Kinetics of Pressure Inactivation at Subzero and Elevated Temperature of Lipoxygenase in Crude Green Bean (*Phaseolus vulgaris* L.) Extract

Indrawati, Ann M. Van Loey, Linda R. Ludikhuyze, and Marc E. Hendrickx*

Department of Food and Microbial Technology, Laboratory of Food Technology, Katholieke Universiteit te Leuven, Kardinaal Mercierlaan 92, B-3001 Heverlee, Belgium

Lipoxygenase (LOX) in crude green bean extract was irreversibly inactivated by pressure treatments combined with subzero or elevated temperature. LOX inactivation was described accurately assuming a first-order reaction. In the entire pressure−temperature domain studied (200 to 700 MPa and −10 to 60 °C), an increase in pressure at constant temperature enhanced the LOX inactivation rate, whereas at constant pressure, an increase in reaction rate was obtained by either increasing or decreasing temperature at 20 °C. At elevated pressure, LOX exhibited the greatest stability around 20 °C. Also the pressure dependence of the inactivation rate constants for LOX was the highest around 20 °C. On the basis of the estimated LOX inactivation rate constants, an iso-rate contour diagram as a function of pressure and temperature was constructed, and an empirical mathematical model describing the combined pressure−temperature dependence of the LOX inactivation rate constants was formulated.

Introduction

The effect of pressure on the solid−liquid phase diagram of water offers several potential applications in food technology, e.g., (i) pressure-assisted freezing, (ii) pressure-assisted thawing, (iii) nonfrozen storage at subzero temperature under pressure, and (iv) modification of ice polymorphs instead of ice I (*1*). Stimulating results in food quality retention upon high-pressure, low-temperature processing were noticed (*2*−*5*). Reported effects of combining high pressure with subzero temperatures include microorganism inactivation (*6*), yeast inactivation (*7*), protein denaturation (*8*−*10*), enzyme inactivation (*11*), and pressure-induced gel formation (*12*−*13*).

Identification of a process concept based on combining pressure and temperature that may substitute hot water or steam blanching is being investigated (*14*). Blanching is an important pretreatment of frozen vegetables directed at inactivation of enzymes that reduce quality during frozen storage. Hence, in investigating the potential of pressure blanching, the effect of high pressure and temperature on the activity of food quality related enzymes should be studied. Studies on enzyme inactivation in model systems with high pressure combined with elevated temperatures were investigated by several authors (*15*−*17*), including kinetic inactivation studies (*18*−*21*). In real food systems, only qualitative studies on the effect of high-pressure treatment on enzymes at room or elevated temperatures are available (*22*−*23*). Little attention was previously directed to the combined effect of pressure and subzero temperatures on enzyme stability (*11*). Kinetics of enzyme inactivation in food model systems due to high pressure treatments at low temperature are completely lacking.

* Ph: 32-16-321572.Fax: 32-16-321960.E-mail: Marc.Hendrickx@agr.kuleuven.ac.be.

Lipoxygenase (LOX) catalyzes polyunsaturated fatty acids containing a *cis*,*cis*-1,4-pentadiene system to conjugated hydroperoxydiene derivatives by the addition of molecular oxygen. Its enzymatic reactions result in the development of an undesired aroma in frozen vegetables, especially in green beans and green peas, and currently lipoxygenase is considered as a blanching index rather than peroxidase (*24*−*26*). Therefore, the aim of this study was to investigate on a kinetic basis the effects of high pressure (up to 700 MPa) combined with subzero and elevated temperatures (−10 to 60 °C) on lipoxygenase in a crude green bean extract. Because a crude extract of green bean is selected as a model system, the effect of the intrinsic food complexity, e.g., the presence of natural substrates, pH, and the presence of enhancing or inhibiting components (i.e., for enzyme activity and high-pressure inactivation), on enzyme stability was taken into account. In addition, the formulation of a mathematical model describing the combined pressure and temperature dependence of lipoxygenase inactivation was undertaken.

Materials and Methods

Green Bean Extraction. Whole fresh green beans (*Phaseolus vulgaris* L.) grown locally and harvested in July, 1997 were purchased in a local auction (St. Katelijne Waver, Belgium). The whole beans were rinsed in distilled water before squeezing in a juice centrifuge (Braun AG type MP 32, Frankfurt, Germany). A supernatant obtained by centrifuging the crude extract for 15 min at 25900*g* and 4 °C (Beckman J2-HS, Palo Alto, CA) was divided into small portions (15−20 mL), instantaneously frozen, and stored in liquid nitrogen until use. Thawing of the frozen extract was standardized at 25 °C for 2 h. Hydrogen potential (pH) of the green bean juice was not influenced by the nitrogen storage, and the pH of the thawed extract was 6.1 ± 0.2.

10.1021/bp9901419 CCC: $19.00
Published on Web 01/15/2000

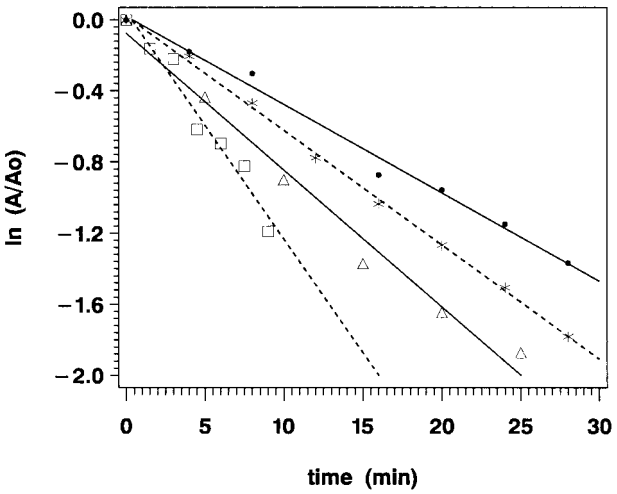

Figure 1. Isobaric isothermal inactivation of LOX in crude green bean extract at constant pressure (650 MPa) combined with various temperatures in subzero/low (—) and elevated (- - -) temperatures: ●, −5 °C; △, 0 °C; *, 40 °C; and □, 50 °C.

Lipoxygenase Activity Measurement. In the presence of oxygen, LOX catalyzes the conversion of substrate containing *cis*,*cis*-1,4-pentadienes to produce conjugated hydroperoxydiene derivatives. In the present study, sodium linoleic acid was used as a substrate and was prepared by dissolving 0.2 mL of Tween 20 (polyoxyethylenesorbotanmonolaurat) as an emulsifier and 1 mL of NaOH (1 M) in 20 mL of distilled water. Next, 0.2 mL of linoleic acid ($C_{18}H_{32}O_2$, *cis*-9,*cis*-12-octadecodienoic acid) was added, and the total volume was adjusted to 50 mL with distilled water. The substrate solution was stable for 2 days under N_2 at 4 °C.

A polarographic assay (Strathkelvin Instruments model 781B oxymeter, Glasgow, Scotland) following oxygen consumption due to LOX reaction was appropriate to determine LOX activity in this investigation using a crude extract of green beans. The assay was performed at 25 °C by homogenizing 1.25 mL of substrate solution and 2.5 mL of air-saturated phosphate buffer solution (0.01 M; pH 6) in a reaction cell (4 mL). Because the measurement of oxygen concentration and the enzymatic reaction are temperature-dependent, the substrate and air-saturated buffer solutions, the reaction cell, and the oxygen electrode were thermostated at the same temperature (25 °C) as the assay (Haake K20-DC3, Karlsruhe, Germany). At first, the oxygen concentration in the homogenized solution was monitored (10 s), and subsequently 0.125 mL of green bean extract was added. The linear curve of oxygen consumption as a function of reaction time was recorded as a measure of LOX activity (ppm O_2/s).

Isobaric Isothermal Inactivation. The pressure treatment was performed in laboratory scale multivessel high-pressure equipment (HPIU-10000, Resato, Roden, The Netherlands) that allows pressurization up to 1000 MPa combined with temperatures of −30 to 100 °C. An oil−glycol mixture (TR15, Greenpoint oil, Resato, Roden, The Netherlands) is used as the pressure-transmitting fluid. The equipment consists of eight single (8 mL) vessels connected to a central pressure circuit with T-joints and valves. Each vessel is enclosed in a thermostated casing connected to a cryostat (Haake F6-C40, Karlsruhe, Germany) circulating distilled water for temperature control above 5 °C and 56% ethylene glycol for temperatures below 5 °C. Each vessel can be provided with a thermocouple (type J) to monitor temperature inside the vessel (Cobra 7-10 Mess+system Technic GmbH, data logger). The high-pressure equipment is designed to perform kinetic experiments and maintain a selected pressure and temperature for selected time periods.

The green bean extract solution was transferred into flexible microcups (0.375 mL) (Elkay, Basingstoke-Hants, England) without creating air bubbles by using a syringe (10 mL), and the cups were covered with parafilm to avoid contamination of the pressure medium. For pressure experiments at subzero temperature, the microcups were wrapped in vacuumed (0.11 Pa) polyethylene pockets (0.5 μm) to protect the extract solution from pressure medium contamination. Samples were incorporated in the preheated or precooled pressure vessels. Pressurization rate was standardized at 100 MPa/min. After achievement of the desired pressure, vessel valves were closed to isolate the pressure in the vessels, and the central circuit was decompressed. After an equilibrium time of 5 min to ensure isobaric−isothermal conditions, the valve of the first vessel was opened, and the residual LOX activity of the green bean juice in this vessel was taken as reference value (A_0), i.e., the LOX activity at $t = 0$. From this moment on, the treatment was considered as an isobaric−isothermal treatment. Afterward, the vessels were decompressed as a function of time. Upon depressurization, the microcups were kept in the vessel for 1 min and stored at 25 °C until determination of the residual enzymatic activity. No reactivation of LOX activity during storage at 25 °C was noticed in less than 3 h. Combined high-pressure and temperature inactivation of LOX was studied in a pressure range from 200 to 700 MPa and a temperature range from −10 to 60 °C.

Data Analysis. The irreversible inactivation of LOX in crude green bean extract due to a combined pressure−temperature treatment can be accurately described by a first-order reaction (*27*). In the case of isobaric and isothermal conditions, i.e., the LOX inactivation rate constant being time-independent, a first-order model can be represented as eq 1.

$$\ln(A) = \ln(A_0) - kt \tag{1}$$

The rate constant (k) at selected pressure−temperature conditions was estimated by linear regression analysis (*28*) of the natural logarithm of the residual enzymatic activity after treatment (A) versus inactivation time at a specified constant temperature and pressure (t).

Arrhenius and Eyring relations (eqs 2 and 3)

$$k = k_{\mathrm{ref-P}} \exp\left(\frac{-E_a}{R_t}\left(\frac{1}{T} - \frac{1}{T_{\mathrm{ref}}}\right)\right) \tag{2}$$

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

are frequently applied to estimate the temperature and pressure dependency of the LOX inactivation rate constants, as expressed by E_a (activation energy) and V_a (activation volume) values, respectively.

The kinetic parameters (E_a and V_a) were determined by a linear regression approach (*28*) of the natural logarithm of the LOX inactivation rate constant versus the reciprocal of the absolute temperature or versus pressure, respectively.

Formulation of a Mathematical Model. An empirical mathematical model describing the combined pressure and temperature dependence of the LOX inactivation rate constant was constructed to fit to the entire kinetic inactivation data set. As a measure for the ability

Table 1. Adiabatic Heating and Cooling (°C) for Selected Preset *P*/*T* Combinations and Selected Pressurization Rates (Instantaneous Decompression)

temp (°C)	pressure (MPa)	pressurization rate (MPa/min)	adiabatic heating (°C)	adiabatic cooling (°C)
−15	300	90	10.50	−12.78
	400	90	10.84	−14.27
−10	200	90	11.94	−11.19
	300	90	9.65	−12.40
	400	90	10.91	−13.32
	400	110	17.64	−21.71
−5	200	90	11.84	−13.21
	300	90	11.56	−11.48
	400	90	11.41	−15.32
	400	170	23.07	−22.00
	500	200	27.02	−22.02
0	300	150	17.51	−15.79
	500	220	26.52	−25.20
10	800	90	13.76	−25.25
	400	160	16.77	−16.37
	600	320	18.05	−22.31

of a model to fit all experimental data (i) a visual inspection of residual plots was performed, and (ii) the corrected r^2 (eq 4) and (iii) the model standard deviation (eq 5) were calculated, taking into account the number of observations (m) and the number of model parameters estimated (j). In addition, for each parameter estimate of the models, the residual standard error (RSE) was calculated on the basis of eq 6.

$$\text{corrected } r^2 = 1 - \frac{(m-i)\left(1 - \frac{\text{SSQ}_{\text{regression}}}{\text{SSQ}_{\text{total}}}\right)}{(m-j)} \quad (4)$$

$$\text{SD} = \sqrt{\frac{\text{SSQ}_{\text{residual}}}{(m-j)}} \quad (5)$$

$$\text{RSE (\%)} = \frac{\text{asymptotic (SE)}}{\text{estimated} - \text{value}} \cdot 100 \quad (6)$$

Results and Discussion

Isobaric−Isothermal Inactivation of LOX in Crude Green Bean Extract. In the case of pressure inactivation at low temperature, it is of importance to have an idea of the temperature evolution of a sample during pressurization and decompression, as it can influence the solid−liquid phase history of the sample. A typical evolution of pressure and temperature during the pressurization, holding, and decompression phase demonstrated that (i) pressure buildup is accompanied by a temperature increase due to adiabatic heating, (ii) to achieve a constant temperature within the vessel after attaining the desired pressure, an equilibrium time is needed, and (iii) pressure decompression results in adiabatic cooling (a decrease in temperature). The effect of pressurization rate and of preset pressure and/or temperature on either the adiabatic heating or the adiabatic cooling in the low-temperature area (<20 °C) is presented in Table 1. At a constant pressurization rate (e.g., 90 MPa/min), the pressure level did not influence the resulting adiabatic heating observed for the pressure range of 200−400 MPa. Therefore, in this experiment, the rate of compression was standardized.

LOX in crude green bean extract was irreversibly inactivated by a combined pressure−temperature treatment in a pressure range of 200 to 700 MPa and a temperature range of −10 to 60 °C. In the whole pressure and temperature domain studied (200 to 700 MPa and −10 to 60 °C), the inactivation of LOX is described accurately using a first-order reaction (Figure 1). A single phase in the pressure−temperature inactivation curves was observed, in contrast with the biphasic thermal inactivation behavior of LOX in crude green bean extract (*27*). Inactivation rate constants were derived from the slope of the regression lines when plotting the natural logarithm of the relative residual enzymatic activity against inactivation time at constant pressure and temperature. The estimated LOX inactivation rate constants are summarized in Table 2. Regression coefficients varied between 0.958 and 0.998.

At constant temperature, the LOX inactivation rate was enhanced by increasing pressure as noticed in the inactivation of *Bacillus subtilis* α-amylase (*18*) and of soybean LOX (*19*). The pressure dependence of the LOX inactivation rate constants are accurately described by the Eyring model (eq 3). At all temperatures studied, estimated activation volumes (V_a values) were negative (Table 3), indicating an acceleration of the inactivation under pressure. A relatively constant activation volume was observed at temperatures above 50 °C. The highest sensitivity of LOX inactivation rate constants to pressure was observed at and slightly below 20 °C. Ludikhuyze and co-workers (*19*), investigating pressure−temperature

Table 2. Estimated Inactivation Rate Constants ($\times 10^{-2}$ min^{-1}) for the Isobaric Isothermal Inactivation of LOX in Crude Green Bean Extract

pressure (MPa)	temperature (°C)									
	−10	−5	0	10	20	30	40	50	55	60
200								0.93 ± 0.05	1.38 ± 0.08	1.54 ± 0.20
300									2.14 ± 0.17	3.25 ± 0.26
350										4.96 ± 0.67
400								3.68 ± 0.24		5.35 ± 0.35
450								3.98 ± 0.31		
500	1.88 ± 0.13[a]	1.64 ± 0.11	1.18 ± 0.08			1.18 ± 0.08	2.07 ± 0.08	3.97 ± 0.11	4.73 ± 0.39	8.32 ± 0.23
550	2.72 ± 0.20	3.05 ± 0.22	1.97 ± 0.26		1.25 ± 0.13		2.58 ± 0.08	6.33 ± 0.16	10.48 ± 0.57	13.22 ± 0.68
575			2.31 ± 0.14		1.33 ± 0.03	2.40 ± 0.09				
586.5				2.13 ± 0.05						
600		3.28 ± 0.20	3.91 ± 0.16		2.11 ± 0.07	3.47 ± 0.23	4.27 ± 0.17	8.17 ± 0.47	12.49 ± 0.39	
625			4.11 ± 0.26	2.80 ± 1.64		3.34 ± 0.09				
637.5				3.56 ± 0.22						
650	4.38 ± 0.46	4.96 ± 0.22	7.69 ± 0.52	4.13 ± 0.18	4.61 ± 0.45	4.58 ± 0.12	6.43 ± 0.10	12.78 ± 1.13		
675			8.92 ± 0.88	5.44 ± 0.30	4.97 ± 4.05	6.27 ± 0.19				
688.5				5.66 ± 0.41						
700	4.73 ± 0.32			8.74 ± 0.26	6.95 ± 0.20	7.22 ± 0.33	8.76 ± 0.10			

[a] Standard error.

Table 3. Pressure Dependence, Expressed as V_a Value, of Inactivation Rate Constant of LOX in Crude Green Bean Extract at Selected Temperatures

temp (°C)	V_a (cm^3/mol)	r^2
−10	−10.16 ± 1.43[a]	0.96
−5	−15.12 ± 3.16	0.92
0	−27.21 ± 2.04	0.97
10	−27.52 ± 2.69	0.95
20	−29.81 ± 2.36	0.98
30	−22.80 ± 1.39	0.98
40	−19.77 ± 1.25	0.99
50	−14.63 ± 1.20	0.97
55	−14.95 ± 1.84	0.96
60	−15.77 ± 1.33	0.97

[a] Standard error.

Table 4. Temperature Dependence of Rate Constants for LOX Inactivation in Crude Green Bean Extract at Various Pressures, Restricted Temperatures of 20−60 °C

pressure (MPa)	E_a (kJ/mol)
200	45.23 ± 14.31;[a] $r^2 = 0.91$
500	52.19 ± 4.20; $r^2 = 0.98$
550	49.03 ± 6.95; $r^2 = 0.94$
600	38.42 ± 4.85; $r^2 = 0.95$
650	26.37 ± 9.17; $r^2 = 0.81$
700	8.76 ± 3.58; $r^2 = 0.86$

[a] Standard error.

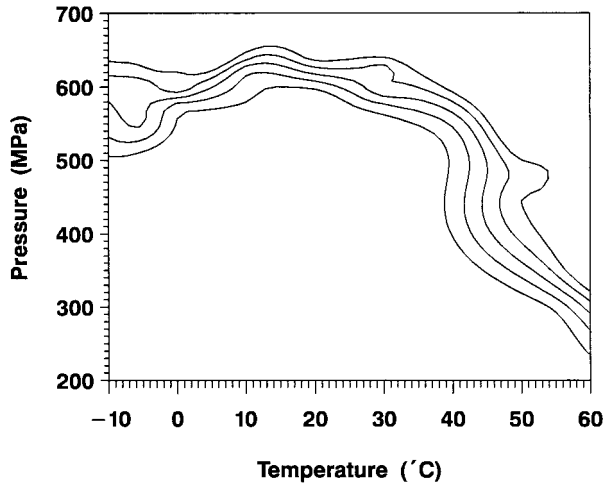

Figure 2. Iso-rate contour diagram of isobaric isothermal inactivation of LOX in crude green bean extract as function of pressure and temperature. The inner and outer lines represent *P*/*T* combinations for *k*-values of 0.02 and 0.04 min^{-1}, respectively.

inactivation of soybean LOX dissolved in Tris-HCl buffer (0.01 M; pH 9) in the pressure−temperature range of 50−650 MPa and 10−64 °C, observed reduced pressure sensitivity of inactivation constants at high temperature, as observed in this study. In addition, by expanding the temperature range to low temperatures, at subzero temperatures the pressure sensitivity of the rate constants for pressure−temperature inactivation of LOX in crude green bean extract is also reduced.

At all pressure levels studied, LOX was most stable around room temperature (Table 2). Authors (*19*) also observed a temperature of maximal stability of soybean LOX at elevated pressure (>500 MPa) situated somewhat above room temperature (30−40 °C). Inactivation data in Table 2 show that in the pressure area greater than 500 MPa, an acceleration of the inactivation rate of LOX was caused by either increasing temperature above 20 °C or decreasing temperature below 20 °C. As a consequence, the Arrhenius model (eq 2), assuming reaction

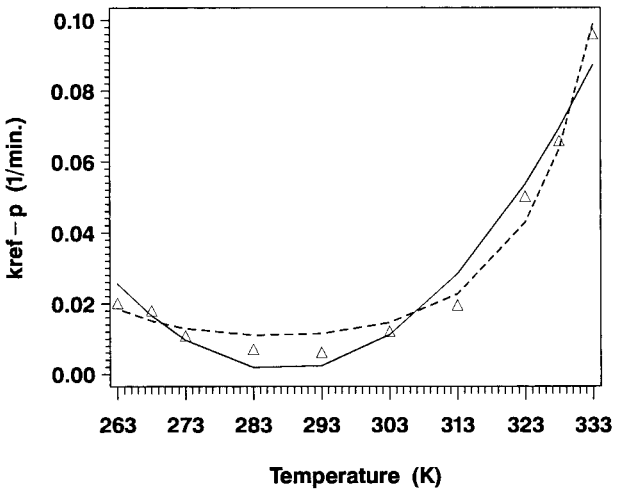

Figure 3. Temperature dependence of k_{ref-P} at reference pressure (500 MPa) described by quadratic model (solid line) and an exponential function of a quadratic model (dash line).

rates increase consistently with temperature, was not valid at elevated pressure over all temperatures investigated. This phenomenon was previously reported for, e.g., pressure-induced denaturation of egg albumin (*29*) and pressure-induced soybean LOX inactivation (*19*). To determine the temperature dependence of the LOX inactivation rate constants, the temperature range was restricted from 20 to 60 °C. Estimated activation energies (E_a) are reported in Table 4. In the elevated temperature range of 20 to 60 °C, a pressure increase resulted in a decrease in temperature sensitivity of the rate constants.

On the basis of the estimated *k*-values, a pressure−temperature diagram describing the same LOX inactivation rate constants (i.e., an iso-rate contour diagram) was constructed (Figure 2). A temperature increase slightly retarded the pressure−temperature inactivation of LOX.The highest pressure dependence of the inactivation rate constants around room temperature can also be deduced from the density of the iso-rate contours at selected temperature. At elevated temperatures up to 60 °C, no antagonistic effect of pressure and temperature was observed in green bean LOX, an effect frequently reported for other proteins due to low-pressure retardation of thermal denaturation/inactivation (*20, 30*−*32*). Authors (*19*) did not observe an antagonistic effect (i.e., pressure retards the LOX inactivation) at temperatures above 50 °C for inactivation of soybean LOX. On the contrary, Heinisch and co-workers (*33*) concluded that low pressure exerted a slightly thermostabilizing effect for soybean LOX in the high-temperature area.

Formulation of a Mathematical Model. Kinetic models describing pressure−temperature dependence of the inactivation rates of several enzymes dissolved in buffer solutions are reported in the literature, including *B. subtilis* α-amylase (*18*), avocado polyphenoloxidase (*21*), and soybean LOX (*19*). Weemaes and co-workers (*21*) described pressure−temperature inactivation of avocado polyphenoloxidase using the Arrhenius equation (eq 2) as a starting point for kinetic modeling because this equation was valid at all pressures studied. The two pressure-dependent parameters in the Arrhenius equation, namely, E_a and k_{ref-T}, were consecutively replaced by a mathematical expression, reflecting their pressure dependencies. In a similar way, an empirical mathematical model describing the LOX inactivation rate constant as a function of temperature and pressure was constructed. The Eyring equation (eq 3), unlike the Arrhe-

Table 5. Estimated Model Parameters for Temperature Dependence of k_{ref-P}, Temperature Dependence of V_a, and Pressure−Temperature Dependence of the LOX Inactivation Rate Constant of the Isobaric Isothermal Inactivation of LOX in Crude Green Bean Extract

parameter	est model parameters (eq 7)	est model parameters (eq 8)	est model parameters (eq 9)
a_1	4.14 ± 0.44[a] ($\times 10^{-5}$)		3.53 ± 0.20 ($\times 10^{-5}$); 5.60[b]
b_1	-2.38 ± 0.26 ($\times 10^{-2}$)		-2.03 ± 0.12 ($\times 10^{-2}$); 5.80
c_1	3.42 ± 0.39		2.93 ± 0.18; 6.01
a_2		3.01 ± 0.32	2.75 ± 0.48; 17.55
b_2		-3.85 ± 0.27 ($\times 10^{-2}$)	-4.01 ± 0.33 ($\times 10^{-2}$); 8.22
SF		259.50 ± 1.16	
		Quality of Fitting	
SD	0.60×10^{-2}	2.43	1.16×10^{-2}
corrected r^2	0.98	0.99	0.96

[a] Asymptotic standard error. [b] RSE (in %).

nius equation, was valid in the entire pressure−temperature range studied (Table 3) for isobaric−isothermal inactivation of green bean LOX. Therefore, the Eyring model was used as starting point for kinetic modeling, and mathematical expressions to describe the temperature dependence of the Eyring model parameters k_{ref-P} and V_a were constructed.

The temperature dependence of k_{ref-P} at a reference pressure of 500 MPa was accurately described by a quadratic equation (eq 7) depicted in Figure 3 (solid line).

$$k_{ref-P} = a_1 T^2 + b_1 T + c_1 \qquad (7)$$

Model parameters a_1, b_1, and c_1 were estimated by a nonlinear regression analysis (*28*) and are represented in Table 5. Ludikhuyze and co-workers (*19*) investigated the pressure−temperature inactivation of soybean LOX in Tris-HCl buffer (0.01 M; pH 9), and the natural logarithm of the LOX inactivation rate constant at reference pressure was a quadratic function of temperature (dash line, Figure 3). In the case of pressure−temperature inactivation of LOX from green beans, a better fitting model was obtained using eq 7 (Figure 3).

The temperature dependence of V_a (activation volume) was described by eq 8, where SF is a scaling factor for

$$V_a = -a_2 (T - \mathrm{SF}) \exp(b_2 (T - \mathrm{SF})) \qquad (8)$$

the temperature axis (Figure 4). Parameter estimates of a_2 and b_2 are given in Table 5.

By implementation of the mathematical expressions (eqs 7 and 8) into the Eyring model (eq 3), eq 9 is

$$k = (a_1 T^2 + b_1 T + c_1) \exp\left(\frac{a_2 (T - \mathrm{SF}) \exp(b_2 (T - \mathrm{SF}))}{R_p T} (P - P_{ref})\right) \qquad (9)$$

obtained. The scaling factor as estimated from eq 8 was considered as a constant in eq 9. Model parameters a_1, b_1, c_1, a_2, and b_2 were estimated by a nonlinear regression analysis of the isobaric−isothermal kinetic inactivation data and are reported in Table 5. The iterative parameter estimation procedure used parameter estimates from the individual equations, expressing the temperature dependence of k_{ref-P} (eq 7) and of V_a (eq 8) as a starting point for predicting estimated kinetic parameters (a_1, b_1, c_1, a_2, and b_2) of eq 9.

The estimated kinetic parameters (a_1, b_1, c_1, a_2, and b_2) and their accuracy are presented in Table 5. The correlation between experimentally determined rate constants and rate constants predicted using eq 9 is depicted in Figure 5 and on the basis of the sufficient accuracy of the model and the parameter estimates, the derived empirical mathematical model describes pressure inactivation of LOX in crude green bean extract at subzero and elevated temperature (−10 up to 60 °C).

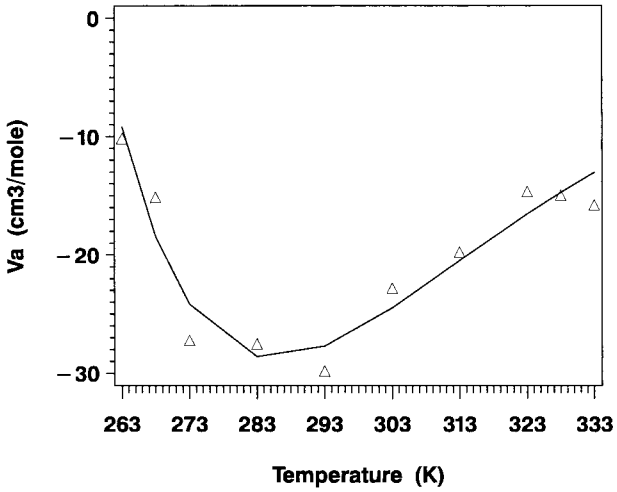

Figure 4. Temperature dependence of activation volume at reference pressure (500 MPa).

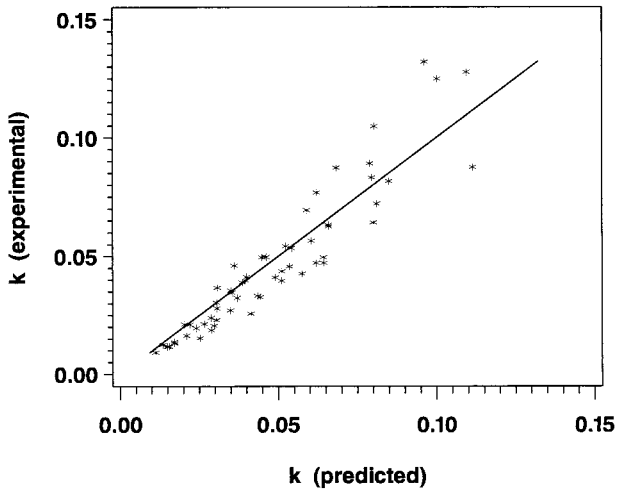

Figure 5. Correlation between experimentally determined rate constants and *k*-values predicted using the empirical model (eq 9).

Conclusions

Application of high pressure at subzero and elevated temperatures resulted in irreversible LOX inactivation according to first-order decay. Maximal stability of LOX in crude green bean extract was observed around room temperature (20 °C) at elevated pressure (500 MPa). Therefore, it is advantageous to combine pressure pro-

cessing with either low (<20 °C) or elevated temperature (>20 °C) to inactivate LOX. A single pressure/subzero temperature process may replace the commonly applied sequential treatment of blanching followed by freezing. A mathematical model describing the pressure and temperature dependence of LOX inactivation rate constants and the pressure temperature diagram describing the same LOX inactivation rate constants provide indispensable information to identify such a combined blanching−freezing concept based on pressure and temperature combination.

Notation

A	enzyme activity at t (ppm O_2/s)
A_0	enzyme activity at $t = 0$ (ppm O_2/s)
a_1	model parameter (min^{-1} K^{-2})
a_2	model parameter (cm^3 mol^{-1} K^{-1})
b_1	model parameter (min^{-1} K^{-1})
b_2	model parameter (K^{-1})
c_1	model parameter (min^{-1})
E_a	activation energy (kJ/mol)
i	0 or 1 depending on the model structure
j	number of model parameters
k	inactivation rate constant at isobaric−isothermal condition (min^{-1})
k_{ref-P}	inactivation rate constant at reference pressure (min^{-1})
k_{ref-T}	inactivation rate constant at reference temperature (min^{-1})
ln	natural logarithm
LOX	(green bean) lipoxygenase
m	number of observations
P	pressure (MPa)
P_{ref}	reference pressure (500 MPa)
r^2	linear correlation coefficient
R_P	universal gas constant (8.31577 cm^3 MPa/K mol)
RSE	residual standard error
R_t	universal gas constant (8.314 J/mol K)
SD	model standard deviation
SE	standard error
SF	scaling factor (K)
t	inactivation time at isobaric isothermal condition (min)
T	temperature (K)
T_{ref}	reference temperature (K)
V_a	activation volume (cm^3 mol^{-1})

Acknowledgment

We acknowledge the Katholieke Universiteit Leuven, the Fund for Scientific Research-Flanders, and the European Commission (FAIR-CT96-1175) for their financial support of this research.

References and Notes

(1) Kalichevsky, M. T.; Knorr, D.; Lillford, P. J. Potential Food Applications of High-Pressure Effects on Ice−Water Transitions. *Trends Food Sci. Technol.* **1995**, *6*, 253−259.

(2) Deuchi, T.; Hayashi, R. High-Pressure Treatments at Subzero Temperature: Application to Preservation, Rapid Freezing and Rapid Thawing of Foods. In *High Pressure and Biotechnology*; Balny, C., Hayashi, R., Heremans, K., Masson, P., Eds.; Colloque INSERM 224, John Libbey Eurotext Ltd.: France, 1992; pp 353−355.

(3) Murakami, T.; Kimura, I.; Yamagishi, T.; Yamashita, M.; Sugimoto, M.; Satake, M. Thawing of Frozen Fish by Hydrostatic Pressure. In *High Pressure and Biotechnology*; Balny, C., Hayashi, R., Heremans, K. , Masson, P., Eds.; Colloque INSERM 224, John Libbey Eurotext Ltd.: France, 1992; pp 329−331.

(4) Yoshioka, K.; Yamada, A.; Maki, T. Application of High Pressurization to Fish Meat: Changes in The Physical Properties of Carp Skeletal Muscle Resulting From High-Pressure Thawing. In *Progress in Biotechnology 13 High-Pressure Bioscience and Biotechnology*; Hayashi, R., Balny, C., Eds.; Elsevier Science: Amsterdam, 1996; pp 369−374.

(5) Fuchigami, M.; Kato, N.; Teramoto, A. High-Pressure-Freezing Effects on Textural Quality of Chinese Cabbage. *J. Food Sci.* **1998**, *63*, 122−125.

(6) Takahashi, K. Sterilization of Microorganisms by Hydrostatic Pressure at Low Temperature. In *High Pressure and Biotechnology*; Balny, C.; Hayashi, R.; Heremans, K., Masson, P., Eds.; Colloque INSERM 224, John Libbey Eurotext Ltd.: France, 1992; pp 303−307.

(7) Hashizume, C.; Kimura, K.; Hayashi, R. Kinetic Analysis of Yeast Inactivation by High-Pressure Treatment at Low Temperatures. *Biosci. Biotech. Biochem.* **1995**, *59*, 1455−1458.

(8) Foguel, D.; Weber, G. Pressure-Induced Dissociation and Denaturation of Allophycocyanin at Subzero Temperatures. *J. Biol. Chem.* **1995**, *270*, 28759−28766.

(9) Zhang, J.; Peng, X.; Jonas, A.; Jonas, J. NMR Study of The Cold, Heat and Pressure Unfolding of Ribonuclease. *Biochemistry* **1995**, *34*, 8631−8641.

(10) Nash, D.; Lee, B. S.; Jonas, J. Hydrogen-Exchange Kinetics in The Cold Denatured State of Ribonuclease A. *Biochim. Biophys. Acta* **1996**, *1297*, 40−48.

(11) Indrawati; Van Loey, A.; Denys, S.; Hendrickx, M. Enzyme Sensitivity Towards High Pressure at Low Temperature. *Food Biotechnol.* **1998**, *12(3)*, 263−277.

(12) Pérez-Mateos, M.; Montero, P. High-Pressure Induced Gel of Sardine (*Sardina pilchardus*) Washed Mince as Affected by Pressure−Time−Temperature. *J. Food Sci.* **1997**, *62*, 1183−1188.

(13) Dumoulin, M.; Ozawa, S.; Hayashi, R. Textural Properties of Pressure-Induced Gels of Food Proteins Obtained under Different Temperatures Including Subzero. *J. Food. Sci.* **1998**, *63*, 92−95.

(14) Knorr, D.; Böttcher, A.; Dörnenburg, H.; Eshtiaghi, M.; Oxen, P.; Richwin, A.; Seyderhelm, I. High Pressure Effects on Microorganisms, Enzyme Activity and Food Functionality. In *High Pressure and Biotechnology*; Balny, C.; Hayashi, R.; Heremans, K.; Masson, P. Eds.; Colloque INSERM 224, John Libbey Eurotext Ltd.: France, 1992; pp 211−218.

(15) Suzuki, K.; Kitamura, K. Inactivation of Enzyme Under High Pressure: Studies on The Kinetics of Inactivation of α-Amylase of *Bacillus subtilis* Under High Pressure. *J. Biochem.* **1963**, *54*, 214−219.

(16) Anese, M.; Nicoli., M. C.; Dall'Aglio, G.; Lerici, C. R. Effect of High-Pressure Treatments on Peroxidase and Polyphenoloxidase Activities. *J. Food Biochem.* **1995**, *18*, 285−293.

(17) Seyderhelm, I.; Boguslawski, S.; Michaelis, G.; Knorr. D. Pressure Induced Inactivation of Selected Food Enzymes. *J. Food Sci.* **1996**, *61*, 308−310.

(18) Ludikhuyze, L. R.; Van den Broeck, I.; Weemaes, C. A.; Herremans, C. H.; Van Impe, J. F.; Hendrickx, M. E.; Tobback, P. P. Kinetics for Isobaric−Isothermal Inactivation of *Bacillus subtilis* α-amylase. *Biotechnol. Prog.* **1997**, *13*, 532−538.

(19) Ludikhuyze, L.; Indrawati; Van den Broeck, I.; Weemaes, C.; Hendrickx, M. Effect of Combined Pressure and Temperature on Soybean Lipoxygenase: II. Modeling Inactivation Kinetics under Static and Dynamic Conditions. *J. Agric. Food. Chem.* **1998**, *46(10)*, 4081−4086.

(20) 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.

(21) Weemaes, C. A.; Ludikhuyze, L. R.; Van den Broeck, I.; Hendrickx, M. E. Kinetics of Combined Pressure−Tempera-

ture Inactivation of Avocado Polyphenoloxidase. *Biotechnol. Bioeng.* **1998**, *60(3)*, 292−300.
(22) Quaglia, G. B.; Paoletti, F. Study of The Effects of High-Pressure Treatments on Peroxidase Activity in Green Peas. *Ital. Food Beverage Technol.* **1995**, *VI*, 14−15.
(23) Cano, M. P.; Hernandez, A.; De Ancos, B. High Pressure and Temperature Effects on Enzyme Inactivation in Strawberry and Orange Product. *J. Food Sci.* **1997**, *62*, 85−88.
(24) Williams, D. C.; Lim, M. H.; Chen, A. O.; Pangborn, R. M.; Whitaker, J. R. Blanching of Vegetables for Freezing-Which Indicator Enzyme to Choose. *Food Technol.* **1986**, *40(6)*, 130−140.
(25) Sheu, S. C.; Chen, A. O. Lipoxygenase as Blanching Index for Frozen Vegetable Soybeans. *J. Food Sci.* **1991**, *56(2)*, 448−451.
(26) Barrett, D. M.; Theerakulkait, C. Quality indicators in Blanched, Frozen, Stored Vegetables. *Food Technol.* **1995**, *49-(1)*, 62 and 64−65.
(27) Indrawati; Van Loey. A. M.; Ludikhuyze, L. R.; Hendrickx, M. E. Single, Combined or Sequential Action of Pressure and Temperature on Lipoxygenase in Green Beans (*Phaseolus vulgaris* L.): A Kinetic Inactivation Study. *Biotechnol. Prog.* **1999**, *15*, 273−277.
(28) SAS Institute Inc. *SAS user's guide: Statistics*; SAS Institute Inc.: Cary, U.S., 1995.
(29) Suzuki. K. Studies on The Denaturation of Egg Albumin Under High Pressure. *Rev. Phys. Chem. Jpn.* **1958**, *28*, 32−38.
(30) Suzuki, K. Studies on The Kinetics of Protein Denaturation Under High Pressure. *Rev. Phys. Chem. Jpn.* **1960**, *29*, 49−55.
(31) Suzuki, K.; Kitamura, K. Denaturation of Hemoglobin Under High-Pressure II. *Rev. Phys. Chem. Jpn.* **1960**, *29*, 44−48.
(32) 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.
(33) Heinisch, O.; Kowalski, E.; Goosens, K.; Frank, J.; Heremans, K.; Ludwig, H.; Tauscher, B. Pressure Effects on The Stability of Lipoxygenase: Fourier Transform-Infrared Spectroscopy (FT-IR) and Enzyme Activity Studies. *Z. Lebensm.-Unters.-Forsch.* **1995**, *201*, 562−565.

Accepted November 30, 1999.

BP9901419