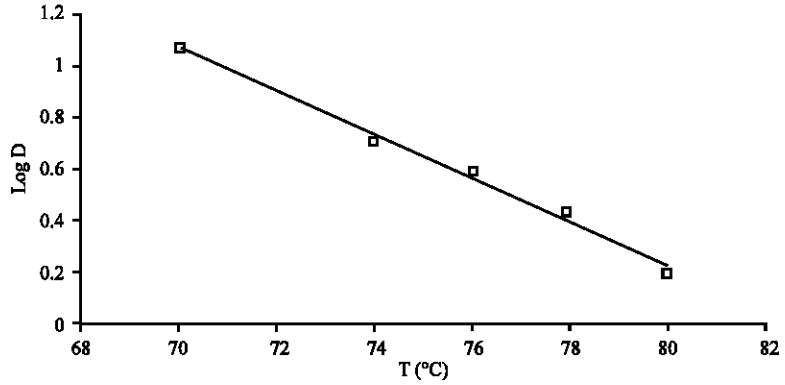

Fig. 5: Borago officinalis polyphenol oxidase D values (log Dt, vs. Temperature)

These experiments corroborate that the thermodestruction kinetic of the PPO follows a first-order kinetics. The z value may be calculated from those data plotting the log of each D value against its corresponding temperature (Fig. 5). The value z thus calculated is 11.7°C ($\sigma = 0.123$). The high values of thermoresistance of PPO from borage indicate that, to obtain a minimally processed pasteurized products, the use of additional hurdles would be required. The application of chilling after processing and the establishment of vacuum atmosphere during the packaging seems to be mandatory.

In the unsteady state approach experiment, with linear increment of the temperature, the activity is obtained while the temperature increases at a rate of 1.44°C per minute. (Fig. 6). The z value obtained using the norlinear regression program Enzfitter is 12.5°C. This value is close enough to the obtained with the isothermal experiment to be considerate as valid for a practical purpose. This result confirms the high thermal resistance of the PPO of borage and shows the adaptation of the method proposed by López *et al.* (1994b) for the determination of PPO thermoresistance.

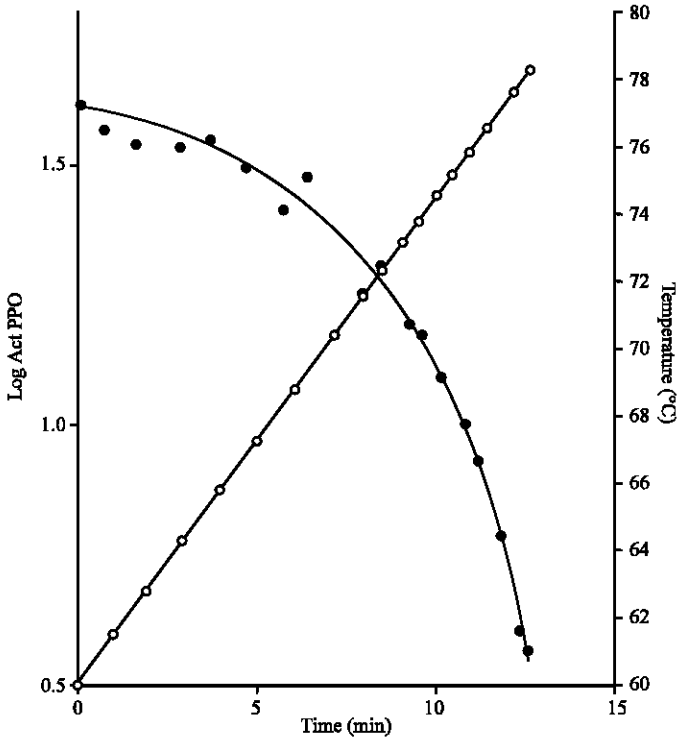

Fig. 6: Heat inactivation of Borago officinalis polyphenol oxidase in increasing temperature sweeps. Enzyme activity (●); temperature (○)

Furthermore, and making an attempt to simplify still more the calculation of thermostability parameters, we have make the adjust of the obtained data with a general purpose software like Microsoft Excel (Microsoft Corp., Seattle, WA), following the minimum square criteria. The z value thus calculated is 12.5°C, references that easy software can be used to the adjustment of kinetic data.

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