Influence of pH, Benzoic Acid, Glutathione, EDTA, 4-Hexylresorcinol, and Sodium Chloride on the Pressure Inactivation Kinetics of Mushroom Polyphenol Oxidase

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Pressure inactivation of mushroom PPO was studied for pH values ranging from 4 to 8, and the effect of some antibrowning agents on the pressure stability of mushroom PPO at pH 6.5 was evaluated. pH reduction below 6.5 resulted in a lowered inactivation threshold pressure and an increase of the absolute value of the activation volume (or a decrease of the z_p value), the latter two parameters reflecting the pressure dependency of the inactivation rate constant. An increase in pH from 6.5 to 8, on the other hand, did only marginally affect the pressure stability of the enzyme. Mushroom PPO at pH 6.5 was markedly sensitized toward pressure by the presence of 2.5 mM 4-hexylresorcinol and slightly stabilized by the presence of 5 mM EDTA. The presence of 5 mM glutathione, sodium chloride, or benzoic acid caused no significant alteration of the enzyme pressure stability. Only in the presence of 4-hexylresorcinol, significant changes of the activation volume and z_p value were noticed.

Keywords: *Polyphenol oxidase; pressure stability; pH; antibrowning agents; mushroom (Agaricus bisporus)*

INTRODUCTION

Damaged fruits, vegetables, and fungi (e.g. mushrooms) are extremely prone to enzymatic browning. This polyphenol oxidase-catalyzed reaction refers to a process in which naturally occurring phenols are oxidized to *o*-quinones, which undergo further enzymatic and nonenzymatic reactions, leading to polymeric pigmented material (Cheynier and Moutounet, 1992; Nicolas et al., 1994). Although the darkening of vegetable products and fungi is thought to be innocuous to consumers, enzymatic browning is, with a few exceptions, highly undesirable due to the development of unpleasant colors and flavors and a lowering of the nutritional value (Vámos-Vigyázó, 1981; Golan-Goldhirsh et al., 1984; Eskin, 1990). Because of this decrease in market value and the concomitant economic losses, control of enzymatic browning is very important to food manufacturing industries (Vámos-Vigyázó, 1981; Matheis and Whitaker, 1984).

A common approach for enzymatic browning prevention is the use of antibrowning agents, which act primarily on the enzyme or react with substrates and/or products of enzymatic catalysis so that pigment formation is inhibited (Iyengar and McEvily, 1992; McEvily et al., 1992). The use of these antibrowning agents in the food industry is however constrained by considerations such as toxicity, effects on taste, flavor, color, and texture, and, cost (Vámos-Vigyázó, 1981; McEvily et al., 1992; Ferrar and Walker, 1996). Next to these chemical browning prevention treatments, physical processes, such as temperature or pressure processing, can be used to inactivate polyphenol oxidase, thus avoiding the occurrence of enzymatic browning.

Thermal processing is often regarded as the most efficient and convenient method to inactivate polyphenol oxidase (Golan-Goldhirsh et al., 1984; McEvily et al., 1992). This processing technique is however associated with considerable sensorial and nutritional food quality loses (Lund, 1975; Villota and Hawkes, 1986). Increasing consumer demand for safe, high-quality, freshlike products that are shelf-stable and minimally processed stimulated the interest of food manufacturing industries in novel processing techniques, such as high-pressure processing (Mertens, 1992; Earnshaw et al., 1995). High-pressure processing has already been shown to be able to inactivate food spoilage enzymes (Anese et al., 1995; Basak and Ramaswamy, 1996; Seyderhelm et al., 1996; Ludikhuyze et al., 1998) and spoilage microorganisms as well as pathogens (Shigehisa et al., 1991; Styles et al., 1991; Raffalli et al., 1994), while leaving food quality attributes *quasi* unaffected (Hayashi, 1989; Eshtiaghi et al., 1994; Kimura et al., 1994). Polyphenol oxidases however seem to be rather pressure stable (Butz et al., 1994; Castellari et al., 1997; Weemaes et al., 1997b, 1998b). Hence, there is a need to screen several factors that could bring about a lowering of the pressure stability of polyphenol oxidase.

In the reported research, it is investigated whether the addition of antibrowning agents can modulate the pressure stability (at room temperature) of polyphenol oxidase from mushrooms. Since the antibrowning agents interact in one way or another with the enzyme (e.g. complexation of the active site copper), it is indeed possible that these agents bring about an alteration of the enzymes pressure stability. The ability of antibrowning agents to change the temperature resistance of mushroom polyphenol oxidase at both atmospheric

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and elevated pressure has already been shown (Weemaes et al., 1997a). The antibrowning agents used in this study are benzoic acid, EDTA, glutathione, sodium chloride, and 4-hexylresorcinol. The intrinsic factor pH was also included in this study, since it mimics the effect of acidulants.

MATERIALS AND METHODS

Enzyme and Media. Mushroom polyphenol oxidase ("PPO") was purchased from Sigma (production lot 24H9542; St. Louis, MO) as a dry powder containing 4400 units/mg of solid. One activity unit causes, at 25 °C and pH 6.5, an absorption increase of 0.001/min at 280 nm. The enzyme was dissolved in buffer at a concentration of 0.08 mg/mL.

To study the effects of antibrowning agents, PPO was dissolved in phosphate buffer (0.1 M, pH 6.5), containing 5 mM benzoic acid (Aldrich Chemie, Steinheim, Germany), EDTA (Sigma, St. Louis, MO; free acid), glutathione (Janssen Chimica, Geel, Belgium), or sodium chloride (Merck, Darmstadt, Germany) or 2.5 mM 4-hexylresorcinol (Aldrich Chemie, Steinheim, Germany). The latter antibrowning agent was used at a lower concentration, since the addition of 5 mM of the compound resulted in a *quasi* complete inhibition of the enzymatic browning reaction. To study the influence of pH, the enzyme was dissolved in 0.1 M phosphate buffers (pH 8−6.5) or McIlvaine buffers (pH 6.5−4).

Activity Assay. The polyphenol oxidase activity was measured spectrophotometrically at 23 °C (Biochrom 4060, UV−visible spectrophotometer, Pharmacia LKB Biochrom Ltd., Cambridge, England). A certain amount of the enzyme solution was added to 1 mL of 0.01 M catechol solution [in phosphate buffer (0.1 M, pH 6.5)], so that the absorption increase (ΔOD/min) of the blank ($t = 0$) equaled maximally 0.6. Up to this value, the relationship between enzyme concentration and enzyme activity was linear. The absorption increase, resulting from the oxidation of catechol to *o*-benzoquinone, was measured at 411 nm. To calculate the enzyme activity (ΔOD/min) using linear regression, only the initial linear part of the absorption curve was taken into account. The occurrence of reaction inactivation (Ludwig and Nelson, 1939; Yamaguchi et al., 1969) led to plateau formation in the absorption curve.

Pressure Treatment. Kinetic parameters for pressure inactivation at room temperature (25 °C) were derived on the basis of isobaric treatments and determination of the residual polyphenol oxidase activity. Microcentrifuge tubes (Elkay, Hants, England; 250 μL) filled with enzyme solution were pressure treated during preset times in a thermostated multivessel high-pressure apparatus (HPIU-10.000 serial no. 95/1994; Resato, Roden, The Netherlands). An oil−glycol mixture (TR15, Greenpoint Oil, Roden, The Netherlands) was used as pressure transmitting fluid.

The initial enzyme activity (A_0; $t = 0$) was defined as the activity of the enzyme sample when entering the time domain where pressure and temperature remained constant in time, as was explained in a previous article (Weemaes et al., 1997a). In this way, the variable pressure−temperature conditions, resulting from pressure buildup and accompanied adiabatic heating, was excluded from the inactivation experiment. The first-order pressure inactivation kinetics of mushroom PPO allowed the use of this "zero-point" approach.

After decompression of the individual pressure vessels, the enzyme samples were stored on ice water ($t < 60$ min) until determination of the polyphenol oxidase activity. No reactivation was observed during this storage period.

Data Analysis. As was observed for pressure inactivation (at room temperature) of apple, avocado, grape, and pear polyphenol oxidase at pH 6−7 (Weemaes et al., 1998b), pressure inactivation of mushroom PPO exhibited first-order kinetics (eq 1) under all conditions tested.

$$\frac{dA}{dt} = -kA \tag{1}$$

Table 1. Activity Change (%) of Mushroom PPO Due to Changes in pH or the Addition of Antibrowning Agents[a]

condition	activity change	condition	activity change
pH 4; MB	−20	5 mM benzoic acid	−5
pH 5; MB	−8	5 mM EDTA	0
pH 6.5; MB	+20	5 mM glutathione	−65
pH 6.5; PB	0	5 mM NaCl	−25
pH 8; PB	+20	5 mM 4-HR	−97
		2.5 mM 4-HR	−60

[a] The activity of mushroom PPO in phosphate buffer (pH 6.5; 0.1 M) is considered as 100%.

Next to inactivation rate constants, first-order inactivation processes can be characterized by decimal reduction times. This so-called *D* value reflects the time (minutes) required, at a certain pressure, to reduce the initial enzyme activity to one-tenth of its original value and equals ln(10)/*k*.

Pressure dependence of the inactivation rate constants and decimal reduction times are respectively expressed by the activation volume (V_a) and the z_p value. The z_p value has the physical meaning of the pressure increase needed to obtain a 10-fold reduction of the decimal reduction time (eq 2). The activation volume, on the other hand, can be derived from the Eyring equation (eq 3).

$$D = D_{ref}10^{\left(\frac{(P_{ref}-P)}{z_p}\right)} \tag{2}$$

$$k = k_{ref}\exp\left(\frac{-V_a}{RT}(P - P_{ref})\right) \tag{3}$$

A two-step procedure was used to derive the kinetic parameters for the first-order pressure inactivations of mushroom PPO. First, the natural or ten-based logarithms of the activity retentions (A/A_0) were regressed versus pressurizing time to calculate *k* or *D* values, respectively. Second, the pressure coefficients were derived. The activation volume was derived from a plot of ln(*k*) versus *P*, whereas the z_p value was obtained by regressing the ten-based logarithms of the decimal reduction times versus pressure.

RESULTS AND DISCUSSION

Effect of pH and Antibrowning Agents on the Activity of Mushroom PPO. Before we proceeded to the pressure inactivation studies, it was tested to which extent the PPO activity was reduced by the presence of the selected antibrowning agents or by the changes in pH. Furthermore, it was evaluated whether the PPO activity changed as a function of incubation time, due to the medium pH or the presence of the antibrowning agents. The changes in PPO activity are summarized in Table 1. From this table it is clear that the presence of 5 mM 4-hexylresorcinol resulted in an almost complete activity loss. Consequently, a lower 4-hexylresorcinol concentration, namely 2.5 mM, was used in the subsequent pressure inactivation experiments. None of the pH values considered caused inactivation of mushroom PPO at atmospheric pressure and room temperature during a period of 2 days at least. These observations are in agreement with a previous study, in which activity loss under the conditions mentioned was only observed at pH values below 4 (Weemaes et al., 1997a). Likewise, none of the antibrowning agents selected was found to reduce the PPO activity in time at atmospheric pressure and room temperature.

Effect of pH on the High-Pressure Inactivation of Mushroom PPO. At all pH values studied, the pressure inactivation of the fungal polyphenol oxidase could accurately be described by a first-order kinetic

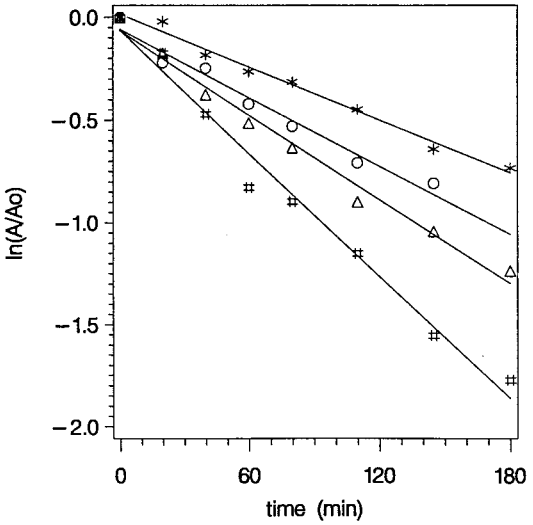

Figure 1. First-order pressure inactivation of mushroom PPO in phosphate buffer (pH 6.5) at 25 °C and 750 (*), 800 (○), 850 (△), or 900 (#) MPa.

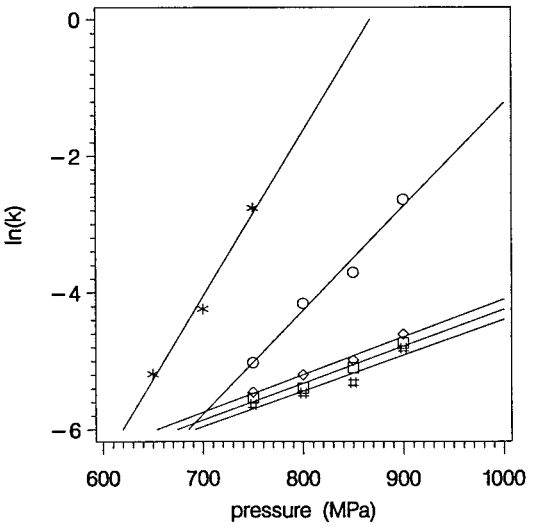

Figure 2. Pressure dependence of the inactivation rate constants of mushroom PPO at pH 4 (*), 5 (○), 6.5 [MB (□); PB (◇)], and 8 (#).

Table 2. Estimated Activation Volumes and z_p Values for the Pressure Inactivation of Mushroom PPO (0.08 mg/mL) at Different pH Values

pH	buffer	V_a (cm^3/mol)	z_p (MPa)
4	McIlvaine buffer	−60.08 ± 7.48[a]	94.97 ± 11.81[a]
5	McIlvaine buffer	−37.70 ± 3.94	151.52 ± 15.84
6.5	McIlvaine buffer	−13.35 ± 1.64	427.53 ± 52.61
6.5	phosphate buffer	−13.63 ± 1.22	418.41 ± 37.29
8	phosphate buffer	−12.95 ± 2.86	440.53 ± 97.23

[a] Standard error.

model. As an example, the pressure inactivation of mushroom PPO at pH 6.5 is illustrated in Figure 1. The pressure dependency of the first-order inactivation rate constants could in all cases adequately be described by the Eyring equation (Figure 2). Corresponding activation volumes and z_p values are summarized in Table 2. These pressure coefficients appeared to vary widely with pH. The absolute value of the activation volume decreased with increasing pH from about 60 cm^3/mol at pH 4 to about 13 cm^3/mol at pH 8, whereas the z_p value increased with increasing pH from about 95 MPa at pH 4 to about 440 MPa at pH 8.

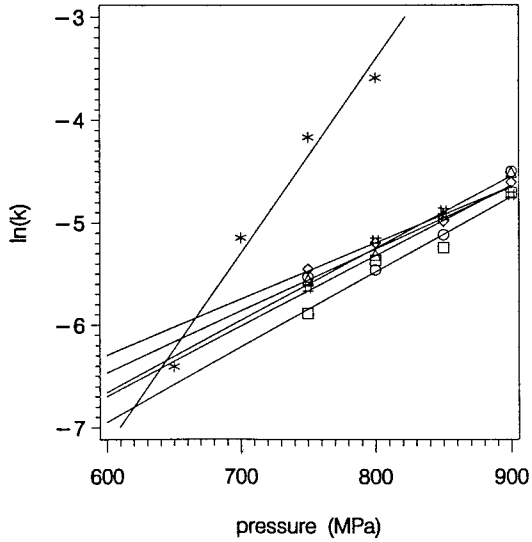

Figure 3. Pressure dependence of the inactivation rate constants of mushroom PPO (pH 6.5) in absence (◇) or presence of 5 mM benzoic acid (○), EDTA (□), NaCl (#), and glutathione (△) or 2.5 mM 4-HR (*).

From Figure 2, it can furthermore be derived that mushroom PPO at pH 4 is far more pressure sensitive than the enzyme system at pH 5, which is in turn considerably more pressure sensitive than the enzyme systems at pH 6.5 and 8. Only minor differences in pressure stability between the latter two enzyme systems were noticed. The inactivation of the fungal enzyme was furthermore not or only very slightly influenced by the type of buffer used (phosphate vs McIlvaine buffer). From the decrease in threshold inactivation pressure (interpreted as pressure needed for a k value of 0.01 min^{-1}) when lowering the pH of the enzyme solution below pH 6.5, it is clear that the microenvironmental parameter pH is a powerful tool to reduce the pressure stability of mushroom polyphenol oxidase.

The results reported here are in good agreement with the results of a previous study of avocado PPO (Weemaes et al., 1998a), since for the latter enzyme also a decrease of the threshold inactivation pressure and an increase of the absolute value of the activation volume or a decrease of the z_p value with decreasing pH (pH 8−5) was observed. A further reduction of the pH to a value of 4 brought along a further decrease of the threshold inactivation pressure of avocado PPO but resulted in a deviation from first-order inactivation kinetics. An analogous change in the pressure inactivation model was not observed for mushroom PPO.

Effect of Antibrowning Agents on the High-Pressure Inactivation of Mushroom PPO. In presence of 5 mM benzoic acid, EDTA, glutathione, or sodium chloride or 2.5 mM 4-hexylresorcinol, the pressure inactivation of mushroom PPO (phosphate buffer, pH 6.5) likewise followed first-order inactivation kinetics. Figure 3 depicts the inactivation rate constants for pressure inactivation of the fungal enzyme in the presence of each of the antibrowning agents studied. From this figure it is clear that the addition of 2.5 mM 4-hexylresorcinol resulted in a marked decrease of the threshold inactivation pressure. As was observed for thermal inactivation of the enzyme (McCord and Kilara, 1983; Weemaes et al., 1997a), the presence of EDTA resulted in a slight stabilization of mushroom PPO toward pressure. This effect of EDTA could not be

Table 3. Estimated Activation Volumes and z_p Values for the Pressure Inactivation of Mushroom PPO (0.08 mg/mL; 0.1 M Phosphate Buffer; pH 6.5) in the Presence and Absence of Antibrowning Agents

antibrowning agent	V_a (cm^3/mol)	z_p (MPa)
none	-13.63 ± 1.22^a	418.41 ± 37.29^a
5 mM benzoic acid[b]	-17.05 ± 4.29	334.45 ± 84.00
5 mM EDTA	-18.19 ± 2.82	313.48 ± 48.45
5 mM glutathione	-17.49 ± 1.43	326.80 ± 26.81
5 mM sodium chloride	-15.02 ± 2.44	380.23 ± 61.73
2.5 mM 4-hexylresorcinol	-46.70 ± 5.39	122.25 ± 14.11

[a] Standard error. [b] r^2 for this linear regression equaled only 0.888.

ascribed to a pH effect, since on the basis of the pH reduction, a slight destabilizing effect would be expected. The hypothesis put forward by McCord and Kilara (1983) for the increased thermal stability of mushroom PPO in the presence of EDTA, i.e. the tertiary structure of mushroom PPO is stabilized by the inclusion of the chelating agent EDTA, possibly also accounts for the increased pressure stability of the enzyme. The antibrowning agents glutathione and sodium chloride did not seem to exert a stabilizing or sensitizing effect on mushroom PPO.

Except for the presence of benzoic acid, the pressure dependency of the inactivation rate constants could reasonably be described by the Eyring equation (Figure 3). The corresponding activation volumes and z_p values are summarized in Table 3. Only in the presence of 2.5 mM 4-hexylresorcinol, the pressure dependency of the inactivation rate constants was significantly altered ($p < 0.05$) as compared to the situation in the absence of antibrowning agent.

CONCLUSION

The presence of antibrowning agents (including acidulants) may alter the pressure stability of polyphenol oxidases. For food applications, especially a reduction of the pH value and the addition of antibrowning agents that bring about a reduction of the enzymes resistance toward pressure (e.g. 4-hexylresorcinol) are valuable since these agents in fact exert a dual enzymatic browning control effect, namely enhancement of the inactivation of polyphenol oxidase and control of its enzymatic activity during and after processing. Even the addition to foods of antibrowning agents that do not stabilize or sensitize the enzyme toward pressure has a positive effect since it delays the onset of and/or reduces the rate of the undesirable enzymatic browning reaction, during as well as after processing.

ABBREVIATIONS USED

EDTA, ethylenediaminetetraacetic acid; MB, McIlvaine buffer; OD, optical density; PB, phosphate buffer; PPO, polyphenol oxidase; 4-HR, 4-hexylresorcinol; A, enzyme activity at time t (OD/min); A_0, enzyme activity at $t = 0$, i.e. when entering the constant P-T phase (OD/min); D, decimal reduction time (min); D_{ref}, decimal reduction time at P_{ref} (min); k, first-order inactivation rate constant (min^{-1}); k_{ref}, first-order inactivation rate constant at P_{ref} (min^{-1}); P, pressure (MPa); P_{ref}, reference pressure (MPa); R, universal gas constant; r^2, coefficient of determination; T, absolute temperature (K); t, processing time (min); V_a, activation volume (cm^3/mol); z_p, z value for high-pressure processing (MPa).

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