Effect of Combined Pressure and Temperature on Soybean Lipoxygenase. 1. Influence of Extrinsic and Intrinsic Factors on Isobaric−Isothermal Inactivation Kinetics

L. Ludikhuyze, Indrawati, I. Van den Broeck, C. Weemaes, and M. Hendrickx*

Department of Food and Microbial Technology, Faculty of Agricultural and Applied Biological Sciences, Katholieke Universiteit Leuven, Kardinaal Mercierlaan 92, B-3001 Heverlee, Belgium

Both isothermal and isobaric−isothermal inactivation of soybean lipoxygenase (LOX) could be accurately described by a first-order kinetic model. Thermal inactivation of LOX at atmospheric pressure proceeded in the temperature range 60−70 °C, whereas pressure−temperature inactivation occurred in the pressure range 50−650 MPa at temperatures between 10 and 64 °C. With increasing pressure at constant temperature, a consistent increase in inactivation rate constants was noted, pointing out a negative activation volume. At constant elevated pressure, on the other hand, a temperature of maximum stability was observed, generally situated somewhat above room temperature, indicating the Arrhenius law not to be valid over the entire temperature range. Finally, the influence of some food-relevant intrinsic factors on thermal and pressure−temperature stability of LOX was screened. Where enzyme concentration only slightly affected thermal and pressure stability of LOX, both pH and flushing with CO_2 effected significant stability changes.

Keywords: *Soybean lipoxygenase; thermal stability; pressure−temperature stability; intrinsic factors*

INTRODUCTION

Most raw vegetables can be stored for only a short period of time, because several enzymes are still active after harvest. These enzymatic actions can result in changes in texture, flavor, color, and nutrient content. For lipoxygenase (LOX), which catalyzes the oxygenation of fatty acids containing methyl-interrupted double bonds into the corresponding hydroperoxides (Eskin et al., 1977; Galliard and Chan, 1980), at least three detrimental effects in foods are known (Whitaker, 1972): (i) There is a destruction of the essential fatty acids linoleic, linolenic, and arachidonic acid. (ii) Further degradation of the primarily formed hydroperoxides results in the formation of volatile compounds such as aldehydes, ketones, and alcohols (Hugues et al., 1994). This causes the development of off-flavor, which has been characterized as a haylike flavor in beans and peas. This is a particularly serious problem in unblanched frozen peas and beans, because LOX can continue its action even at low temperature. In some cases, LOX also contributes to the development of bitterness (Baur et al., 1977). (iii) The free radicals produced can damage other compounds, including vitamins and proteins. Also pigments such as chlorophyll and carotenoids are damaged, resulting in color changes (Eskin et al., 1977). Hitherto in vegetable processing, blanching was pre-eminently the method to inactivate enzymes and hence increase the shelf life of frozen vegetables. The term blanching (70−105 °C) most often designates heat treatments that prevent deteriorative changes during frozen storage; that is, blanching ensures the reduction of enzyme activity. Nevertheless, there are some disadvantages linked to blanching. These include loss in texture and nutritional quality by the heating process, formation of a cooked taste, change in color, and loss of soluble solids (Williams et al., 1986). Because of the consumer demand for fresh foods with little heat-induced degradation of nutritional and organoleptic properties, research for new nonthermal and combined processes has been stimulated (Hoover et al., 1989; Mertens, 1995). In this framework, the use of high pressure can be investigated as an alternative for traditional water blanching. High pressure has been shown to inactivate vegetative microorganisms and some spoilage enzymes while leaving most quality carriers such as color, flavor, and nutrient/vitamin content unaffected (Hoover, 1993; Mozhaev et al., 1994).

Nevertheless, application of high-pressure technology in the food industry is awaiting a generic scientific basis to assess quantitatively the impact of combined pressure−temperature processes. From this viewpoint, kinetic studies on the effects of pressure and temperature on food quality related aspects, including enzymes, are indispensable.

In the present paper, the kinetics of inactivation of soybean LOX by combined pressure and temperature are investigated. Moreover, as it is well established that kinetics largely depend on both extrinsic (temperature, pressure) and intrinsic (state of the enzyme, properties of the surrounding medium) factors, a case study was performed concerning the influence of enzyme concentration, type of buffer, and pH on the inactivation kinetics of soybean LOX.

MATERIALS AND METHODS

Enzyme and Activity Measurement. Lipoxygenase type IB from soybean was purchased as a lyophilized powder, containing 60% protein and 1.8×10^5 units/mg of protein

* Author to whom correspondence should be addressed (telephone 32-16-32.15.85; fax 32-16-32.19.60; e-mail Marc.Hendrickx@agr.Kuleuven.ac.be).

10.1021/jf980256c CCC: $15.00
Published on Web 09/03/1998

(Sigma, product L7395) as well as NaCl and stabilizers. The commercial sample was used without further purification. One unit of enzyme causes an increase in A_{234} of 0.001/min at pH 9 and 25 °C, when linoleic acid is used as substrate in a volume of 3 mL. One A_{234} unit is equivalent to the oxidation of 0.12 μmol of linoleic acid. The enzyme was dissolved in a 0.01 M Tris-HCl buffer at pH 9 at a concentration of 0.4 mg/mL, unless the influence of enzyme concentration or pH was examined.

The activity of LOX was measured spectrophotometrically at 234 nm (LKB UV−visible spectrophotometer, Biochrom 4060) using sodium linoleate as substrate. The latter was prepared as follows: 280 mg of linoleic acid and 280 mg of the solubilizer Tween 20 were added to 4 mL of O_2-free water. After homogenization, 0.5 N NaOH was added to clear the solution. Finally, the solution was made up with distilled water to a total volume of 25 mL. The solution was flushed with N_2, divided in small portions (1.5 mL), and stored frozen until used. The reaction was carried out at 25 °C in a quartz cuvette. The assay mixture contained 45 μL of enzyme solution, 25 μL of substrate solution, and 2.9 mL of 0.0125 M borate buffer at pH 9. The absorption at 234 nm was recorded as a function of reaction time (3 min), and the activity was determined from the slope of the linear portion of the curve. The linear relationship between measured activity and enzyme concentration was verified in a preliminary test.

Isothermal Treatment. Isothermal inactivation experiments were performed in a thermostatic water bath at constant temperature. To ensure direct heating and direct cooling, the enzyme solution was filled in capillary tubes (Hirschmann labogeräte; diameter = 15 mm, length = 15 cm). After filling, the tubes were placed in the water bath and the heating time was exactly measured using a stopwatch. Immediately after withdrawal from the hot water bath, the samples were transferred to a water bath at 25 °C and stored for 0.5 h before activity measurement, allowing slight reactivation (~10%) to be completed.

Isobaric−Isothermal Treatment. Isobaric−isothermal inactivation treatments were performed in a laboratory pilot scale, multivessel high-pressure equipment (HPIU-10000, 95/1994, Resato). High pressure (up to 1000 MPa) is generated using a pressure intensifier in the central pressure circuit. Eight individual vessels (volume = 8 mL, diameter = 10 mm, length = 100 mm), surrounded by a thermostatic mantle linked to a cryostat, are connected to the central pressure circuit using T-joints and valves. The pressure transferring liquid is a glycol/oil mixture (TR15, Resato).

This equipment allows performance of kinetic experiments because eight samples can be submitted simultaneously to treatments at the same pressure and temperature but for different preset treatment times. After the samples are enclosed in the vessels (filled in 0.25 mL microtubes, Elkay), only the central valve is closed and pressure is built up slowly (100 MPa/min) until the desired value is reached. Once the maximum pressure is reached, the individual vessels are isolated from the central pressure tubing and the central valve is opened. As a function of time, pressure can now be released in the individual vessels. To ensure treatment of the samples at constant pressure and temperature, the activity of the sample from the first vessel, where pressure is released the moment the central valve is opened (after 4 min), is considered as the reference (A_0, blank activity).

Data Analysis. On the basis of literature data (Kermasha et al., 1993; Heinisch et al., 1995; Lopez and Burgos, 1995), both isothermal and isobaric−isothermal inactivations of soybean LOX were assumed to follow first-order kinetics.

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

For constant extrinsic/intrinsic factors, integration of this differential equation between initial and final conditions leads to the following expression of enzyme activity as a function of treatment time:

$$\ln(A/A_0) = -kt \tag{2}$$

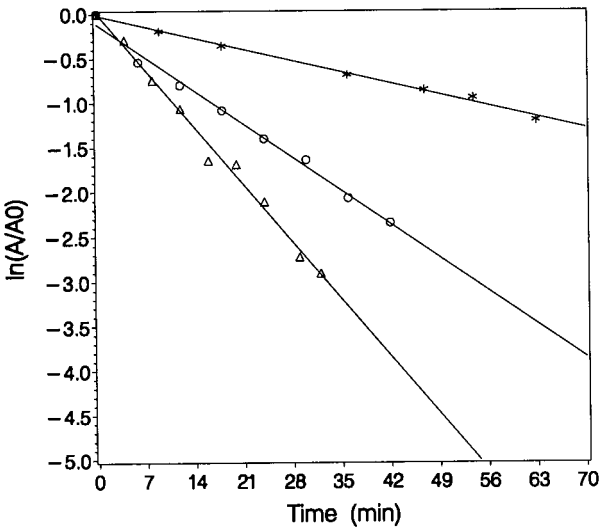

Figure 1. Inactivation of LOX, 0.4 mg/mL in 0.01 M Tris-HCl at pH 9, at atmospheric and elevated pressure: (*) 20 °C/525 MPa; (○) 62 °C/200 MPa; (△) 66 °C/0.1 MPa.

To verify the validity of this first-order kinetic model, semilogarithmic plots of residual activity as a function of time at constant pressure and temperature were drawn. As a measure for linearity, regression coefficients were calculated and the residual plots were analyzed on their randomness, that is, the absence of eventual systematic errors.

Temperature and pressure dependence of the inactivation rate constants were calculated according to the Arrhenius and Eyring equations, respectively.

$$\ln(k) = \ln(k_{refT}) - \frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right) \tag{3}$$

$$\ln(k) = \ln(k_{refP}) - \frac{V_a}{RT}(P - P_{ref}) \tag{4}$$

The kinetic parameters (k, E_a, V_a) were calculated using a two-step linear regression approach on the linearized (logarithmic) data. Linear regression procedures were performed in the statistical software package from SAS Institute (1982).

In a second phase, the influence of some intrinsic factors on the thermal stability of soybean LOX at atmospheric and elevated pressure was kinetically investigated. The systems were ranked according to their k values. When E_a values of the system under various conditions are different, specification to temperature is required because an isokinetic point exists at which the stability ranking is reversed (Weemaes et al., 1997).

RESULTS AND DISCUSSION

Isothermal Inactivation Kinetics of Soybean LOX. Thermal stability of soybean LOX at atmospheric pressure was studied in the temperature range 62−68 °C. In this temperature range, isothermal inactivation of LOX followed first-order kinetics, allowing inactivation rate constants to be determined from semilogarithmic plots of the activity retention as a function of time (Figure 1). The kinetic parameters describing isothermal inactivation of soybean LOX, together with standard errors and regression coefficients, are presented in Table 1.

Formerly, first-order kinetics for thermal inactivation of soybean LOX have been frequently reported in the literature (Kermasha et al., 1993; Lopez and Burgos, 1995; Srinivasulu and Appu Rao, 1995). However, somewhat contradictory results were obtained as to the

Table 1. Kinetic Parameters Describing Isothermal Inactivation of Soybean LOX, 0.4 mg/mL in 0.01 M Tris-HCl at pH 9

temp (°C)	k value (min^{-1})	r^2
62	$(2.02 \pm 0.09^a) \times 10^{-2}$	0.987
64	$(4.94 \pm 0.16) \times 10^{-2}$	0.993
66	$(9.18 \pm 0.32) \times 10^{-2}$	0.992
68	$(15.5 \pm 0.52) \times 10^{-2}$	0.992
$E_a = 319.8 \pm 27.3$ kJ/mol		0.986

[a] Standard error.

thermal stability and temperature dependence of the inactivation rate. Srinivasulu and Appu Rao (1995) reported the midpoint of thermal inactivation to be 44 ± 1 °C, referring to a low thermal stability, whereas Kermasha et al. (1993) reported high thermostability as inactivation was achieved in the temperature range 60−90 °C. The latter authors derived an activation energy of 119 kJ/mol, pointing to low temperature sensitivity of the k values. On the other hand, Lopez and Burgos (1995) observed high temperature sensitivity for inactivation of soybean LOX at temperatures between 69 and 74 °C, as indicated by an activation energy value exceeding 500 kJ/mol. Likewise, inactivation of LOX from many different sources such a green peas, green beans, potatoes, asparagus, wheat germ, and germinated barley could be described either by a simple first-order model or by a two-fraction first-order model (Svensson and Eriksson, 1972; Park et al., 1988; Ganthavorn et al., 1991; Henderson et al., 1991; Zhang et al., 1991; Bhirud and Sosulski, 1993; Günes and Bayindirli, 1993; Hugues et al., 1994). The latter model refers to the existence of two fractions (isozymes) with different thermal stability. However, temperature ranges for inactivation of LOX from different sources (40−100 °C) as well as activation energy values (65−655 kJ/mol) varied significantly.

Isobaric−Isothermal Inactivation Kinetics of Soybean LOX. Isobaric−isothermal inactivation of LOX was studied in the pressure range 50−650 MPa at temperatures between 10 and 64 °C. By analogy with isothermal inactivation, first-order inactivation kinetics were assumed to analyze isobaric−isothermal inactivation of LOX and verified on semilogarithmic plots of the activity retention as a function of time (Figure 1). Regression coefficients varied between 0.930 and 0.998. First-order rate constants for isobaric−isothermal inactivation of LOX together with standard errors are summarized in Table 2, showing the latter to be situated between 1 and 10%.

This table immediately indicates an antagonistic effect at low temperature (T < 40 °C) and high pressure (P > 475 MPa). In this low-temperature region, temperature increase causes the inactivation rate constant to decrease. Minimal inactivation rate constants are observed in the temperature range 30−40 °C, showing LOX to exhibit maximal pressure stability at temperatures slightly higher than room temperature. Hence, both heat and cold denaturations of LOX are feasible at elevated pressure. This antagonistic effect is likewise apparent from the shape of the pressure−temperature kinetic diagram for inactivation of soybean LOX (Figure 2). Though antagonistic effects of pressure and temperature have been frequently reported in the literature (Johnson and Campbell, 1945; Suzuki and Kitamura, 1960; Heremans, 1993; Mozhaev et al., 1996; Weemaes et al., 1998), mainly retardation of thermal inactivation

Table 2. First-Order Rate Constants ($\times 10^{-2}$ min^{-1}) for Isobaric−Isothermal Inactivation of LOX, 0.4 mg/mL in 0.01 M Tris-HCl at pH 9

	T (°C)														
P (MPa)	10	15	20	25	30	35	40	45	50	55	62	64
50												5.94 ± 0.28[a]
100											5.47 ± 0.38	7.03 ± 0.80
150											6.55 ± 0.45	8.36 ± 0.81
200											8.17 ± 0.64	8.58 ± 0.61
250											8.52 ± 0.57	9.92 ± 0.61
300										3.01 ± 0.24		11.7 ± 0.17
400										4.18 ± 0.26		
475									1.48 ± 0.09			
500	2.02 ± 0.09	0.90 ± 0.04	0.88 ± 0.04					1.33 ± 0.06	2.18 ± 0.16			
525	3.48 ± 0.21	2.68 ± 0.25	1.81 ± 0.06	1.18 ± 0.08	0.79 ± 0.05	1.19 ± 0.07	1.71 ± 0.09	1.98 ± 0.16	2.74 ± 0.11			
550	6.91 ± 0.46	5.72 ± 0.23	4.18 ± 0.11	2.17 ± 0.14	1.61 ± 0.08		2.50 ± 0.16	3.11 ± 0.24	2.94 ± 0.26	7.46 ± 0.81		
575	14.5 ± 1.45	7.37 ± 0.42	7.24 ± 0.64	7.73 ± 0.37	3.90 ± 0.18	3.83 ± 0.28	3.20 ± 0.15	3.31 ± 0.18	3.67 ± 0.17			
600			13.5 ± 0.65	11.5 ± 0.56	9.84 ± 0.88	8.35 ± 0.43	4.77 ± 0.29	7.95 ± 0.39				
625				18.8 ± 1.19	16.9 ± 1.70	14.8 ± 0.48	11.6 ± 0.98	12.1 ± 0.29				
650				34.5 ± 5.38	17.8 ± 1.10		16.9 ± 0.24					

[a] Standard error.

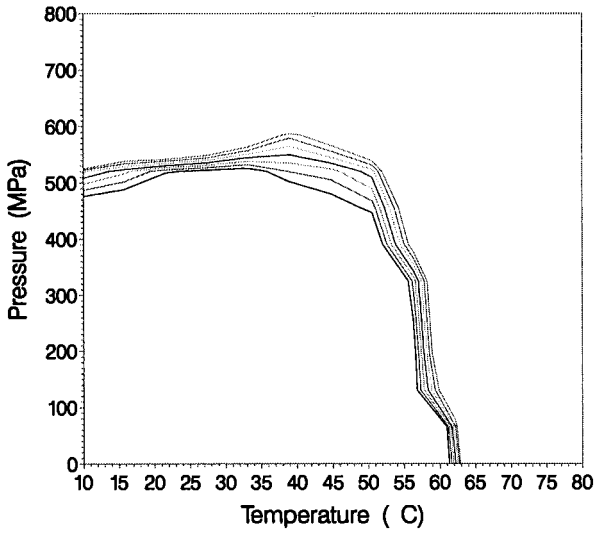

Figure 2. Pressure−temperature kinetic diagram for inactivation of LOX, 0.4 mg/mL in 0.01 M Tris-HCl at pH 9: (upper line) $k = 0.07$ min^{-1}; (lower line) $k = 0.01$ min^{-1}.

Table 3. Activation Volume for Isobaric−Isothermal Inactivation of LOX, 0.4 mg/mL in 0.01 M Tris-HCl at pH 9, at Different Constant Temperature Levels

temp (°C)	V_a (cm^3/mol)	r^2
10	−62.6 ± 3.1[a]	0.995
15	−67.7 ± 12.1	0.935
20	−66.5 ± 2.8	0.995
25	−67.2 ± 5.9	0.970
30	−67.9 ± 7.5	0.955
35	−65.3 ± 2.5	0.997
40	−48.9 ± 4.4	0.963
45	−45.6 ± 4.5	0.961
50	−22.7 ± 3.3	0.941
55	−9.9 ± 0.4	0.998
62	−8.6 ± 1.4	0.944
64	−7.1 ± 0.5	0.976

[a] Standard error.

by low pressure is involved, a phenomenon that is not observed for LOX. At all temperatures studied, inactivation was enhanced by pressure increase. In this respect, our results are somewhat conflicting with those published by Heinisch et al. (1995), who investigated inactivation of soybean LOX in the pressure range 0.1−650 MPa at temperatures varying from 0 to 65 °C and revealed low pressure to exert a slightly thermostabilizing effect at high temperature.

Pressure dependence of the inactivation rate constant could be analyzed using the Eyring equation (eq 4). Activation volumes together with standard errors and regression coefficients are presented in Table 3, denoting the Eyring equation to be valid in the entire temperature range. At all temperatures studied, the activation volume was negative, as expected from Table 2. The absolute value of the activation volume seemed rather constant up to 35 °C but decreased with further increase in temperature, pointing to reduced pressure sensitivity at high temperature. Similar results were reported for egg albumin, carbonylhemoglobin, chymotrypsin, Taka amylase A, and PPO (Suzuki et al., 1958; Suzuki and Kitamura, 1960; Miyagawa and Suzuki, 1963b, 1964; Taniguchi and Suzuki, 1983; Weemaes et al., 1998), whereas for trypsin the opposite results, that is, an increase in absolute activation volume with higher temperature, were noted (Miyagawa and Suzuki, 1963a).

Obviously, the Arrhenius equation (eq 3) could not be applied to describe the temperature dependence of the inactivation rate constant over the entire temperature region. Therefore, it was investigated whether activation energies could be calculated for distinguishable low- and high-temperature regions. An additional problem arose because these regions could not be demarcated unambiguously as the temperature of maximal stability varied with pressure. The results are presented in Table 4. Activation energies are negative in the low-temperature area but positive at high temperature. In addition, on a qualitative basis, a tendency for the temperature sensitivity to decrease with increasing pressure was observed, both in the low- and high-temperature areas. The poor statistical results ($r^2 = 0.800-0.982$, errors = 7−30%), however, indicate that quantitative interpretation of these results could lead to erroneous conclusions.

Table 4. Activation Energy for Isobaric−Isothermal Inactivation of LOX, 0.4 mg/mL in 0.01 M Tris-HCl at pH 9, at Different Constant Pressure Levels

	low-temperature area		high-temperature area	
P (MPa)	E_a (kJ/mol)	r^2	E_a (kJ/mol)	r^2
525	−53.9 ± 3.1[a]	0.990	48.8 ± 3.8	0.982
550	−55.2 ± 6.6	0.956	42.9 ± 12.3	0.802
575	−34.1 ± 5.7	0.879	11.5 ± 3.4	0.916
600	−36.4 ± 7.4	0.889		
625	−24.4 ± 3.7	0.956		

[a] Standard error.

Influence of Food-Relevant Intrinsic Factors on Thermal and Pressure Inactivation Kinetics of Soybean LOX. It is well established that kinetics largely depend on intrinsic factors (e.g., state of the food component, properties of the surrounding medium). Therefore, a case study was performed concerning the influence of some food-relevant intrinsic factors (enzyme concentration, type of buffer, pH) on the kinetics of thermally induced and pressure-induced inactivation of LOX. Because the interest in the use of high pressure to inactivate LOX mainly arises from the possibility of avoiding detrimental effects caused by heat, the influence of these factors on pressure inactivation was studied at low temperature (10−25 °C) and a constant pressure of 525 MPa. Former results on the inactivation of LOX, 0.4 mg/mL in 0.01 M Tris-HCl at pH 9, were used as reference.

Influence of Enzyme Concentration. The influence of a reduced enzyme concentration (0.1 mg/mL) on thermal and pressure inactivation kinetics of LOX was investigated. Under all conditions studied, first-order kinetics could analyze the inactivation of LOX at a concentration of 0.1 mg/mL. Whereas the thermal stability of LOX was slightly enhanced at higher enzyme concentration, no such effect was noted with respect to pressure stability, the k values being not significantly different in the latter case. Activation energies for thermal and pressure inactivation were respectively calculated as 300.4 ± 35.5 and −51.4 ± 7.1 kJ/mol, indicating that temperature sensitivity of the inactivation rate constants at atmospheric and elevated pressure is not affected by enzyme concentration. According to Putman (1953), proteins are known to be more resistant against heat and other denaturing agents in concentrate than in dilute solutions. Generally this protective effect is considered to be nonspecific and ascribed to various causes. Lopez and Burgos (1995), however, reported heat stability of soybean LOX to be independent of the enzyme concentration in the range 0−6 mg/mL.

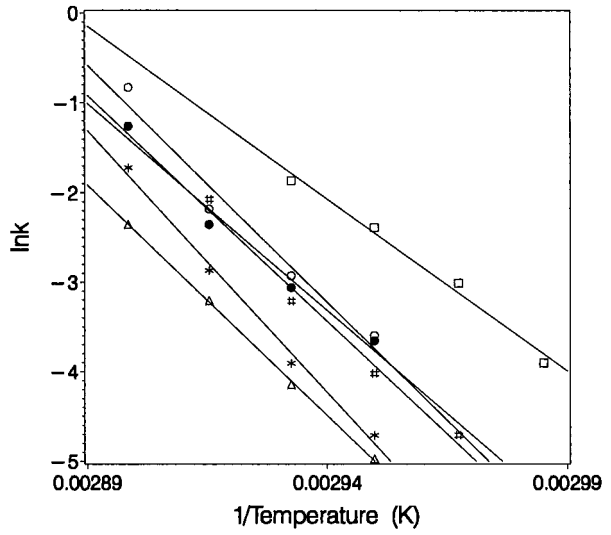

Figure 3. Influence of pH and type of buffer on isothermal inactivation kinetics of soybean LOX, 0.4 mg/mL: (*) Tris buffer, pH 9, flushed with CO_2; (●) McIlvaine buffer, pH 5.4; (○) McIlvaine buffer, pH 6.6; (△) Tris buffer, pH 6.6; (#) Tris buffer, pH 8; (□) Tris buffer, pH 9.

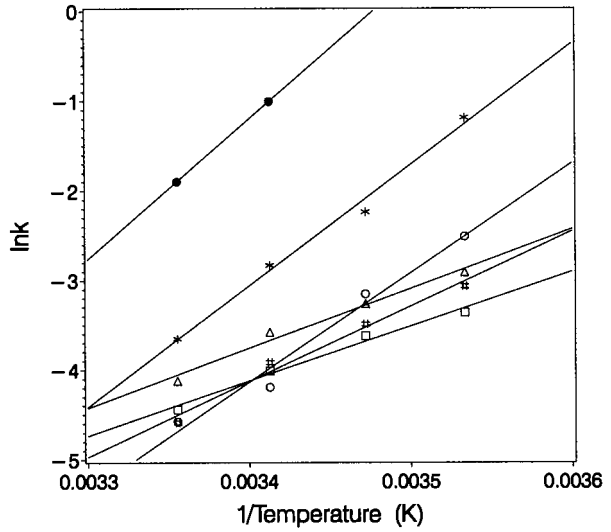

Figure 4. Influence of pH and type of buffer on isobaric–isothermal inactivation kinetics of soybean LOX, 0.4 mg/mL: (*) Tris buffer, pH 9, flushed with CO_2 at 400 MPa; (●) McIlvaine buffer, pH 5.4, at 400 MPa; (○) McIlvaine buffer, pH 6.6, at 400 MPa; (△) Tris buffer, pH 6.6, at 525 MPa; (#) Tris buffer, pH 8, at 525 MPa; (□) Tris buffer, pH 9, at 525 MPa.

Influence of Type of Buffer and pH. The influence of pH was investigated in the range 4.2–9. Because of restrictions set to buffering capacity of Tris-HCl, a McIlvaine buffer was used in the low-pH range (4.2–6.6). As solubility problems were encountered at pH 4.2, this pH value was not considered in further experiments. For each of the pH values studied (5.4, 6.6, 8, and 9), thermal as well as pressure inactivation could be accurately described by a first-order kinetic model (Figure 3; Figure 4).

Both thermal and pressure stabilities were largely affected by the type of buffer. LOX seemed to be much more stable in Tris buffer as compared to McIlvaine buffer. Inactivation rate constants for isothermal inactivation at pH 6.6 were quadrupled on switching from Tris to McIlvaine buffer (Figure 3). The type of buffer, on the other hand, did not affect the activation energy.

Table 5. E_a Values for Thermal and Pressure Inactivation of LOX, 0.4 mg/mL, in Different Buffers with Different pH Values

system	P/T range for inactivation	E_a (kJ/mol)
Isothermal Inactivation		
Tris-HCl buffer, pH 9	0.1 MPa, 62–68 °C	319.8 ± 27.3[a]
Tris-HCl buffer, pH 8	0.1 MPa, 64–70 °C	419.5 ± 36.7
Tris-HCl buffer, pH 6.6	0.1 MPa, 66–72 °C	428.7 ± 7.7
McIlvaine buffer, pH 6.6	0.1 MPa, 66–72 °C	439.8 ± 57.6
McIlvaine buffer, pH 5.4	0.1 MPa, 66–72 °C	383.8 ± 77.1
Tris-HCl buffer, pH 9, flushed with CO_2	0.1 MPa, 66–72 °C	486.7 ± 25.7
Isobaric–Isothermal Inactivation		
Tris-HCl buffer, pH 9	525 MPa, 10–25 °C	−50.9 ± 4.4
Tris-HCl buffer, pH 8	525 MPa, 10–25 °C	−67.8 ± 10.6
Tris-HCl buffer, pH 6.6	525 MPa, 10–25 °C	−55.5 ± 5.4
McIlvaine buffer, pH 6.6	400 MPa, 10–25 °C	−101.7 ± 11.5
McIlvaine buffer, pH 5.4	400 MPa, 20–25 °C	−129.5
Tris-HCl buffer, pH 9, flushed with CO_2	400 MPa, 10–25 °C	−112.4 ± 7.9

[a] Standard error.

Pressure stability in McIlvaine buffer was reduced to such an extent that inactivation could not be studied at the preset pressure (525 MPa), because after pressure buildup, the blank activity became too low to investigate sufficiently accurately the progressive decrease in activity with time. Therefore, the influence of pH in McIlvaine buffer was investigated at 400 MPa (Figure 4). These results are somewhat conflicting with the ones published by Heinisch et al. (1995), who observed a similar pressure-dependent decrease of LOX activity in both Tris and borate buffer.

For both buffer systems used, thermostability increased with decreasing pH, although this effect became less pronounced at pH 5.4. Thermostability of LOX seems maximal at a pH value corresponding to its isoelectric point (Ludikhuyze et al., 1998). Although the temperature sensitivity of the inactivation rate constant seemed lowest in Tris buffer at pH 9, no significant differences in E_a values for the enzyme solutions in McIlvaine or Tris buffer at different pH values could be detected, on the basis of the 95% confidence interval (Table 5). The same findings, that is, enhanced thermostability of LOX at lower pH, were often reported in the literature. The susceptibility to heat of LOX in whole soybeans was greatly enhanced as pH was increased from 6.8 to 8 (Borhan and Snyder, 1979). Furthermore, thermal and manothermosonication resistances of soybean LOX were intensified with decreasing pH in the range 8 to 5.2, but more for thermal than for manothermosonication treatment. The fact that thermal stability keeps growing as the pH of the medium moves away from the optimum (pH 9) toward more acidic values seems a rather unusual phenomenon. It could be explained by the fact that electrostatic interactions between amino acid residues located in parts of the molecule away from the active site play an important role in protecting the native structure against heat (Lopez and Burgos, 1995). For pea LOX, inactivation was studied in the pH range 4–8 and maximal thermal stability was observed at pH 6 (Svensson and Eriksson, 1972). The two isozymes of germinated barley showed opposite behavior in the pH range 7 to 4.65: the thermostability of LOX1 decreased, whereas that of LOX2 increased (Hugues et al., 1994).

As opposed to thermal stability, pressure stability increased with increasing pH. Although the inactiva-

tion rates of LOX in Tris buffer at pH 9 and 8 were very similar, lowering the pH to 6.6 could accelerate inactivation (Figure 4). In McIlvaine buffers, this pH effect was even more pronounced. For both buffer systems, no significant influence of pH on the activation energy value was noted (Table 5). These results could possibly indicate mechanistic differences between thermal and pressure inactivation because at elevated pressure the enzyme is no longer protected against inactivation by pH values near the isoelectric point. Whereas the existence of such mechanistic differences has previously been postulated on the basis of FTIR studies on heat- and pressure-denatured LOX, it could not be confirmed by gel electrophoretic studies (Goossens et al., 1992; Ludikhuyze et al., 1998)

Finally, the pH effect caused by flushing with CO_2 was examined. The enzyme solution (0.4 mg/mL LOX in 0.01 M Tris-HCl at pH 9) was flushed with CO_2 for 5 min at 25 °C and afterward allowed to stand for 10 min to avoid supersaturation. Dissolution of CO_2 in water results in the formation of H_2CO_3 that lowers the pH to ~6.8. Indeed, thermostability was on the same order of magnitude as for LOX in Tris-HCl at pH 6.6 (Figure 3). Pressure stability, on the other hand, was largely reduced as compared to LOX in Tris-HCl at pH 6.6. After building up pressure to 525 MPa, activity was reduced below the detection limit. Therefore, experiments were carried out at 400 MPa, in agreement with those in McIlvaine buffer. Even at this lower pressure level, k values were ~2−10 times higher as compared to those for LOX in Tris-HCl at pH 6.6. The activation energy for pressure inactivation was on the same order of magnitude as that for pressure inactivation in McIlvaine buffer (Figure 4; Table 5). This may be explained by taking into account the distinct dissociation volumes of the buffers used. A Tris-HCl buffer is known to be very insensitive to pressure variations as it is characterized by a dissociation volume (ΔV^D) of ~1 cm^3/mol (Morild, 1981). For McIlvaine buffer and H_2CO_3, ΔV^D values in the range −25 to −30 cm^3/mol were derived (Morild, 1981; Kitamura and Itoh, 1987). Therefore, strong pH changes under pressure must be expected using the latter buffers. This way, the extreme sensitization when using McIlvaine buffer or CO_2-bubbled Tris buffer, as well as the close agreement between the results of pressure inactivation in the respective buffers, may be explained.

Conclusions. The advantage of performing isobaric−isothermal inactivation experiments on simple enzymatic model systems is twofold. First, contributions of pressure and temperature to changes in the inactivation rate constant can be separated, allowing temperature and pressure dependence of the inactivation rate constant to be expressed by individual mathematical models. Second, the use of simple model systems to study pressure inactivation of enzymes allows changes in the kinetic parameters to be attributed to specific and controlled variations in experimental conditions. When one is working with complex food components, variations in the sample, which are not readily known, can interfere. A disadvantage, however, is that the kinetic models and concomitant parameters are strongly restricted to the specific model system (i.e., the enzyme in its surrounding medium) as kinetic parameters seem to be influenced by extrinsic and intrinsic factors. Otherwise, it can be stated that kinetic models, established for simple systems, can be refined using synthetic media simulating the conditions of real food products by adjustment of food-relevant intrinsic factors such as pH, enzyme concentration, ionic strength, macromolecules, and oxygen concentration while still reducing the analytical difficulties linked to working with real food products. Moreover, knowledge about how medium properties influence the kinetics of enzyme inactivation may be useful in regard to destabilization, allowing required pressure and temperature to be reduced without affecting the global impact of the process. In view of pressure application as an alternative to blanching, sensitization of LOX toward pressure may be important. Whereas enzyme concentration induces only minor changes in the stability of LOX, promising results have been obtained with respect to the extreme sensitization of LOX toward pressure treatment by lowering the pH, including flushing with CO_2.

ABBREVIATIONS USED

A, activity at time t; A_0, activity at time zero; E_a, activation energy (kJ/mol); k, inactivation rate constant (min^{-1}); k_{refT}, inactivation rate constant at reference temperature (min^{-1}); k_{refP}, inactivation rate constant at reference pressure (min^{-1}); LOX, lipoxygenase; P, pressure (MPa); P_{ref}, reference pressure (500 MPa); R, universal gas constant (8.314 J/mol·K); T, absolute temperature (K); T_{ref}, absolute reference temperature (323 K); t, time (min); Tris, tris(hydroxymethyl)aminomethane; V_a, activation volume (cm^3/mol).

LITERATURE CITED

Baur, C.; Grosch, W.; Wieser, H.; Jugel, H. Enzymatic oxidation of linoleic acid: formation of bittering fatty acids. *Z. Lebens. Unters. Forsch.* **1977**, *164*, 171−176.

Bhirud, P. R.; Sosulski, F. W. Thermal inactivation kinetics of wheat germ lipoxygenase. *J. Food Sci.* **1993**, *58* (5), 1095−1098.

Borhan, M.; Snyder, H. E. Lipoxygenase destruction in whole soybeans by combinations of heating and soaking in ethanol. *J. Food Sci.* **1979**, *44* (2), 586−590.

Eskin, M. N. A.; Grossman, S.; Pinsky, A. Biochemistry of lipoxygenase in relation to food quality. *Crit. Rev. Food Sci. Nutr.* **1977**, Apr, 1−40.

Galliard, T.; Chan, H. W.-S. In *The Biochemistry of Plants, Lipids: Structure and Function*; Stumpf, P. K., Ed.; Academic Press: New York, 1980; Vol. 4, Chapter 5 (Lipoxygenases), pp 131−161.

Ganthavorn, C.; Nagel, C. W.; Powers, J. R. Thermal inactivation of asparagus lipoxygenase and peroxidase. *J. Food Sci.* **1991**, *56* (1), 47−49.

Goossens, K.; Smeller, L.; Heremans, K.; Frank, J. Pressure effect on lipoxygenase: a FTIR study with the diamond anvill cell. In *High Pressure and Biotechnology*; Balny, C., Hayashi, R., Heremans, K., Masson, P., Eds.; John Libbey Eurotext: Montrouge, 1992; Vol. 24, pp 171−174.

Günes, B.; Bayindirli, A. Peroxidase and lipoxygenase inactivation during blanching of green beans, green peas and carrots. *Lebensm.-Wiss. -Technol.* **1993**, *26*, 406−410.

Heinisch, O.; Kowalski, E.; Goossens, K.; Frank, J.; Heremans, K.; Ludwig, H.; Tauscher, B. Pressure effects on the stability of lipoxygenase: Fourier Transform Infrared spectroscopy and enzyme activity study. *Z. Lebensm. Unters. Forsch.* **1995**, *201*, 562−565.

Henderson, H. M.; Blank, G.; Sustackova, H. Thermal inactivation of pea flour lipoxygenase. *J. Food Biochem.* **1991**, *15*, 107−115.

Heremans, K. The behavior of proteins under pressure. In *High-Pressure Chemistry, Biochemistry and Material Science*; Winter, R., Jonas, J., Eds.; Kluwer Academic Publishers: Dordrecht, 1993; pp 443−469.

Hoover, D. G. Pressure effects on biological systems. *Food Technol.* **1993**, *47* (6), 150−155.

Hoover, D. G.; Metrick, C.; Papineau, A. M.; Farkas, D. F.; Knorr, D. Biological effects of high hydrostatic pressure on food microorganisms. *Food Technol.* **1989**, *43* (3), 99−107.

Hugues, M.; Bovin, P.; Gauillard, F.; Nicolas, J.; Thiry, J.-M.; Richard-Forget, F. Two lipoxygenases from germinated barley: Heat and killing stability. *J. Food Sci.* **1994**, *59* (4), 885−889.

Johnson, F. H.; Campbell, D. H. Retardation of protein denaturation by high pressure. *J. Cell. Comp. Physiol.* **1945**, *26*, 43−46.

Kermasha, S.; Bisakowski, B.; Ramaswamy, H.; Van de Voort, F. R. Thermal and microwave inactivation of soybean lipoxygenase. *Lebensm.-Wiss. -Technol.* **1993**, *26*, 215−219.

Kitamura, Y.; Itoh, T. Reaction volume of protonic ionization for buffering agents. Prediction of pressure dependence of pH and pOH. *J. Solution Chem.* **1987**, *16* (9), 715−725.

Lopez, P.; Burgos, J. Lipoxygenase inactivation by manothermosonication: effects of sonication physical parameters, pH, KCl, sugars, glycerol and enzyme concentration. *J. Agric. Food Chem.* **1995**, *43*, 620−625.

Ludikhuyze, L. R.; Indrawati; Van den Broeck, I.; Weemaes, C. A.; Hendrickx, M. E. High pressure and thermal denaturation kinetics of soybean lipoxygenase: a study based on gel electrophoresis. *Lebensm.-Wiss. -Technol.* **1998**, in press.

Mertens, B. Hydrostatic pressure treatment of food: equipment and processing. In *New Methods of Food Preservation*; Gould, G. W., Ed.; Blackie Academic and Professional: Glasgow, 1995; pp 135−158.

Miyagawa, K.; Suzuki, K. Pressure inactivation of enzyme: some kinetic aspects of pressure inactivation of trypsin. *Rev. Phys. Chem. Jpn.* **1963a**, *32*, 43−50.

Miyagawa, K.; Suzuki, K. Pressure inactivation of enzyme: some kinetic aspects of pressure inactivation of chymotrypsin. *Rev. Phys. Chem. Jpn.* **1963b**, *32*, 88−90.

Miyagawa, K.; Suzuki, K. Studies on Taka amylase A under high pressure: some kinetic aspects of pressure inactivation of Taka amylase A. *Arch. Biochem. Biophys.* **1964**, *105*, 297−302.

Morild, E. The theory of pressure effects on enzymes. *Adv. Protein Chem.* **1981**, *34*, 93−166.

Mozhaev, V. V.; Heremans, K.; Frank, J.; Masson, P.; Balny, C. Exploiting the effects of high hydrostatic pressure in biotechnological applications. *Trends Biotechnol.* **1994**, *12* (12), 493−501.

Mozhaev, V. V.; Lange, R.; Kudryashova, E. V.; Balny, C. Application of high hydrostatic pressure for increasing activity and stability of enzymes. *Biotechnol. Bioeng.* **1996**, *52*, 320−331.

Park, K. H.; Kim, Y. M.; Lee, C. W. Thermal inactivation kinetics of potato tuber lipoxygenase. *J. Agric. Food Chem.* **1988**, *36*, 1012−1015.

Putman, F. W. Protein denaturation. In *The Proteins*; Neurath, H., Bailey, K., Eds.; Academic Press: New York, 1953; Vol. 1 (Part B), pp 807−892.

SAS Institute Inc. *SAS User's Guide: Statistics*; SAS Institute Inc.: Cary, NC, 1982.

Srinivasulu, S.; Appu Rao, A. G. Structure and kinetic thermal stability studies of the interaction of monohydric alcohols with lipoxygenase I from soybean (*Glycine max*). *J. Agric. Food Chem.* **1995**, *43*, 562−567.

Suzuki, K.; Kitamura, K. Denaturation of hemoglobin under high pressure, II. *Rev. Phys. Chem. Jpn.* **1960**, *29* (2), 86−90.

Suzuki, K.; Kitamura, K.; Kagawa, S.; Tamura, K. Thermal denaturation of egg albumin under moderate pressure. *Mem. Res. Inst. Sci. Eng., Ritsumeikan Univ.* **1958**, *3*, 1−4.

Svensson, S. G.; Eriksson, C. E. Thermal inactivation of lipoxygenase from peas: Time−temperature relationships and pH dependence. *Lebensm.-Wiss. -Technol.* **1972**, *5* (4), 118−123.

Taniguchi, Y.; Suzuki, K. Pressure inactivation of α-chymotrypsin. *J. Phys. Chem.* **1983**, *87*, 215−223.

Weemaes, C. A.; De Cordt, S. V.; Ludikhuyze, L. R.; Van den Broeck, I.; Hendrickx, M. E.; Tobback, P. P. Influence of pH, benzoic acid, EDTA and glutathione on the pressure and/or temperature inactivation kinetics of mushroom polyphenoloxidase. *Biotechnol. Prog.* **1997**, *13*, 25−32.

Weemaes, C.; Ludikhuyze, L.; Van den Broeck, I.; Hendrickx, M. Kinetics of combined pressure-temperature inactivation of avocado polyphenol oxidase. *Biotechnol. Bioeng.* **1998**, in press.

Whitaker, J. R. Lipoxygenase. In *Principles of Enzymology for the Food Sciences*; Dekker: New York, 1972; Chapter 25, pp 607−617.

Williams, D. C.; Lim, M. H.; Chen, A. O.; Pangborn, R. M.; Withaker, J. R. Blanching of vegetables for freezing: which indicator enzyme to choose. *Food Technol.* **1986**, *40* (6), 130−140.

Zhang, Q.; Cavalieri, R. P.; Powers, J. R.; Wu, J. Measurement of lipoxygenase activity in homogenized green bean tissue. *J. Food Sci.* **1991**, *56* (3), 719−721.

Received for review March 13, 1998. Revised manuscript received July 20, 1998. Accepted July 23, 1998. This research has been supported by the Belgian Fund for Scientific Research (FWO), the European Commission as a part of Project FAIR-CT96-1175, the KULeuven Research Council as a part of Project OT/94/19, and the Prime Minister's Office of the Federal Office for Scientific, Technical and Cultural Affaires as a part of Project N0/01/010 (within the program of Scientific Support for Standardization Procedures).

JF980256C