Soybean Lipoxygenase Inactivation by Pressure at Subzero and Elevated Temperatures

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Soybean lipoxygenase (LOX) inactivation [0.4 mg/mL in Tris-HCl buffer (0.01 M, pH 9)] was studied quantitatively under constant pressure (up to 650 MPa) and temperature (−15 to 68 °C) conditions and kinetically characterized by rate constants, activation energies, and activation volumes. The irreversible LOX inactivation followed a first-order reaction at all pressure−temperature combinations tested. In the entire pressure−temperature area studied, LOX inactivation rate constants increased with increasing pressure at constant temperature. On the contrary, at constant pressure, the inactivation rate constants showed a minimum around 30 °C and could be increased by either a temperature increase or decrease. On the basis of the calculated rate constants at 102 pressure−temperature combinations, an iso-rate contour diagram was constructed as a function of pressure and temperature. The pressure−temperature dependence of the LOX inactivation rate constants was described successfully using a modified kinetic model of Hawley (*Biochemistry* **1971**, *10*, 2436−2442).

Keywords: *Lipoxygenase; soybean; inactivation; pressure; thermal; subzero temperature; kinetics*

INTRODUCTION

High-pressure research has concentrated on three aspects: the adaptation of living organisms to the depths of the oceans, the use of pressure as a tool to perturb physical and chemical systems to study the behavior of matter under pressure, and the possibility of using high pressure as a unit operation in food material treatment (Heremans, 1992). Corresponding to the latter aspect, the key effects of high pressure on food systems involve (i) inactivation of microorganisms; (ii) modification of biopolymers including enzyme activation and inactivation, protein denaturation, and gel formation; (iii) susceptibility to enzymatic action as well as quality retention such as stability of color, flavor, or palatability; and (iv) product functionality as exemplified by density changes, freezing and melting temperatures, or textural attributes (Knorr, 1993).

The effect of high pressure on the phase transitions of water has been studied in detail by Bridgman (1912), but until recent years little attention has been paid to food applications. Because pressure reduces the freezing point of water and the melting point of ice, several potential applications in food technology, for example, (i) pressure-assisted freezing, (ii) pressure-assisted thawing, and (iii) nonfrozen storage at subzero temperature (under pressure), are currently put forward (Kalichevsky et al., 1995). For frozen legumes and vegetables, blanching is necessary to inactivate some undesired enzymes such as lipoxygenase and peroxidase. Because lipoxygenase is currently considered as a blanching index rather than peroxidase (Williams et al., 1986; Sheu and Chen, 1991; Barret and Theerakulkait, 1995), the effect of pressure and temperature on LOX inactivation should be studied systematically to evaluate, on a scientific basis, the feasibility of a combined pressure−temperature process as an alternative to the currently applied thermal blanching and freezing process. The purpose of this study was to investigate kinetically the effect of pressure (up to 650 MPa), combined with subzero and elevated temperatures (−15 to 68 °C), on LOX.

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MATERIALS AND METHODS

Enzyme and Substrate Preparation. Lipoxygenase type 1B from soybean (LOX, Sigma, EC 1.13.11.12, lot 118F03422), 0.4 mg/mL in Tris-HCl buffer (0.01M; pH 9) was used as an enzymatic model system. One unit of enzyme caused an absorbance increase at 234 nm of 0.001/min at pH 9 and 25 °C when linoleic acid was used as a substrate in 3.0 mL volume (1 cm light path).

The substrate was prepared according to a modified method of Axelrod et al. (1981): 280 mg of linoleic acid ($C_{18}H_{32}O_2$, *cis*-9,*cis*-12-octadecodienoic acid) and an equal amount of Tween 20 (polyoxyethylenesorbitanmonolaurat) as an emulsifier were homogenized in 4 mL of oxygen-free distilled water by drawing back and forth in a Pasteur pipet, avoiding air bubbles. To clear the solution after homogenization, NaOH (0.5 N) was added and the total volume was made up to 25 mL by distilled water. After flushing with N_2, the solution was divided into small portions (1−1.5 mL) and kept frozen until usage.

Measurement of Enzyme Activity. A spectrophotometric assay (Biochrom 4060, UV−visible spectrophotometer, Pharmacia LKB−Biochrom Ltd.) was used to measure LOX activity at 25 °C following a modified procedure as described by Axelrod et al. (1981). The assay mixture contained 2.9 mL of sodium borate buffer (0.0125 M, pH 9), 0.025 mL of substrate solution, and 0.045 mL of enzyme solution. At low residual enzyme activity after treatment, the amount of enzyme was increased to be (0.045 + *x*) mL (maximal up to 0.3 mL of the total amount) to allow a detectable change in absorbance at 234 nm and, consequently, the volume of buffer solution was adjusted to (2.9 − *x*) mL. The absorbance at 234 nm was

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recorded as a function of reaction time (3 min). The enzyme activity was derived from the slope of the linear portion of the absorption versus reaction time curve.

Thermal Treatment at Ambient Pressure. Thermal treatments were designed to be isothermal to facilitate kinetic analysis. To avoid heating lags, the enzyme solution was contained in thin capillary tubes (Hirschmann, 1.15 mm i.d. and 150 mm length) and heated in a temperature-controlled water bath (Haake DC1-B3, cryostat) during preset time intervals. The samples were immediately cooled in ice water upon their withdrawal from the water bath to stop thermal inactivation. The activity measurement was performed after ice bath storage for 20–30 min. No reactivation was noticed during storage.

Combined Pressure and Temperature Treatment. Pressure–temperature (*P–T*) treatments were carried out in a multivessel (eight vessels with a volume of 8 mL) high-pressure equipment (HPIU-10000, 95/1994, Resato, Roden, Holland) with a maximum pressurization capacity of 1000 MPa. A mixture of oil–glycol (TR15, Resato) was used as pressure-transferring liquid. The equipment was thermostated by a cryostat (Haake N8-KT50W (56% ethylene glycol as cooling medium) or Haake F3-K (distilled water as heating medium), respectively, for subzero and elevated temperatures). Temperature (thermocouple type J) and pressure could be recorded (data logger Cobra 7–10 Mess+system Technic GmbH) continuously during treatment.

The enzyme solution was filled in flexible microcups (Elkay, 0.375 mL) without creating air bubbles by using a syringe and closed with Parafilm to avoid pressure medium contamination. For pressure treatments at subzero temperature, microcups were double-wrapped in a vacuumed polyethylene pocket to protect the enzyme solution from oil–glycol contamination because of microcups opening upon freezing. Samples were incorporated in the preheated or precooled pressure vessels. Pressure buildup was standardized at a constant rate of 90 MPa/min. After achievement of the desired pressure, all vessel valves were closed to isolate the pressure in the vessels and the central circuit was decompressed. As the experiments were designed to be "isobaric and isothermal", an equilibration time of 5 min (Indrawati et al., 1998a) was needed to ensure the preset temperature was constant. After this equilibration time, one vessel was decompressed and the enzyme activity referring to this vessel was considered as A_0 (enzyme activity at $t = 0$). Afterward, the other vessels were decompressed as a function of time. After depressurization, the samples were kept in the vessel for 4 min and afterward stored at 25 °C (Indrawati et al., 1998b). For pressure treatments at high temperature (above 30 °C), samples were kept in the vessel for only 1 min after decompression, and upon their withdrawal, samples were directly cooled in an ice bath for 5 min to stop thermal inactivation. No activity increase was noticed during storage at 25 °C for 3 h.

Estimation of Kinetic Parameters. Inactivation of enzymes is frequently assumed to obey a first-order kinetic model (eq 1) describing a simple conversion of native or active enzyme (E_N) to denatured or inactive enzyme (E_I) with a specific rate constant k (eq 2). For constant extrinsic (temperature and pressure) and intrinsic factors, the inactivation rate constant is independent of time and eq 1 can be integrated as eq 3.

$$\frac{d[E_N]}{dt} = -k[E_N] \quad \text{or} \quad \frac{dA}{dt} = -kA \tag{1}$$

$$E_N \xrightarrow{k} E_I \tag{2}$$

$$\ln\frac{[E_N]}{[E_{N0}]} = -kt \quad \text{or} \quad \ln\frac{A}{A_0} = -kt \tag{3}$$

On the basis of linear regression analysis (SAS, 1995), the inactivation rate constant (k) can be derived from the slope of the regression line obtained by plotting the natural logarithm of relative residual activity as a function of inactivation time.

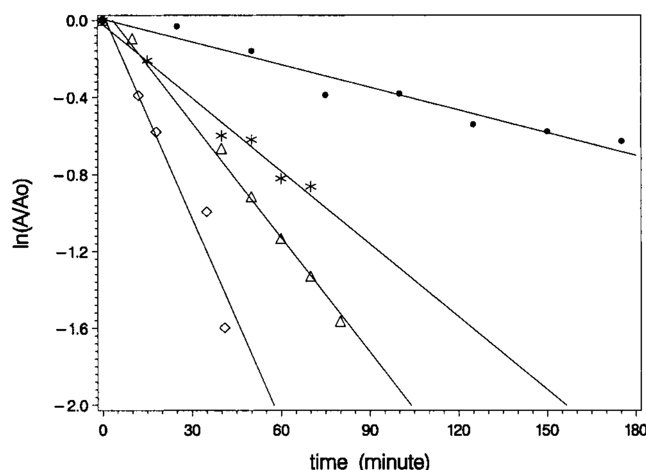

Figure 1. Isobaric and isothermal inactivation of LOX (0.4 mg/mL) in Tris-HCl buffer (0.01 M, pH 9) at −12 °C and different constant pressures: 300 (●), 350 (*), 375 (△), and 400 (◇) MPa.

The Arrhenius equation is the most common mathematical expression to describe the temperature effect on the (inactivation) rate constants at constant pressure. The Arrhenius relation can be linearized by a logarithmic transformation, expressed as

$$\ln(k) = \ln(k_{ref-T}) - \left[\frac{E_a}{R_t}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right] \tag{4}$$

To describe the pressure dependence of protein denaturation or enzyme inactivation rate constants at constant temperature, the Eyring relation is often used (Suzuki, 1958; Suzuki and Kitamura, 1963; Ludikhuyze et al., 1997; Weemaes et al., 1998). The Eyring relation at a defined reference pressure can be rewritten as eq 5 by a logarithmic conversion.

$$\ln(k) = \ln(k_{ref-P}) - \left[\frac{V_a}{R_p T}(P - P_{ref})\right] \tag{5}$$

The kinetic parameters, activation energy (E_a) and activation volume (V_a), are estimated on the basis of a linear regression analysis (SAS, 1995) of ln(k) versus the reciprocal of the absolute temperature or ln(k) versus pressure, respectively.

RESULTS AND DISCUSSION

Pressure treatment of lipoxygenase (0.4 mg/mL) in Tris-HCl buffer (0.01 M, pH 9) at subzero and elevated temperatures resulted in an irreversible enzyme inactivation. The inactivation followed a first-order reaction (Figure 1) as previously reported for thermal and combined pressure and elevated temperature treatments (Svensson and Ericksson, 1972; Al-Obaidy and Siddiqi, 1981; Henderson et al., 1991; Ludikhuyze et al., 1998). Factors causing enzyme inactivation in the case of pressure treatments at subzero temperature could be the extrinsic factors pressure and temperature but also mechanical damage, by entering different solid–liquid water phase transitions.

Unfortunately, the behavior of enzymes at low temperature is less well-known, and the phenomenon of cold denaturation under pressure is not yet understood. Several studies show protein denaturation, for example, ribonuclease A (Zhang et al., 1995; Nash et al., 1996) and allophycocyanin (Foguel and Weber, 1995), and enzyme inactivation (Indrawati et al., 1998b) at low temperature under pressure. Some hypotheses based on

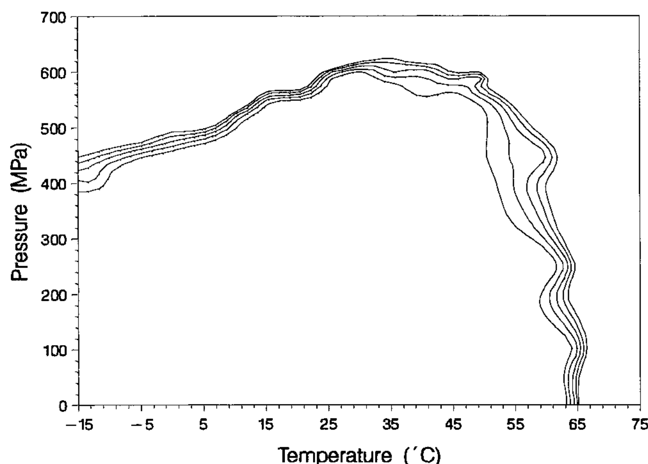

Figure 2. Iso-rate contour diagram of LOX inactivation (0.4 mg/mL) in Tris-HCl buffer (0.01 M, pH 9) as a function of pressure and temperature. The upper line indicates k value of 0.07 min^{-1}, and the lower line indicates k value of 0.03 min^{-1}.

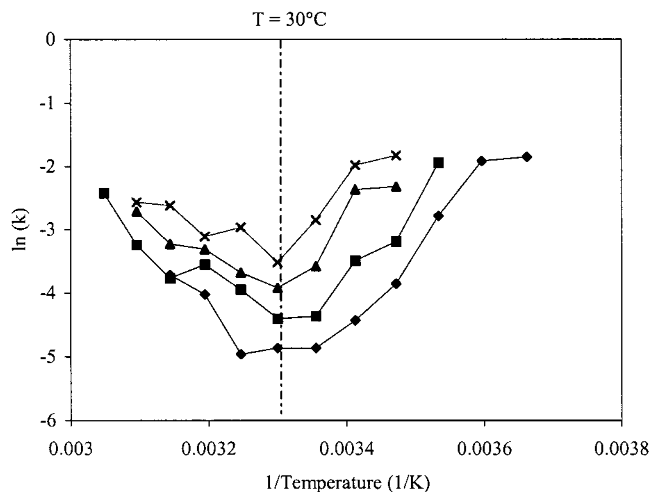

Figure 3. Natural logarithm of inactivation rate constant versus the reciprocal of absolute temperature for various constant pressures: 525 (◆), 550 (■), 575 (▲), and 600 (×) MPa.

the interaction between protein nonpolar groups and adjacent water have been postulated to explain this phenomenon (Richardson and Hyslop, 1985; Silva et al., 1996).

The reproducibility of determination of inactivation rate constants (k values) was investigated, mainly in the low-temperature area. The percentage difference in LOX inactivation rate constants between two replicate identical pressure–temperature treatments ranged between 2 and 15%. For all tested pressure–temperature combinations, the estimated LOX inactivation rate constants, derived from eq 3, are summarized in Table 1. On the basis of the calculated k values, an iso-rate contour diagram indicating pressure–temperature combinations resulting in the same inactivation rate constants was constructed (Figure 2) by interpolation. From Figure 2, it can be seen that the same LOX inactivation rate constant can be achieved by combining a certain pressure level with either subzero or elevated temperature.

Temperature Dependence of Inactivation Rate Constant. No linear relationship was observed by plotting the natural logarithm of inactivation rate constants as a function of reciprocal absolute temperature, indicating that the Arrhenius model was not valid in the entire pressure and temperature domain studied. At elevated pressure, the LOX inactivation rate constant was minimal around 30 °C, indicating the highest pressure stability of LOX at that temperature, and it could be enhanced by a temperature increase as well as by a temperature decrease (Figure 3). To apply the Arrhenius model, the temperature area was divided into two parts, namely, low temperature (LT) and high temperature (HT) for temperatures below and above 30 °C, respectively. The predicted E_a values at various constant pressures in both temperature domains are given in Table 2. It was noticed that at some pressure levels (e.g., 300 and 550 MPa), the Arrhenius model was less appropriate to describe the temperature dependence of the inactivation rate constants, as indicated by a lower correlation coefficient. Especially at these pressure levels, extrapolation of the rate constants to higher or lower temperatures should be avoided. The negative activation energies in the low-temperature area imply an increase in the inactivation rate constant with decreasing temperature. When the absolute values of E_a were compared, it was noticed that in the pressure range from 500 to 575 MPa, a certain temperature change influenced the LOX inactivation rate constant in the low-temperature area to a larger extent as compared to the change in inactivation rate constant in the high-temperature area. In general, in the high-temperature area, the activation energy of LOX inactivation at constant pressure decreases as pressure increases as previously reported by Ludikhuyze et al. (1998). On the contrary, in the low-temperature area, the highest thermosensitivity of the inactivation rate constants was observed at pressure around 525–550 MPa; both increasing and lowering that pressure level result in a decrease in thermosensitivity.

Pressure Dependence of Inactivation Rate Constants. The Eyring model (eq 5) was not valid for soybean LOX inactivation in the entire $P-T$ area studied, but only in the low- and subzero-temperature region from −15 to 35 °C, although at −10 and −15 °C the correlation coefficients of linear regression of ln(k) versus pressure were somewhat lower. In that region (−15 to 35 °C), estimated activation volumes at constant temperature had a negative sign (Table 3), indicating an acceleration of the LOX inactivation by increasing pressure. The highest pressure sensitivity of the inactivation rate constants, or alternatively the temperature at which a certain increase in pressure has the greatest acceleration effect, occurs at temperatures around 20–25 °C. From the shape of the pressure–temperature kinetic diagram for inactivation of soybean LOX (Figure 2), an antagonistic effect of pressure and temperature in the low-temperature area can be observed, as was the case for LOX of green beans (Indrawati et al., 1998a): a temperature increase retarded the pressure–temperature inactivation of LOX. From Figure 2 no clear retardation effect of low pressure on thermal inactivation was observed, in contrast to a previous study by Heinisch et al. (1995). In the temperature area above 35 °C, it was noticed that the relation between inactivation rate constants at constant temperature and pressure could be expressed by eq 6. This relation is empirical in nature, and no theoretical information can be derived from the model parameters (a and b) (Table 4).

Table 1. Inactivation Rate Constant Values ($\times 10^{-2}$ in min^{-1}) of LOX (0.4 mg/mL) in Tris-HCl Buffer (0.01 M, pH 9) under Isobaric Isothermal Conditions (with $r^2 > 0.950$)

T (°C)	P (MPa)																			
	0.1	50	100	150	200	250	300	325	350	375	400	425	450	475	500	525	550	575	600	625
68	18.28 ± 0.55[a]		10.76 ± 0.26	11.00 ± 1.09	13.24 ± 0.63	17.86 ± 0.81														
65	6.12 ± 0.31		3.82 ± 0.22	6.35 ± 0.69	9.10 ± 0.85	7.69 ± 0.59														
62	1.98 ± 0.06																			
60	1.22 ± 0.05	1.22 ± 0.12	1.92 ± 0.08		3.53 ± 0.26				6.18 ± 0.43		6.97 ± 0.36		4.96 ± 0.33							
58	0.78 ± 0.03																			
55		1.42 ± 0.12			1.65 ± 0.15		2.47 ± 0.19				4.11 ± 0.14		4.22 ± 0.22		4.87 ± 0.22		8.88 ± 0.69			
50			1.48 ± 0.11				2.16 ± 0.15				2.37 ± 0.20				2.85 ± 0.19		3.91 ± 0.20	6.63 ± 0.49	7.69 ± 0.59	
45							0.94 ± 0.08				1.47 ± 0.06				2.04 ± 0.24	2.43 ± 0.11	2.31 ± 0.21	3.96 ± 0.28	7.29 ± 0.50	
40											1.35 ± 0.14				1.63 ± 0.14	1.79 ± 0.16	2.86 ± 0.18	3.64 ± 0.22	4.45 ± 0.51	9.57 ± 0.19
35																0.70 ± 0.04	1.93 ± 0.10	2.53 ± 0.18	5.14 ± 0.62	6.97 ± 0.25
30																0.77 ± 0.03	1.22 ± 0.08	1.98 ± 0.21	2.96 ± 0.18	
25																0.77 ± 0.04	1.27 ± 0.08	2.78 ± 0.12	5.77 ± 0.28	16.93 ± 1.64
20															0.59 ± 0.06	1.19 ± 0.04	3.04 ± 0.18	9.36 ± 0.68	13.77 ± 0.89	
15															1.04 ± 0.05	2.12 ± 0.06	4.11 ± 0.23	9.80 ± 0.30	16.05 ± 0.34	
10													0.65 ± 0.04	1.41 ± 0.10	3.32 ± 0.13	6.18 ± 0.41	14.29 ± 0.44			
5											0.50 ± 0.04	0.59 ± 0.05	1.02 ± 0.07	3.51 ± 0.19	7.56 ± 0.30	14.71 ± 0.45				
0												0.79 ± 0.05	2.16 ± 0.22	5.04 ± 0.23	8.13 ± 0.55	15.67 ± 0.61				
−5											0.99 ± 0.05	1.72 ± 0.12	3.39 ± 0.29	7.21 ± 0.79						
−10										1.02 ± 0.05	2.65 ± 0.16	3.32 ± 0.28								
−12							0.39 ± 0.04		1.32 ± 0.15	2.07 ± 0.17	3.90 ± 0.18									
−15							0.49 ± 0.06	1.42 ± 0.14	1.78 ± 0.12		3.66 ± 0.31									

[a] Standard error.

Table 2. Temperature Dependence of Inactivation Rate Constants of LOX (0.4 mg/mL) in Tris-HCl Buffer (0.01 M, pH 9) at Various Constant Pressures

P (MPa)	E_a at LT area (kJ/mol)	E_a at HT area (kJ/mol)
0.1		$+300.56 \pm 20.12$[a] $r^2 = 0.987$
200		$+152.69 \pm 6.67$ $r^2 = 0.996$
300		$+84.08 \pm 34.19$ $r^2 = 0.858$
400	-66.61 ± 10.04 $r^2 = 0.936$	$+74.44 \pm 9.86$ $r^2 = 0.950$
425	-72.62 ± 8.09 $r^2 = 0.976$	
450	-71.93 ± 5.42 $r^2 = 0.989$	
475	-66.04 ± 12.77 $r^2 = 0.930$	
500	-95.82 ± 14.82 $r^2 = 0.937$	$+62.59 \pm 10.72$ $r^2 = 0.945$
525	-104.26 ± 8.92 $r^2 = 0.979$	$+101.61 \pm 28.83$ $r^2 = 0.926$
550	-106.22 ± 16.62 $r^2 = 0.953$	$+56.10 \pm 17.50$ $r^2 = 0.774$
575	-87.07 ± 22.32 $r^2 = 0.884$	$+49.12 \pm 9.43$ $r^2 = 0.931$
600	-85.94 ± 15.84 $r^2 = 0.936$	

[a] Standard error.

Table 3. Pressure Dependence of Inactivation Rate Constants of LOX (0.4 mg/mL) in Tris-HCl Buffer (0.01 M, pH 9) at Various Constant Temperatures

T (°C)	V_a (cm^3/mol)	T (°C)	V_a (cm^3/mol)
−15	-39.71 ± 9.25[a] $r^2 = 0.902$	10	-72.09 ± 1.81 $r^2 = 0.998$
−12	-49.38 ± 1.80 $r^2 = 0.997$	15	-67.10 ± 2.74 $r^2 = 0.995$
−10	-51.62 ± 18.42 $r^2 = 0.887$	20	-81.50 ± 6.17 $r^2 = 0.983$
−5	-59.15 ± 2.87 $r^2 = 0.995$	25	-76.27 ± 5.56 $r^2 = 0.984$
0	-66.29 ± 5.27 $r^2 = 0.981$	30	-46.25 ± 2.83 $r^2 = 0.993$
5	-68.15 ± 6.67 $r^2 = 0.963$	35	-57.13 ± 6.97 $r^2 = 0.957$

[a] Standard error.

Table 4. Estimated Kinetic Model Parameters To Describe the Relation between Inactivation Rate Constant and Pressure Based on Equation 6 for Temperatures >35 °C

T (°C)	a	b	corr r^2
40	-2.57 ± 0.21[a]	-652.06 ± 2.69	0.995
45	-2.38 ± 0.28	-633.08 ± 4.65	0.990
50	-6.00 ± 1.16	-677.27 ± 18.29	0.983
55	-8.17 ± 1.03	-644.37 ± 14.82	0.989
60	-17.21 ± 9.80	-715.60 ± 184.58	0.908
65	-20.34 ± 16.05	-485.59 ± 218.45	0.947
68	-33.71 ± 5.81	-442.57 ± 39.45	0.997

[a] Asymptotic standard error.

$$k = \frac{a}{b + P} \quad (6)$$

Combined Pressure and Temperature Dependence of Inactivation Rate Constants. An attempt was made to develop a mathematical model describing the observed LOX inactivation rate constants as a function of pressure and temperature. As described above, neither the temperature nor the pressure dependence of the LOX inactivation rate constants could be described over the entire pressure−temperature area studied, by the Arrhenius and the Eyring models, respectively. As a consequence, the approach for kinetic modeling set forth by Weemaes et al. (1998), namely, using the Arrhenius (Eyring) model as a starting point and replacing the model parameters by empirical mathematical expressions reflecting their pressure (temperature) dependence, could not be applied. This approach has, however, been proven successful for kinetic modeling of pressure−temperature inactivation of *Bacillus subtilis* α-amylase and of avocado polyphenol oxidase (Ludikhuyze et al., 1997; Weemaes et al., 1998).

Currently, no general applicable kinetic model that describes pressure−temperature enzyme inactivation data is available in the literature. Therefore, the modeling was approached on a thermodynamic basis. The basic equation governing the behavior of a system during a pressure and temperature change can be represented as

$$d(\Delta G) = -\Delta S\, dT + \Delta V\, dP \quad (7)$$

Because the entropy change (ΔS) and the volume change (ΔV) vary with pressure and temperature as shown by eqs 8 and 9, eq 7 can be reformulated as eq 10 (Morild, 1981).

$$d(\Delta S) = \left(\frac{\partial \Delta S}{\partial T}\right)_P dT + \left(\frac{\partial \Delta S}{\partial P}\right)_T dP \quad (8)$$

$$d(\Delta V) = \left(\frac{\partial \Delta V}{\partial T}\right)_P dT + \left(\frac{\partial \Delta V}{\partial P}\right)_T dP \quad (9)$$

$$\Delta G = \Delta G_0 + \Delta V_0(P - P_0) - \Delta S_0(T - T_0) + \frac{1}{2}\Delta\kappa(P - P_0)^2 + 2\Delta\zeta(P - P_0)(T - T_0) - \Delta C_p\left[T\left(\ln\frac{T}{T_0} - 1\right) + T_0\right] \quad (10)$$

Equation 10, previously described by Hawley (1971) and Morild (1981), is often used to understand a system's response toward pressure and temperature. This thermodynamic model can be converted into a kinetic model through the transition state theory of Eyring, suggesting that enzyme inactivation is accompanied by the formation of a metastable activated state (‡), which exists in equilibrium with the native enzyme. This conversion is based on the substitution of eqs 11 and 12 in eq 10, yielding eq 13.

$$\Delta G^{\ddagger} = -R_t T \ln(K^{\ddagger}) \quad (11)$$

$$K^{\ddagger} = kh/rk_B T \quad (12)$$

$$\ln(k) = \ln(k_0) - \frac{\Delta V_0^{\ddagger}}{R_t T}(P - P_0) + \frac{\Delta S_0^{\ddagger}}{R_t T}(T - T_0) - \frac{1}{2}\frac{\Delta\kappa^{\ddagger}}{R_t T}(P - P_0)^2 - 2\frac{\Delta\zeta^{\ddagger}}{R_t T}(P - P_0)(T - T_0) + \frac{\Delta C_p^{\ddagger}}{R_t T}\left[T\left(\ln\frac{T}{T_0} - 1\right) + T_0\right] \quad (13)$$

The model parameters are estimated using a nonlinear regression analysis, involving an iterative numerical procedure based on the minimal sum of squares (SAS, 1995). The estimated values of the model parameters are summarized in Table 5. The good agreement between the natural logarithm of experimentally deter-

Table 5. Predicted Model Parameters for LOX Inactivation (0.4 mg/mL) in Tris-HCl Buffer (0.01 M, pH 9) Based on Equation 13 at Reference Pressure 50 N·cm^{-2} and Reference Temperature 298 K

parameter	estimated value	quality of fitting
k_0 ($\times 10^{-2}$)	1.28 ± 0.11[a]	
$\Delta V_0^\ddagger$	−341.95 ± 22.21	corr r^2 = 0.985
$\Delta S_0^\ddagger$	−20.65 ± 7.20	
$\Delta\kappa^\ddagger$	−1.40 ± 1.06	
$\Delta\zeta^\ddagger$	3.20 ± 0.26	SD = 0.45
$\Delta C_p^\ddagger$	3046.55 ± 207.16	

[a] Asymptotic standard error.

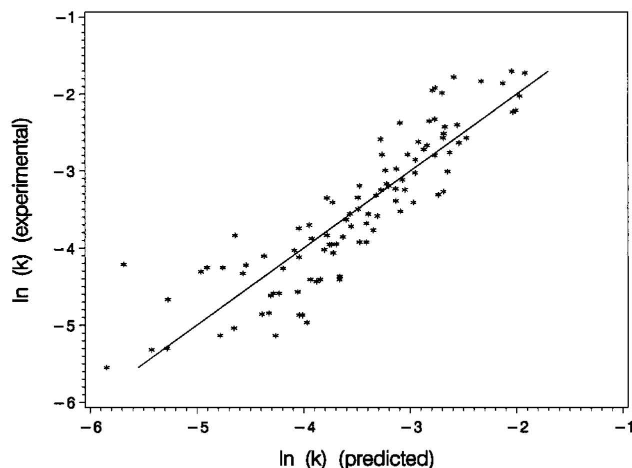

Figure 4. Relation between the natural logarithm of experimental k values from isobaric–isothermal inactivations of LOX (0.4 mg/mL) in Tris-HCl buffer (0.01 M, pH 9) and the natural logarithm of predicted k values according to eq 13.

mined inactivation rate constants and the natural logarithm of the rate constants predicted on the basis of eq 13 is depicted in Figure 4. No trend in residuals (differences between predicted and experimental values) was noticed as a function of temperature, pressure, or experimental inactivation rate constants. Applying eq 13 to describe pressure–temperature inactivation of avocado polyphenoloxidase resulted in a systematic tendency of residuals versus pressure (Weemaes et al., 1998). An equation similar to eq 13 was also applied to study the kinetics of microorganism inactivation [e.g., *Lactobacillus case*i and *Escherichia coli* (Sonoike et al., 1992); yeast (Hashizume et al., 1995)], but it was observed that this model did not result in an accurate prediction of the model parameters. According to Morild (1981), eq 10 should, in principle, be able to describe most phenomena with regard to their pressure and temperature behavior. However, evaluation of the unknown parameters of eq 10, and hence of eq 13, by means of computerized curve-fitting would require data from rather large pressure and temperature intervals, in the range from 500 MPa and 100 °C. From a practical point of view, this requirement may not always be realizable and may partially explain the poor parameter estimates reported in previous studies (Hawley, 1971; Sonoike et al., 1992; Hashizume et al., 1995).

Conclusion. Irreversible inactivation of soybean LOX could be realized by a pressure treatment at subzero to elevated temperatures. As this investigation showed that soybean LOX can be inactivated at elevated pressure and subzero temperature, a single pressure–subzero temperature process might be able to replace the commonly applied sequential thermal blanching–freezing process. At elevated pressure, maximal LOX stability was observed around room temperature. The kinetic version of the pressure–temperature denaturation model of Hawley (1971) was able to describe the experimentally obtained soybean LOX pressure temperature inactivation data set.

ABBREVIATIONS USED

a, model parameter (MPa^{-1}); A, enzymic activity; A_0, enzymic activity at time = 0; b, model parameter; corr r^2, corrected r^2 defined as $1 - [(i-1)(1 - SSQ_{regression}/SSQ_{total})]/(i - j)$; E_a, activation energy (kJ/mol); E_I, inactive enzyme; E_N, native or active enzyme; E_{N0}, native or active enzyme at time = 0; h, Planck's constant (6.626×10^{-34} J·s); HT, high-temperature area; i, number of observations; j, number of model parameters; k, inactivation rate constant (min^{-1} for first-order reaction); k_B, Boltzman constant (1.38×10^{-23} J·K^{-1}); k_0, inactivation rate constant at T_0 and P_0 (min^{-1} for first-order reaction); k_{ref-T}, inactivation rate constant at reference temperature (min^{-1} for first-order reaction); k_{ref-P}, inactivation rate constant at reference pressure (min^{-1} for first-order reaction); $K^\ddagger$, equilibrium constant; ln, natural logarithm; LOX, (soybean) lipoxygenase; LT, low-temperature area; P, pressure (MPa or N·cm^{-2}); P_0, P_{ref} (N·cm^{-2}); P_{ref}, reference pressure (MPa); r, transmission coefficient; r^2, linear correlation coefficient; R_p, universal gas constant (8.31577 cm^3·MPa·K^{-1}·mol^{-1}); R_t, universal gas constant (8.314 J·mol^{-1}·K^{-1}); s, second; SD, model standard deviation defined as $[SSQ_{residual}/(i - j)]^{-1/2}$; SSQ, sum of square; t, inactivation time (min); T, temperature (K); T_0, T_{ref} (K); Tris, tris(hydroxymethyl)aminomethane; T_{ref}, reference temperature (K); V_a, activation volume (cm^3·mol^{-1}); $\Delta\zeta$, thermal expansibility absolute (cm^3·mol^{-1}·K^{-1}); ΔC_p, heat capacity (J·mol^{-1}·K^{-1}); ΔG, free energy change between native and denatured states (J·mol^{-1}); ΔG_0, ΔG at T_0 and P_0 (J·mol^{-1}); $\Delta\kappa$, compressibility factor (cm^6·J^{-1}·mol^{-1}); ΔS, entropy change between native and denatured states (J·mol^{-1}·K^{-1}); ΔS_0, ΔS at T_0 and P_0 (J·mol^{-1}·K^{-1}); ΔV, volume change between native and denatured states (cm^3·mol^{-1}); ΔV_0, ΔV at T_0 and P_0 (cm^3·mol^{-1}); ‡, of the transition state.

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