Lipoxygenase Inactivation in Green Beans (*Phaseolus vulgaris* L.) Due to High Pressure Treatment at Subzero and Elevated Temperatures

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

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

The kinetics of lipoxygenase (LOX) inactivation in green beans due to high-pressure treatment were studied in the pressure−temperature area of 0.1 up to 650 MPa and −10 up to 70 °C for systems with different levels of food complexity, i.e., in green bean juice and intact green beans (in situ study). For both systems, LOX was irreversibly inactivated by high-pressure treatment combined with subzero and elevated temperatures and the inactivation could be described as a first-order reaction. At ambient pressure, in situ LOX was less thermostable than in the juice at temperatures below 68 °C whereas the stability ranking was reverse at temperatures above 68 °C. At temperatures below 63 °C, sensitivity of the inactivation rate constants to temperature changes was on the same order of magnitude in the juice and in situ, while at higher temperature it was lower in situ. The pressure needed to obtain the same rate of LOX inactivation at a given temperature was lower in situ than in the juice. Application of high-pressure treatment at low/subzero temperature resulted in an antagonistic effect on LOX inactivation for both systems, whereas no such effect was found above room temperature. The pressure−temperature dependence of the LOX inactivation rate constants in green beans was successfully modeled.

Keywords: *Green beans; inactivation; in situ; kinetics; lipoxygenase; pressure; subzero temperature; thermal*

INTRODUCTION

Lipoxygenase (LOX) is widespread in the plant kingdom and is the key enzyme responsible for the development of undesirable aroma in legumes such as green beans and green peas. In this respect, lipoxygenase is considered as an index to fulfill the criterion of blanching adequacy next to peroxidase (Williams et al., 1986; Sheu and Chen, 1991; Barret and Theerakulkait, 1995). Previous studies revealed that thermal treatment, such as blanching, are effective to inactivate lipoxygenase either as purified enzyme (Park et al., 1988) or in real foodstuffs (Günes and Bayindirli, 1993). However, thermal treatment simultaneously causes some decrease in food quality aspects such as color, nutritional value, and flavor (Lund, 1977). Hence, in the context of improving food quality retention, new techniques such as high pressure become interesting in food processing and preservation.

In the last decade, the interest in high-pressure research in the food area has grown rapidly since a great number of key effects of high pressure on food components have been demonstrated and several potential food applications either at subzero or elevated temperature have been identified (Cheftel, 1991; Heremans, 1992; Knorr, 1993; Kalichevsky et al., 1995). Currently, high-pressure research is mainly focused on its use for preservation purposes and protein modification. Studies on food quality related enzyme inactivation by pressure combined with either subzero or elevated temperature have been carried out qualitatively and showed the barostability of LOX to be less as compared to other food quality related enzymes (Seyderhelm et al., 1996; Indrawati et al., 1998). So far, most kinetic studies regarding the combined high-pressure and -temperature effect on enzyme inactivation have been concentrating on simple model systems, i.e., use of (partially) purified enzymes dissolved in buffer solutions such as soybean LOX (Ludikhuyze et al., 1998; Indrawati et al., 1999a), avocado polyphenoloxidase (Weemaes et al., 1998), and orange peel pectinmethylesterase (Van den Broeck et al., 1999), hereby not taking into account the intrinsic complexity of food products. As a consequence, available information on the latter could be limited and evaluation in real food systems is needed.

Responding to this situation, this paper presents a study on enzyme inactivation in real foodstuffs by combined high-pressure and -temperature treatment. The purpose was to investigate on a kinetic basis LOX inactivation in systems with different levels of food complexity, namely in green bean juice and intact green beans (in situ study), due to high-pressure treatment (up to 650 and 550 MPa, respectively) at subzero and elevated temperatures (−10 °C up to 70 °C). Such quantitative information is useful for design, evaluation, and optimization of high-pressure preservation processes.

MATERIALS AND METHODS

Green beans (*Phaseolus vulgaris* L.) of same age and freshness, harvested in September 1998 and purchased in a

* Author to whom correspondence should be addressed. Fax: +32-16-32.19.60. Tel: +32-16-32.15.85. E-mail: marc.hendrickx@agr.kuleuven.ac.be.

10.1021/jf990937n CCC: $19.00
Published on Web 04/21/2000

local auction, were used throughout the study. The green beans were sorted according to size (60−90 mm diameter). They were packed in aluminum foil (±200−250 g) and stored in liquid nitrogen until use.

Kinetics of Lipoxygenase Inactivation in Green Bean Juice. *Green Bean Juice Preparation*. The beans were thawed overnight at 5 °C. Crude extract was obtained by squeezing the green beans and centrifuging the juice for 15 min at 4 °C at 25 900*g* as described in Indrawati et al. (1999b). The resulting supernatant was filled in cryogenic vials (Nalgene, Rochester, USA, 15 mL) and stored in liquid nitrogen until use. It was thawed overnight at 5 °C prior to inactivation treatments.

Thermal Treatment at Ambient Pressure. The experiment was designed to be isothermal. Therefore, to ensure direct heating and cooling, the green bean juice was filled in capillary tubes and the samples were heated in a thermostated water bath (Grant Y14, Cambridge, England) at constant temperature during different preset time intervals. After withdrawal, the samples were immediately transferred to an ice bath to avoid a further thermal inactivation and stored (<3 h) at 5 °C until the residual LOX activity was measured (Indrawati et al., 1999b).

Combined High-Pressure and Temperature Treatment. Samples were treated in a laboratory pilot scale, multivessel high-pressure apparatus (HPUI-10000, serial no. 95/1994, Resato, Roden, The Netherlands) that allowed pressurization up to 1000 MPa combined with temperature between −10 and 100 °C. A pressure intensifier was used to generate high pressure. The apparatus allowed performance of kinetic experiments since it consisted of eight individual vessels (8 mL, 10 mm inner diameter) surrounded by a thermostated mantle connected to a cryostat (Haake F6-C40, Karlsruhe, Germany). Distilled water and 56% ethylene glycol solution were used as heating and cooling medium, respectively. An oil−glycol mixture (TR15, Greenpoint oil, Resato, Roden, The Netherlands) was used as pressure transferring medium. Temperature and pressure evolution during treatment could be followed as the apparatus was equipped with thermocouples (type J) for each vessel to monitor temperature of the pressure medium, a pressure sensor, and a data logger (Cobra 7-10 Mess+system Technic GmbH, Germany).

The green bean juice was filled in flexible microtubes (Elkay, Labsystems Group (UK) Ltd., Basingstoke-Hants, England, 0.25 mL, 4.2 mm inner diameter, 33 mm length), excluding air bubbles and closed with Parafilm to avoid pressure medium contamination. The samples were placed in the vessels and pressure was manually built up with a standardized pressurization rate of 100−125 MPa/min. After maximum pressure was achieved, all valves were closed. The valve of the first vessel was opened after an equilibration time of 5 min to ensure achievement of isobaric isothermal conditions in the vessel. After treatment, samples were stored at 5 °C (<3 h) until residual LOX activity was measured.

Kinetics of Lipoxygenase Inactivation in Intact Green Beans (in Situ Study). *Green Beans Preparation*. The beans were thawed overnight at 5 °C prior to each pressure−temperature combination and only unbroken ones were used for the kinetic study.

Thermal Treatment at Ambient Pressure. Samples were prepared at room temperature by placing the beans (15−20 g) in a perforated polyethylene plastic pack (Nasco Whirl-Pak, USA, 175 mm length, 75 mm width). The samples were placed individually in a sample holder to allow appropriate circulation of heating medium among the beans and heated in a temperature controlled water bath (Grant Y14, Cambridge, England) during preset time intervals. Temperature evolution in the beans and in the heating medium during treatment could be followed using thermocouples (type T) and recorded using a data logger (Ellab TR 9216-UMX 693, Copenhagen, Denmark). To stop further thermal inactivation, the samples were immediately cooled in an ice bath after withdrawal from the water bath. Enzyme extraction was executed after storage on ice (<3 h).

Combined High-Pressure and Temperature Treatment. Combined pressure and temperature treatments were performed in a pilot scale single vessel high-pressure equipment (warm isostatic press, Engineered Pressure System Int., Belgium) (590 mL, 5 cm inner diameter, 30 cm length). The apparatus allowed pressurization up to 550 MPa in the range of 0−100 °C; however, the maximum pressure of 450 MPa could be reached at subzero temperatures (−10 to 0 °C). The pressure transmitting medium, a mixture of propylene glycol (60% Dowcal N, The Dow Chemical Co., Horgen, Switzerland), was pumped using an electrically driven high-pressure intensifier with a displacement of 83 mL/min. The system was thermostated by a cryostat (Haake KT50W-N8, Karlsruhe, Germany) using a fluid flow heat exchange from outside of the vessel and 56% ethylene glycol solution was used as heating and cooling medium. The high-pressure machine was provided with three thermocouples (type K) to record temperature evolution in the beans and in the pressure medium, a pressure sensor, and a data logger (SCXI-1000, National Instruments, USA).

Samples were prepared at room temperature by packing the beans (15−20 g) in double polyethylene plastic packs (Medisch Labo Service, Menen, Belgium, 220 mm length, 60 mm width) to avoid direct contact with the pressure medium. They were vacuum-sealed (Multivac A300/16, Wolfertschwenden, Germany) up to 11 mbar and the sample was placed in a sample holder (5 cm diameter, 17 cm length), located 5 cm above the bottom of the vessel. It was pressurized automatically up to preset pressure, and after preset time, the pressure was manually released. The package was opened after withdrawal from the vessel and the treated beans were stored at 5 °C (<3 h) until enzyme extraction. The samples were individually pressurized for different preset times at each pressure temperature combination.

Green Beans Extraction for LOX Activity Measurement. The (treated) samples were cut into small slices (3−5 mm thickness) and frozen in liquid nitrogen. To obtain a fine powder, the frozen beans were ground (IKA Anaytical mill A10, IKA Labortechnik, Janke & Kunkel Gmbh & Co.KG, Staufen, Germany). The bean powder was extracted (1:1 v/w) using phosphate buffer (5 mM; pH 6) containing 0.5% of Tween 20 (Merck, Darmstadt, Germany) and 0.5% of Triton X-100 (Fluka, Buchs, Switzerland). The extract/buffer mixture was homogenized using a high-speed dispersing and emulsifying apparatus (Ultra turrax T25, IKA Labortechnik, Janke & Kunkel Gmbh & Co.KG, Staufen, Germany) successively at 8000 and 13 500 rpm for 10 and 20 min, respectively. The homogenized mixture was filtered through a folded cellulose filter (Schleicher & Schuell 595 $^1/_2$ o.d. 185 mm, Dassel, Germany) at 5 °C and the resulting filtrate was used for LOX activity measurement.

Measurement of Lipoxygenase Activity. A polarographic assay (Oximeter model 781B, Strathkelvin Instruments, Glasgow, Scotland) with sodium linoleic acid as substrate was used to measure enzyme activity. The assay was carried out at 25 °C and the reaction solution consisted of 2.5 mL of phosphate buffer (10 mM; pH 6), 1.25 mL of substrate solution (a mixture of 12.84 mM linoleic acid, 0.4% (v/v) Tween 20 and 20 mM NaOH), and 0.125 mL of green bean extract. The enzyme activity (Δppm of O_2/s) was derived from the slope of oxygen concentration as a function of reaction time (Indrawati et al., 1999b) and LOX activity for each temperature−pressure−time combination was measured in duplo.

Estimation of Kinetic Parameters. A kinetic study of enzyme inactivation relies on its inactivation progress as a function of time under specified conditions. Previous studies showed that thermal and combined pressure temperature inactivation of LOX either in simple model systems (Park et al., 1988; Ludikhuyze et al., 1998) or in real food system (Günes and Bayindirli, 1993; Indrawati et al., 1999b) could be described as a first-order reaction, allowing the decrease of enzyme activity (A) as a function of time under constant pressure and temperature to be expressed as eq 1.

$$\ln(A) = \ln(A_0) - k^*t \quad (1)$$

Inactivation rate constants (k) could be estimated using a linear regression analysis (SAS, 1995). To describe the temperature dependency at constant pressure and the pressure dependency at a constant temperature of the inactivation rate constants, the Arrhenius (2) and Eyring (3) relations were used, respectively.

$$\ln(k) = \ln(k_{T_{ref}}) - \left(\frac{E_a}{R_T}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right) \quad (2)$$

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

Activation energy (E_a) and activation volume (V_a) values could be determined based on a linear regression analysis (SAS, 1995) of ln(k) versus the reciprocal of absolute temperature or the pressure, respectively.

RESULTS AND DISCUSSION

Pressure and Temperature Evolution during Treatments. A study on temperature evolution in the medium and at the center of the beans during thermal and combined pressure temperature treatments was accomplished. A typical temperature profile at the center of the beans during thermal treatment at ambient pressure is presented in Figure 1. It was noticed that the time to reach isothermal conditions at the center of the beans was strongly dependent on the bean diameter, i.e. longer equilibration time is needed to achieve isothermal conditions in beans with larger diameter. Therefore, beans with similar diameter were used in each kinetic study of thermal inactivation at ambient pressure to avoid variation in the initial nonisothermal conditions.

An example of pressure and temperature evolution of the pressure medium in the multivessel is shown in Figure 2. During pressurization, temperature increases due to adiabatic heating and isobaric isothermal conditions could be reached after 5 min equilibration. Upon pressure release, adiabatic cooling occurred, resulting in a temperature decrease of the pressure medium. Pressure and temperature profile inside the sample was assumed similar to that of the pressure medium since the green bean juice was filled in micro tubes.

Temperature profiles of the pressure medium inside the single vessel apparatus were analogous. Figure 3 shows typical temperature profiles of the pressure medium and at the center of the beans during pressure treatment (500 MPa) at a temperature of 20 °C. It can be seen that the preset pressure of 500 MPa is initially exceeded by approximately 50 MPa, due to the indirect piston-type compression. Moreover, it could be observed that the temperature evolution at the center of the beans was similar to that of the pressure medium, although the adiabatic heating was somewhat larger in beans. Equilibrium time prior to isobaric isothermal conditions at the center of the beans was on average 30 min after the achievement of maximum pressure. The effect of adiabatic heating and the equilibration time to achieve isobaric isothermal conditions were influenced by the bean diameter (Figure 3). For beans of similar diameter, no significant differences were observed.

Temperature profiles at the center of the beans and of the pressure medium at subzero temperature under pressure are shown in Figure 4. It could be seen that during pressurization and decompression, the effect of adiabatic heating and cooling on temperature profile of the pressure medium was different from on that at the center of the beans. This phenomenon could be explained by the ice−water phase transition under pressure in which a partially or totally frozen sample can be obtained before pressure buildup when the beans are

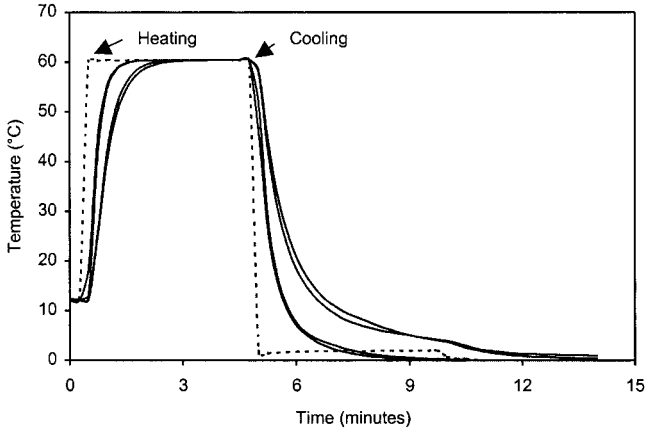

Figure 1. Temperature evolution at the center of green beans with different diameter (6.6, 6.6, 9.0, and 9.2 mm from left to right, respectively) during thermal treatment at 60 °C combined with ambient pressure.

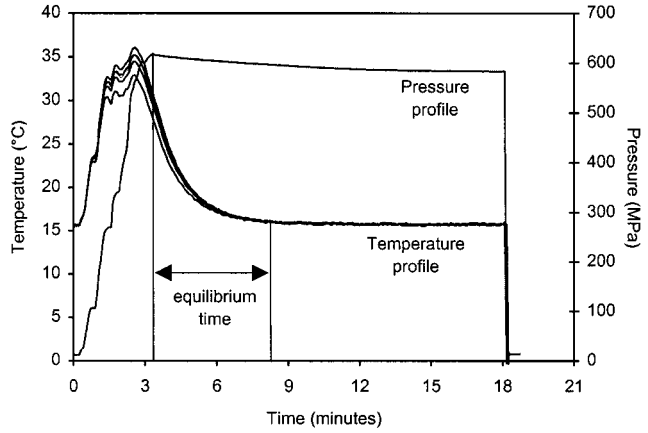

Figure 2. Pressure and temperature profiles of pressure medium in the multivessel high-pressure machine at 600 MPa and 15 °C.

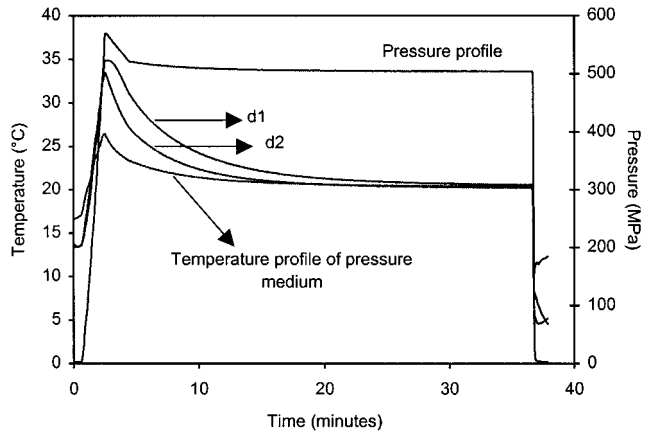

Figure 3. Pressure and temperature profiles of pressure medium and at the center of green beans using different bean diameter ($d_1 = 9.7$ mm; $d_2 = 5.0$ mm) in the single vessel high-pressure machine at 500 MPa and 20 °C.

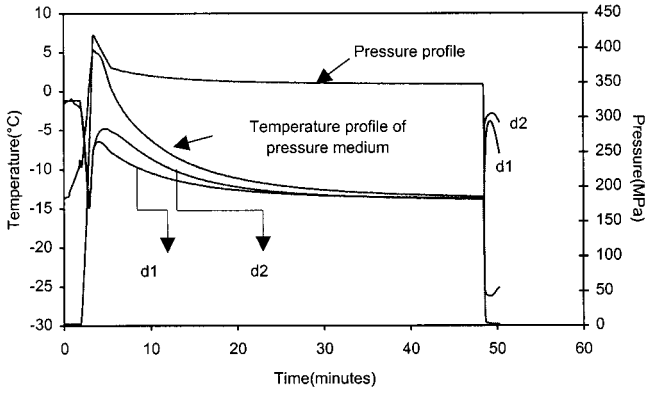

Figure 4. Pressure and temperature profiles of pressure medium and at the center of green beans using different diameter (d_1 = 8.7 mm; d_2 = 9.6 mm) as a function of processing time in the single vessel high-pressure machine at 350 MPa and −13 °C.

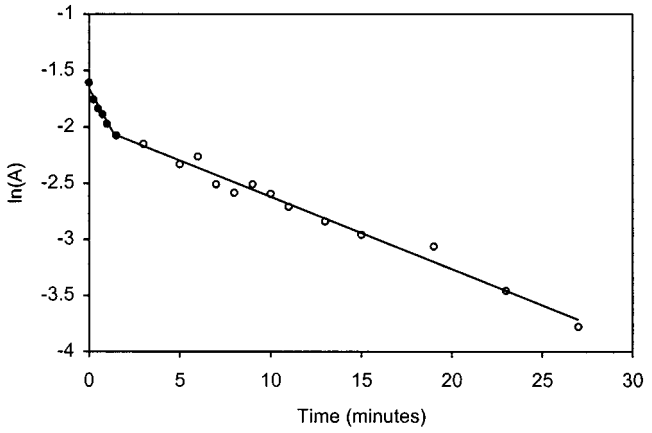

Figure 5. Biphasic behavior (heat labile fraction (●) and heat stable fraction (○) of LOX inactivation in green bean juice under isothermal conditions at 65 °C combined with ambient pressure.

placed in the cooled (below 0 °C) vessel and during pressurization, the compression work is transferred into melting energy resulting in a decrease of temperature inside the green beans.

Lipoxygenase Inactivation Due to Thermal Treatment at Ambient Pressure. Reproducibility of LOX activity measurement in green bean juice and extract was determined. The standard deviation of activity measurement was found to be 4.4% on average (10 replications). During storage at 5 °C and in the ice bath, LOX activity was stable until 8 h of storage. Afterward, the enzyme activity decreased progressively down to 50% of its initial activity after 1 day. As a consequence, LOX activity was measured within 3 h.

LOX activity in green beans was unevenly distributed: the activity ratio in seed and in pod was 1.94:1 whereas the weight ratio was 1:0.12. Reproducibility of LOX extraction depended on seed size and seed/pod proportion among the samples. When using green beans with similar seed size and proportion, standard deviation of LOX extraction (using 10 replications) was situated at 7.6% on average. A progressive decrease in LOX activity in thawed green beans was noticed after 1 day storage at 5 °C, down to 40% of its initial activity on the second day of storage. Therefore, the beans were immediately used for in situ study after overnight thawing (maximum 16−24 h).

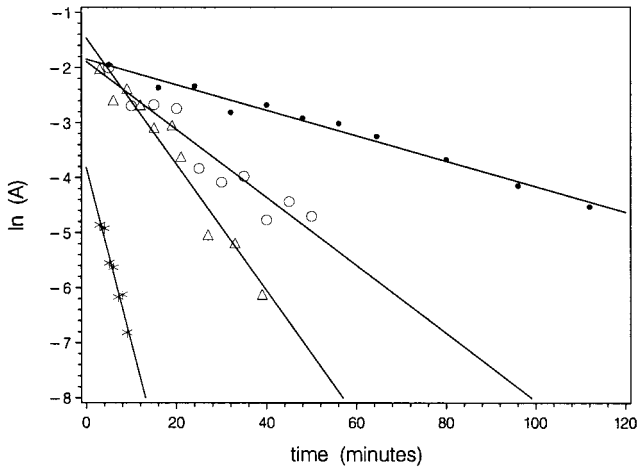

Figure 6. First-order inactivation of LOX in situ under isothermal conditions combined with ambient pressure (●, 57 °C; ○, 60 °C; △, 63 °C; ∗, 70 °C).

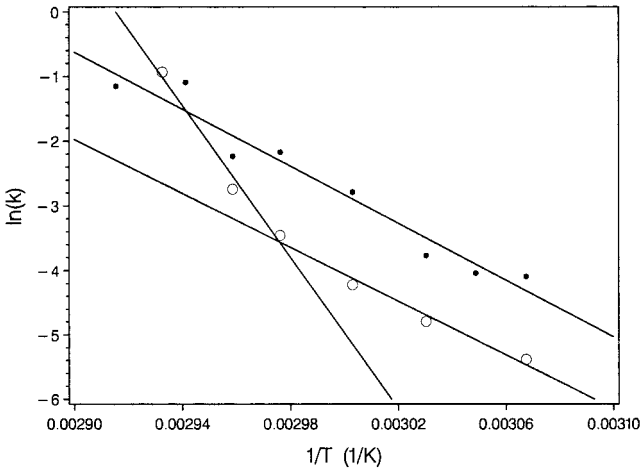

Figure 7. Temperature sensitivity of inactivation rate constants for LOX in green bean juice (○) and in situ (●) at ambient pressure.

Thermal inactivation of LOX either in the green bean juice or in situ was carried out in a temperature range from 53 up to 70 °C. No reactivation of the residual LOX activity was noticed after treatments during storage at 5 °C. Under isothermal conditions, LOX inactivation in the juice showed a biphasic behavior, i.e., a succession of two first-order inactivation reactions, namely of the heat-labile and heat-stable fraction (Figure 5) as previously reported by Indrawati et al. (1999b). It could be attributed to different thermostability of LOX isozymes in green beans. For in situ LOX inactivation, only one fraction, probably a part of the heat stable fraction, following first-order kinetics was observed (Figure 6). Because of the heat transfer limitations, the first data point was only taken after 3 min to ensure the isothermal condition from that moment on. It might be that the major part of the heat labile fraction has already inactivated. To allow comparison of LOX inactivation in both systems, inactivation rate constants were calculated according to the same data analysis method, i.e., linear regression (SAS, 1995) of residual LOX activity as a function of time. The estimated k values are summarized in Table 1. LOX was less thermostable in situ than in the juice at temperatures below 68 °C whereas the thermostability ranking became reverse at temperatures above 68 °C (Figure 7). In the entire

Table 1. Estimated Rate Constants ($\times 10^{-2}$ min^{-1}) of LOX Inactivation in Green Beans Due to Thermal Treatment at Ambient Pressure

temp (°C)	green bean juice[a]	intact green beans (in situ study)[a]
53	0.46 ± 0.07[b]	1.67 ± 0.08[b]
	$r^2 = 0.907$	$r^2 = 0.9828$
55		1.76 ± 0.17
		$r^2 = 0.920$
57	0.83 ± 0.02	2.32 ± 0.11
	$r^2 = 0.986$	$r^2 = 0.980$
60	1.47 ± 0.05	6.17 ± 0.70
	$r^2 = 0.977$	$r^2 = 0.906$
63	3.16 ± 0.07	11.45 ± 0.99
	$r^2 = 0.992$	$r^2 = 0.944$
65	6.45 ± 0.25	10.74 ± 0.96
	$r^2 = 0.982$	$r^2 = 0.962$
68	39.46 ± 1.39	33.67 ± 2.81
	$r^2 = 0.987$	$r^2 = 0.980$
70		31.77 ± 3.34
		$r^2 = 0.948$
E_a (kJ/mol)	173.52 ± 17.08 (53−63 °C)	183.30 ± 16.83
	$r^2 = 0.981$	$r^2 = 0.951$
	488.39 ± 66.35 (63−68 °C)	
	$r^2 = 0.982$	

[a] The k values were estimated from the residual LOX activity after the heating time of 3 min. [b] Standard error of regression.

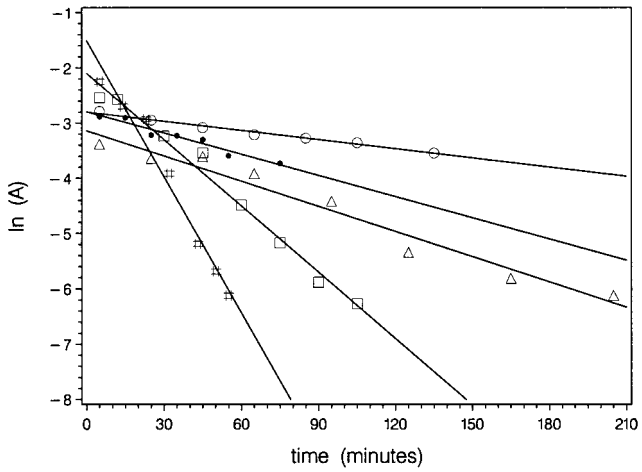

Figure 8. Inactivation of LOX in green bean juice under isobaric isothermal conditions at 450 MPa and different temperature levels (●, −10 °C; ○, 0 °C; △, 30 °C; □, 40 °C; and #, 50 °C).

temperature domain studied, temperature sensitivity of the rate constants for LOX inactivation in situ could be estimated using the Arrhenius relation (2) whereas this relation was inappropriate to predict E_a values of LOX inactivation in the juice. In this case, the Arrhenius plot showed a "breakpoint" at 63 °C (Figure 7), as previously revealed by Indrawati et al. (1999b). Consequently, the estimated E_a values of LOX inactivation in the juice were calculated in two different temperature (above and below 63 °C) domains (Table 1). Activation energy values for LOX inactivation in the juice were on the same order of magnitude as compared to the previous study (Indrawati et al., 1999b). Temperature sensitivity of the inactivation rate constants for LOX in situ was similar to that in the juice at temperatures below 63 °C but higher than that at temperatures above 63 °C (i.e., a lower activation energy).

Lipoxygenase Inactivation Due to Combined Pressure and Temperature Treatment. Under all

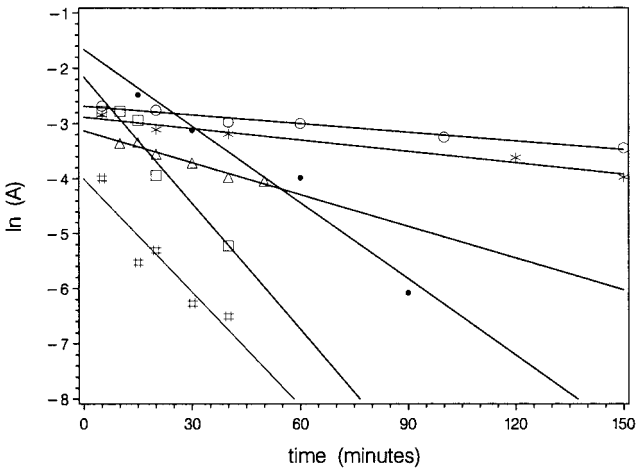

Figure 9. LOX inactivation in situ due to combined pressure and temperature treatments at 450 MPa and different temperature levels (●, −10 °C; ○, 0 °C; ∗, 10 °C; △, 30 °C; □, 40 °C; and #, 50 °C).

experimental conditions studied, it was noticed that combined pressure temperature inactivation of LOX in both systems was irreversible. LOX inactivation in the juice was studied after an equilibration time of 5 min (i.e., initial preset time) to ensure the achievement of isobaric isothermal conditions (Figure 2). The inactivation under isobaric and isothermal conditions could be described as a first-order reaction (Figure 8) and the estimated inactivation rate constants are summarized in Table 2. A single phase of first-order reaction in the pressure−temperature inactivation curves was observed, in contrast with the biphasic LOX thermal inactivation behavior in green bean juice. LOX isozymes seem to exhibit a similar pressure stability. Nevertheless, it should be taken into account that LOX is already partially inactivated during pressure buildup, which is accompanied by a temperature increase due to adiabatic heating of pressure medium, and during equilibrium time (5 min). The degree of LOX inactivation due to pressure built up and equilibrium time depended on the level of pressure temperature combinations (resulting in the y-intercepts variation). As a consequence, the existence of a pressure labile fraction might not be detected; therefore, study on pressure stability of the separate purified LOX isozymes calls a further investigation. Upon pressure increase at constant temperature the rate constants of LOX inactivation consistently increased whereas at constant pressure, minimal k values were found at about 10 °C so that either temperature increase (above 10 °C) or decrease (below 10 °C) could accelerate the inactivation.

Kinetics of LOX inactivation in situ by combined pressure and temperature treatments was studied, likewise taking into account an equilibration time of 5 min. By plotting the natural logarithm of residual LOX activity as a function of inactivation time, a linear correlation was found; i.e., a first-order reaction was valid to describe the kinetics of inactivation (Figure 9). The resulting nonisobaric nonisothermal conditions after 5 min did not influence the kinetics of LOX inactivation (i.e., the reaction order). As shown in Figure 3, isobaric isothermal conditions in the beans are achieved approximately within 30 min. However, by extending the equilibration time up to 30 min, an appropriate kinetic analysis of k value estimation can hardly be performed due to the resulting low residual

Table 2. Estimated Rate Constants ($\times 10^{-2}$ min^{-1}) of LOX Inactivation in Green Bean Juice Due to Combined Pressure and Temperature Treatment

press. (MPa)	temperature (°C)							
	−10	0	10	20	30	40	50	55
50								0.79 ± 0.09 $r^2 = 0.936$
150								1.34 ± 0.02 $r^2 = 0.998$
250								1.54 ± 0.12 $r^2 = 0.963$
300							1.09 ± 0.12 $r^2 = 0.954$	2.68 ± 0.14 $r^2 = 0.987$
350						1.36 ± 0.10 $r^2 = 0.971$	1.57 ± 0.14 $r^2 = 0.960$	4.29 ± 0.32 $r^2 = 0.967$
400	0.45 ± 0.03[a] $r^2 = 0.974$						4.89 ± 0.33 $r^2 = 0.977$	
450	1.28 ± 0.14 $r^2 = 0.946$	0.55 ± 0.03 $r^2 = 0.985$			1.52 ± 0.12 $r^2 = 0.963$	4.00 ± 0.20 $r^2 = 0.985$	8.20 ± 0.63 $r^2 = 0.972$	9.86 ± 0.85 $r^2 = 0.957$
500	5.03 ± 0.66 $r^2 = 0.951$	1.67 ± 0.05 $r^2 = 0.996$	0.73 ± 0.04 $r^2 = 0.983$	1.68 ± 0.09 $r^2 = 0.984$	2.54 ± 0.10 $r^2 = 0.991$	3.47 ± 0.24 $r^2 = 0.971$		
550	7.73 ± 0.56 $r^2 = 0.969$	5.20 ± 0.33 $r^2 = 0.980$	2.44 ± 0.19 $r^2 = 0.963$	2.51 ± 0.15 $r^2 = 0.981$	2.94 ± 0.17 $r^2 = 0.980$	5.12 ± 0.27 $r^2 = 0.984$		
600		8.59 ± 0.51 $r^2 = 0.983$	3.58 ± 0.20 $r^2 = 0.984$	5.01 ± 0.33 $r^2 = 0.975$	3.84 ± 0.36 $r^2 = 0.949$			
650			9.14 ± 0.51 $r^2 = 0.978$	13.03 ± 0.80 $r^2 = 0.982$		10.60 ± 0.59 $r^2 = 0.982$		

[a] Standard error of regression.

Table 3. Estimated Rate Constants ($\times 10^{-2}$ min^{-1}) of LOX Inactivation in Situ Due to Combined Pressure and Temperature Treatment

press. (MPa)	temperature (°C)								
	−10	−5	0	10	20	30	40	50	55
50								1.22 ± 0.23 $r^2 = 0.903$	2.06 ± 0.29 $r^2 = 0.944$
150								2.10 ± 0.22 $r^2 = 0.958$	2.12 ± 0.29 $r^2 = 0.913$
250								2.57 ± 0.34 $r^2 = 0.919$	3.71 ± 3.74 $r^2 = 0.961$
300							1.15 ± 0.18 $r^2 = 0.928$		3.44 ± 0.19 $r^2 = 0.991$
350		0.26 ± 0.04 $r^2 = 0.922$				0.56 ± 0.05 $r^2 = 0.980$	2.10 ± 0.17 $r^2 = 0.976$	4.47 ± 0.35 $r^2 = 0.970$	10.23 ± 1.22 $r^2 = 0.946$
400	0.55± 0.10[a] $r^2 = 0.906$	1.31 ± 0.10 $r^2 = 0.978$				1.45 ± 0.20 $r^2 = 0.946$	3.06 ± 0.34 $r^2 = 0.965$		
450	4.62 ± 0.66 $r^2 = 0.961$		0.52 ± 0.04 $r^2 = 0.975$	0.69 ± 0.07 $r^2 = 0.967$		1.93 ± 0.19 $r^2 = 0.963$	7.63 ± 1.11 $r^2 = 0.940$	6.86 ± 1.47 $r^2 = 0.879$	
500				1.14 ± 0.08 $r^2 = 0.982$	1.20 ± 0.17 $r^2 = 0.945$	5.48 ± 0.57 $r^2 = 0.969$	9.21 ± 1.13 $r^2 = 0.957$		
550				2.18 ± 0.18 $r^2 = 0.974$	3.21 ± 0.40 $r^2 = 0.914$	14.22 ± 2.04 $r^2 = 0.942$			

[a] Standard error of regression.

LOX activity. The estimated k values are given in Table 3. It could be noticed that the inactivation rate constant could be enhanced by pressure increase at constant temperature.

By interpolating the estimated k values (g3grid procedure in SAS program), iso rate contour diagrams as a function of pressure and temperature could be constructed (Figure 10). From the shape of those diagrams, an antagonistic effect at low or subzero temperature was observed but not at elevated temperature, which is in agreement with previous studies on soybean LOX inactivation by Ludikhuyze et al. (1998) and Indrawati et al. (1999a) but in contrary to the findings of Heinisch et al. (1995). It could be seen that the barostability of LOX was somewhat lower in situ than in the juice, i.e. a slightly lower pressure was needed to obtain the same rate of inactivation (Figure 10a,b).

Pressure sensitivities of the inactivation rate constants for both systems were estimated using the Eyring eq 3. The equation was merely valid at temperatures lower than 55 °C, and the estimated activation volumes at different constant temperature levels are given in Table 4. Sensitivity to pressure changes of the rate constants for LOX inactivation in situ could not be investigated in detail since the number of observations was rather low due to the limited pressure capacity of the single vessel machine. The absolute value of the activation volume seemed to increase slightly to reach a maximum at about 30 °C and then decreased with further temperature rise, pointing to a reduced pressure sensitivity at high temperature. Study on LOX inactivation in the juice, however, showed that the absolute value of the activation volume first decreased with increasing temperature up to 30 °C and then again increased pointing to enhanced pressure sensitivity at high temperature, in contrast to LOX inactivation in situ or to some previous studies on other food quality related enzymes, e.g., soybean LOX (Ludikhuyze et al., 1998; Indrawati et al., 1999a), Taka amylase A (Tan-

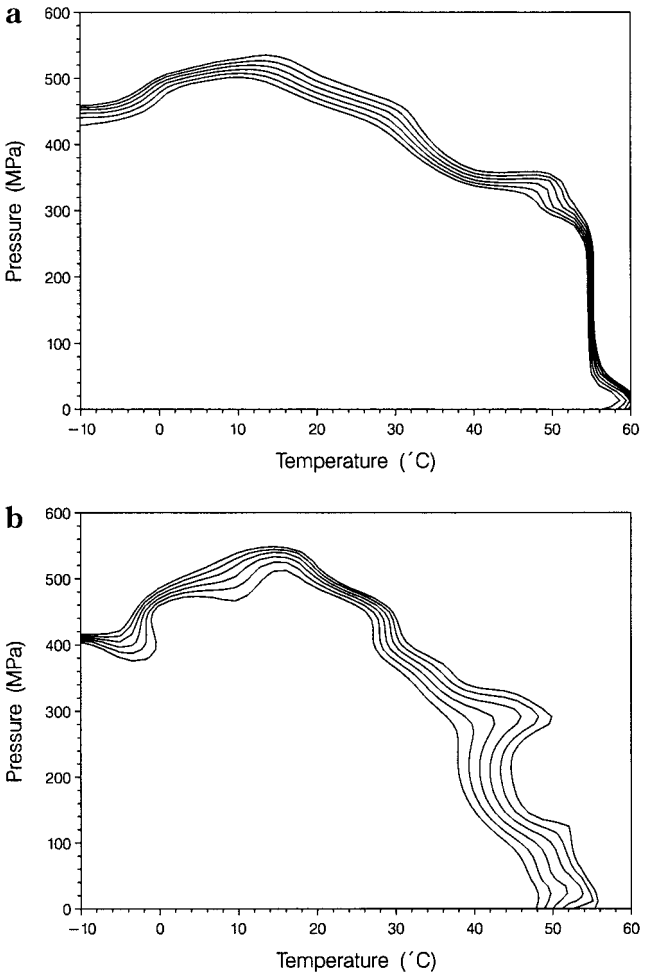

Figure 10. Iso rate contour diagram of LOX inactivation in green bean juice (a) and in situ (b) as a function of pressure and temperature: 0.018, 0.016, 0.014, 0.012, 0.010, and 0.008 min^{-1} (from upper line to lower line).

Table 4. Estimated Activation Volume ($cm^3 \cdot mol^{-1}$) of LOX Inactivation in Green Beans at Different Constant Temperature Levels

temp (°C)	green bean juice	intact green beans (in situ study)
−10	−43.30 ± 5.78[a] $r^2 = 0.966$	
0	−42.59 ± 4.86 $r^2 = 0.975$	
10	−37.49 ± 5.03 $r^2 = 0.966$	−27.07 ± 1.99 $r^2 = 0.995$
20	−33.61 ± 4.27 $r^2 = 0.968$	
30	−14.75 ± 2.62 $r^2 = 0.941$	−39.30 ± 3.54 $r^2 = 0.976$
40	−16.68 ± 2.53 $r^2 = 0.935$	−28.38 ± 2.85 $r^2 = 0.971$
50	−38.62 ± 5.44 $r^2 = 0.962$	−11.31 ± 0.78 $r^2 = 0.986$

[a] Standard error of regression.

iguchi and Suzuki, 1983), and PPO (Weemaes et al., 1998). It was observed that the pressure dependence of the k values in the juice was the lowest at 30 °C. In this study, a reverse tendency between pressure sensitivity of LOX inactivation rate constants in the juice and in situ was observed.

Inactivation data in Tables 2 and 3 show that an acceleration of the inactivation rate of LOX can be caused either by increasing temperature above 10 °C or by decreasing temperature below 10 °C. As a result, Arrhenius eq 2 could not be applied directly for both systems to determine temperature dependency of the inactivation rate constants in the entire temperature region. Therefore, activation energy at constant pressure was calculated in distinct temperature regions. The estimated E_a values for LOX inactivation either in the juice or in situ are summarized in Table 5. The negative value of the activation energy indicated that a temperature decrease under pressure could accelerate the inactivation. For LOX inactivation in the juice, the absolute value of the activation energy at 500 MPa was higher at low temperature (<10 °C) as compared to higher temperature (>10 °C), indicating a high thermal sensitivity at low or subzero temperature. It was also noticed that high-pressure application reduced temperature sensitivity of LOX inactivation rate constants as compared to thermal treatment at ambient pressure (Tables 1 and 5).

Table 5. Estimated Activation Energy ($kJ \cdot mol^{-1}$) of LOX Inactivation in Green Beans in Different Pressure Temperature Ranges of Inactivation

system	P/T range for inactivation	E_a (kJ mol^{-1})
green bean juice	450 MPa, 30−55 °C	62.74 ± 5.30[a] ($r^2 = 0.986$)
	500 MPa, 10−40 °C	37.72 ± 5.52 ($r^2 = 0.959$)
	500 MPa, (−10)−10 °C	−59.80 ± 3.65 ($r^2 = 0.996$)
	550 MPa, (−10)−10 °C	−35.53 ± 7.19 ($r^2 = 0.961$)
intact green beans (in situ)	350 MPa, 30−55 °C	91.01 ± 8.06 ($r^2 = 0.985$)
	450 MPa, 30−50 °C	42.50 ± 6.80 ($r^2 = 0.929$)
	500 MPa, 10−40 °C	57.06 ± 14.49 ($r^2 = 0.886$)
	550 MPa, 10−30 °C	66.38 ± 23.98 ($r^2 = 0.885$)

[a] Standard error of regression.

Combined Pressure and Temperature Dependence of the Inactivation Rate Constants. A general mathematical model describing the pressure−temperature dependence of LOX inactivation rate constants could not be developed using the Eyring or Arrhenius relation as a starting point, a method previously applied by several authors (Ludikhuyze et al., 1998; Weemaes et al., 1998; Van den Broeck et al., 1999) since the variation of pressure and temperature sensitivities of inactivation rate constants was different in both systems. Some authors (Weemaes et al., 1998; Indrawati et al., 1999a) have developed a kinetic model (4) to describe pressure−temperature dependence of the inactivation rate constants, by modifying the thermodynamic model proposed by Hawley (1971) into a kinetic version. Previous study showed that pressure temperature inactivation of soybean LOX in a simple model system could be described by this modified model (Indrawati et al., 1999a). The same model was therefore used to fit the experimentally obtained data on pressure temperature inactivation of LOX in real foodstuffs.

$$\ln(k) = \ln(k_0) - \frac{\Delta V_0^{\ddagger}}{R_T T}(P - P_0) + \frac{\Delta S_0^{\ddagger}}{R_T T}(T - T_0) - \frac{1}{2}\frac{\Delta \kappa^{\ddagger}}{R_T T}(P - P_0)^2 - 2\frac{\Delta \zeta^{\ddagger}}{R_T T}(P - P_0)(T - T_0) + \frac{\Delta C_p^{\ddagger}}{R_T T}\left[T\left(\ln\frac{T}{T_0} - 1\right) + T_0\right] \quad (4)$$

To estimate the model parameters (k_0, $\Delta V_0^{\ddagger}$, $\Delta S_0^{\ddagger}$, $\Delta k^{\ddagger}$, $\Delta \zeta^{\ddagger}$, and $\Delta C_p^{\ddagger}$), a nonlinear regression analysis was used

Table 6. Estimated Model Parameters for LOX Inactivation of Soybean and in Green Beans Based on Eq 4 at Reference Pressure 50 N·cm^{-2} and Reference Temperature 298 K

	estimated value		
model parameter	soybean LOX inactivation in Tris HCl buffer (0.01M; pH9)[b]	LOX inactivation in green bean juice[c]	LOX inactivation in situ[c]
k_0 ($\times 10^{-2}$)	1.28 ± 0.011[a]	1.34 ± 0.16	2.47 ± 0.33
$\Delta V_0^{\ddagger}$	−341.95 ± 22.21	−308.14 ± 24.19	−356.72 ± 44.54
$\Delta S_0^{\ddagger}$	−20.65 ± 7.20	90.63 ± 12.29	139.41 ± 23.95
$\Delta\kappa^{\ddagger}$	−1.40 ± 1.06	−0.54 ± 1.55	−7.34 ± 2.62
$\Delta\zeta^{\ddagger}$	3.20 ± 0.26	2.22 ± 0.52	0.10 ± 0.80
$\Delta C_p^{\ddagger}$	3046.55 ± 207.16	2466.71 ± 323.41	2811.82 ± 394.92
	Quality of Fitting		
cor r^2	0.985	0.988	0.986
SD	0.45	0.40	0.44

[a] Asymptotic standard error. [b] Indrawati et al., 1999a. [c] This study.

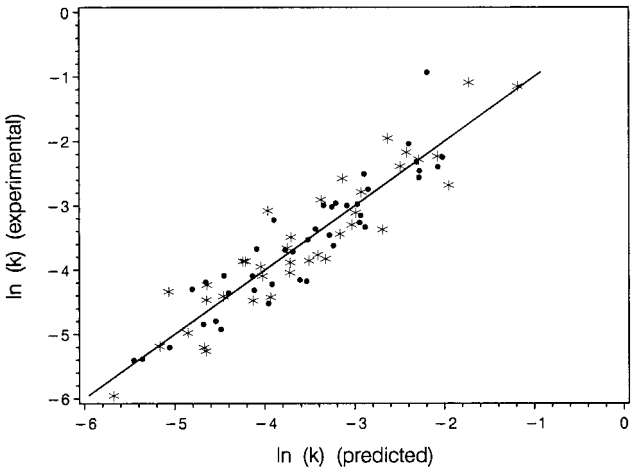

Figure 11. Relation between the natural logarithm of experimental estimated k values of combined pressure and temperature inactivation of LOX in green bean juice (●) and in situ (∗) and the natural logarithm of k values predicted according to eq 4.

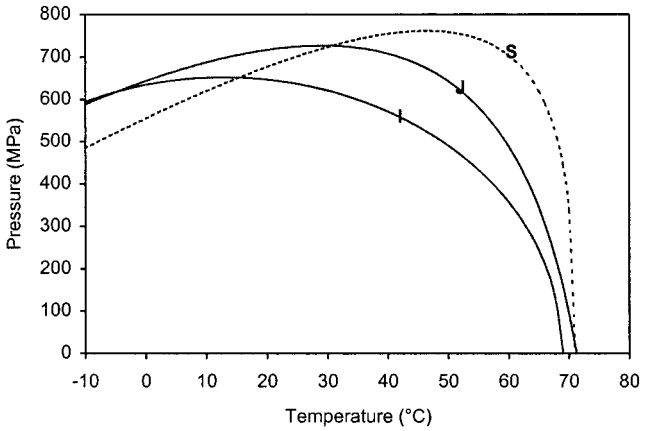

Figure 12. Combined predicted pressure−temperature combination of the same inactivation rate constant (k = 0.2303 min^{-1}) for different sources of LOX, i.e. soybean (dashed line), in green bean juice (full line J) and green beans in situ (full line I).

involving an iterative numerical procedure based on the minimal sum of squares (SAS, 1995). By plotting residuals (differences between predicted and experimental values) as a function of temperature, pressure, experimental values, or inactivation rate constants predicted on the base of eq 4, no significant tendency was found. The estimated model parameters are summarized in Table 6 and a good agreement between the natural logarithm of inactivation rate constants predicted based on eq 4 and the natural logarithm of experimental values was observed for both systems (Figure 11). Nevertheless, it was noticed that this model did not result in an accurate estimation of the model parameters $\Delta k^{\ddagger}$ and $\Delta\zeta^{\ddagger}$,respectively for LOX inactivation in the juice and in situ. According to Morild (1981), eq 4 could in principle describe the pressure and temperature behavior of most phenomena; however, its practical use may be limited due to the many parameters, requiring rather large pressure and temperature intervals, on the order of 500 MPa and 100 K to estimate the unknown parameters by means of computerized curve-fitting. Since the pressure−temperature interval which could be reliably studied from a practical point of view was quite limited, especially in this investigation using real food systems, this might partly explain the poor parameter estimates.

In this study, it was noticed that the kinetic model (4) derived from the thermodynamic equation (Hawley, 1971) could basically describe the pressure−temperature dependence of LOX inactivation either in green beans or of soybean (0.4 mg/mL in Tris HCl buffer (0.01 M; pH 9)) as previously revealed by Indrawati et al. (1999a). The estimated model parameters $\Delta V_0^{\ddagger}$ and $\Delta C_p^{\ddagger}$ of LOX inactivation of soybean and in green beans are in the same order of magnitude whereas the predicted parameter $\Delta S_0^{\ddagger}$ was likely more dependent on the type of vegetables rather than on the level of food complexity (i.e., green bean juice and in situ) (Table 6).

By inserting all estimated model parameters (Table 6) into eq 4, pressure−temperature combinations resulting in a specific preset inactivation rate constant for LOX of soybean and in green beans were predicted and are depicted in Figure 12. It was observed that the estimated pressure−temperature level for inactivation of LOX either in green beans or of soybean was very similar in the high temperature (±70 °C) and low pressure (<300 MPa) area. However, at lower temperature (30−60 °C) and higher pressure, significant differences in stability became noticeable, the stability being reduced from soybean over green bean juice to intact green beans. At low (<30 °C) and subzero temperatures, the ranking became reverse. In the latter case, soybean LOX could be inactivated at lower pressure and temperature as compared to LOX in green beans. Moreover, the antagonistic effect between low temperature and high-pressure became much less pronounced with increasing level of food complexity.

Nevertheless, it has to be stated that the kinetic study at subzero temperature was rather limited because of practical reasons, i.e., detection limit of the enzymatic assay. Consequently, modeling pressure−temperature

dependence in this area became somewhat critical and results have to be interpreted with caution. Moreover, kinetic approach to describe pressure−temperature dependence of LOX inactivation rate constants has been successfully transferred and applied from model system, i.e., soybean LOX (Indrawati, 1999a) to real food system, e.g., green beans.

CONCLUSION

Performing pressure and/or temperature inactivation experiments on enzymes in real foodstuff provides a method to get insight into the influence of the food matrix on the inactivation. Moreover, such experimental approach offers other advantages: (i) the effect of different intrinsic food complexity on enzyme inactivation is studied, considering the intactness of plant tissue; (ii) the kinetic information obtained could be directly applied to optimization of high-pressure applications in real foods, and (iii) since the implementation of high-pressure treatment (in situ study) was simulated in a simple laboratory scale pilot installation the impact of some processing factors, e.g., nonuniformity in the high-pressure vessel, on the kinetics of enzyme inactivation could be considered.

On a kinetic basis, the progress of LOX inactivation as a function of time (i.e., reaction order of inactivation) due to pressure treatment either at subzero or elevated temperature was not influenced by different levels of food complexity, in contrast to the pressure and temperature sensitivities of the inactivation rate constants. In the pressure−temperature area studied, LOX was less pressure stable in situ than in the juice.

In view of using lipoxygenase as a blanching index, pressure treatment as an alternative to water blanching could be explored from subzero up to elevated temperature depending on the desired additional applications. Conventionally, water blanching is established prior to freezing processes of particular vegetables (e.g., green beans) to inactivate some undesired enzymes. The application of high pressure at subzero temperature could propose a single process operation of blanching−freezing replacing conventional blanching−freezing processing with regard to obtain a better quality retention.

NOMENCLATURE

A = enzymic activity at inactivation time = t (Δppm of $O_2 \cdot s^{-1}$)
A_0 = enzymic activity at time = 0 (Δppm of $O_2 \cdot s^{-1}$)
cor r^2 = corrected r^2 defined as $[1 - (i - 1)(1 - SSQ_{regression}/SSQ_{total}/(i - j)]$
E_a = activation energy ($kJ \cdot mol^{-1}$)
i = number of observations
j = number of model parameters
k = inactivation rate constant (min^{-1} for first-order reaction)
k_0 = inactivation rate constant at T_0 and P_0 (min^{-1} for first-order reaction)
$k_{T_{ref}}$ = inactivation rate constant at reference temperature (min^{-1} for first-order reaction)
$k_{P_{ref}}$ = inactivation rate constant at reference pressure (min^{-1} for first-order reaction)
ln = natural logarithm
LOX = lipoxygenase
P = pressure (MPa or $N \cdot cm^{-2}$)
$P_0 = P_{ref}$ ($N \cdot cm^{-2}$)
P_{ref} = reference pressure (MPa)
r^2 = linear correlation coefficient
R_P = universal gas constant ($8.31577\ cm^3 \cdot MPa \cdot K^{-1} \cdot mol^{-1}$)
R_T = universal gas constant ($8.314\ J \cdot mol^{-1} \cdot K^{-1}$)
s = second
SD = model standard deviation defined as $2\sqrt{SSQ_{residual}/(i-j)}$
SSQ = sum of squares
t = inactivation time (min)
T = temperature (K)
$T_0 = T_{ref}$ (K)
T_{ref} = reference temperature (K)
V_a = activation volume ($cm^3 \cdot mol^{-1}$)
$\Delta\zeta$ = thermal expansibility absolute ($cm^3 \cdot mol^{-1} \cdot K^{-1}$)
ΔC_p = heat capacity ($J \cdot mol^{-1} \cdot K^{-1}$)
ΔG = free energy change between native and denatured state ($J \cdot mol^{-1}$)
$\Delta G_0 = \Delta G$ at T_0 and P_0 ($J \cdot mol^{-1}$)
$\Delta\kappa$ = compressibility factor ($cm^6 \cdot J^{-1} \cdot mol^{-1}$)
ΔS = entropy change between native and denatured state ($J \cdot mol^{-1} \cdot K^{-1}$)
$\Delta S_0 = \Delta S$ at T_0 and P_0 ($J \cdot mol^{-1} \cdot K^{-1}$)
ΔV = volume change between native and denatured state ($cm^3 \cdot mol^{-1}$)
$\Delta V_0 = \Delta V$ at T_0 and P_0 ($cm^3 \cdot mol^{-1}$)
‡ = of the transition state

ACKNOWLEDGMENT

This research was supported by Katholieke Universiteit Leuven, the Fund for Scientific Research-Flanders, and the European Commission project (FAIR-CT96-1175).

LITERATURE CITED

Barret, D. M.; Theerakulkait, C. Quality Indicators in Blanched, Frozen, Stored Vegetables. *Food Technol.* **1995**, *49*, 62,64−65.

Cheftel, J. C. Applications des Hautes Pressions en Technologie Alimentaire. *Ind. Aliment. Agric.* **1991**, *108*, 141−153.

Günes, B.; Bayindirli, A. Peroxidase and Lipoxygenase Inactivation During Blanching of Green Beans, Green Peas and Carrots. *Lebens.-wiss. Technol.* **1993**, *26*, 406−410.

Hawley, S. A. Reversible Pressure−Temperature Denaturation of Chymotrypsinogen. *Biochemistry* **1971**, *10*, 2436−2442.

Hashizume, C.; Kimura, K.; Hayashi, R. Kinetic Analysis of Yeast Inactivation by High-Pressure Treatment at Low Temperatures. *Biosci. Biotechnol. Biochem.* **1995**, *59(8)*, 1455−1458.

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.

Heremans, K. From Living Systems to Biomolecules. In *High Pressure and Biotechnology*; Balny, C., Hayashi, R., Heremans, K., Masson, P., Eds.; John Libbey Eurotext Ltd.: Montrouge, France, 1992; Colloque INSERM 224, pp 37−44.

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

Indrawati; Van Loey, A. M.; Ludikhuyze, L. R.; Hendrickx, M. E. Soybean Lipoxygenase Inactivation by Pressure at Subzero and Elevated Temperatures. *J. Agric. Food Chem.* **1999a**, *47 (6)*, 2468−2474.

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.* **1999b**, *15 (2)*, 273−277.

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.

Knorr, D. Effects of High-Hydrostatic-Pressure Processes on Food Safety and Quality. *Food Technol.* June **1993**, 156−161.

Ludikhuyze, L.; Indrawati; Van den Broeck, I.; Weemaes, C.; Hendrickx, M. Effect of Combined Pressure and Temperature on Soybean Lipoxygenase. 2. Modeling Inactivation Kinetics under Static and Dynamic Conditions. *J. Agric. Food Chem*. **1998**, *46*, 4081−4086.

Lund, D. B. Design of thermal processes for maximizing nutrient retention. *Food Technol*. **1977**, *31 (2)*, 71−78.

Morild, E. The Theory of Pressure Effects on Enzymes. *Adv. Protein Chem*. **1981**, *34*, 93−166.

Park, K. H.; Kim, Y. M.; Lee, C. W. Thermal Inactivation Kinetics of Potato Tuber Lipoxygenase. *J. Agric. Food Chem*. **1988**, *36*, 1012−1015.

SAS Institute Inc. *SAS user's guide: Statistics*. SAS Institute Inc. Cary, USA, 1995.

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

Sheu, S. C.; Chen, A. O. Lipoxygenase as Blanching Index for Frozen Vegetable Soybeans. *J. Food Sci*. **1991**, *56 (2)*, 448−451.

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

Van den Broeck, I.; Ludikhuyze, L. R.; Hendrickx, M. E. Inactivation of Orange Pectinesterase by Combined High Pressure and Temperature Treatments: A Kinetic Study. Submitted for publication in *J. Agric. Food Chem*. **1999**.

Weemaes, C. A.; Ludikhuyze, L. R.; Van den Broeck, I.; Hendrickx, M. E. Kinetics of Combined Pressure−Temperature Inactivation of Avocado Polyphenoloxidase. *Biotechnol. Bioeng*. **1998**, *60 (3)*, 292−300.

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*, 130−140.

Received for review August 23, 1999. Revised manuscript received February 10, 2000. Accepted February 23, 2000.

JF990937N