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High pressure inactivation of lipoxygenase in soy milk and crude soybean extract

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Abstract

The high pressure inactivation of lipoxygenase (LOX) in soy milk and crude soybean extract was studied in the pressure range 0.1–650 MPa with temperature varying from 5 to 60 °C. For both systems, the isobaric–isothermal inactivation of LOX was irreversible and followed a first-order reaction at all pressure–temperature combinations tested. In the entire pressure–temperature area studied, the LOX inactivation rate constants increased with increasing pressure at constant temperature for both systems; the rate constants were somewhat smaller in soy milk system than in crude soybean extract. At constant elevated pressure, LOX exhibited the greatest stability around 20 °C in both systems, indicating that the Arrhenius equation was not valid over the entire temperature range. For both systems, the temperature dependence of the LOX inactivation rate constants at high temperature decreased with increasing pressure, while the highest sensitivity of the LOX inactivation rate constants to pressure was observed at about 30 °C. The pressure–temperature dependence of the LOX inactivation rate constants was successfully described either using an empirical mathematical model or using a thermodynamic kinetic model for both systems. On a kinetic basis, neither the reaction order of inactivation nor the pressure and temperature sensitivities of the inactivation rate constants were influenced by the different levels of food complexity between the two systems.

Keywords: Lipoxygenase; Inactivation; Soybean; Pressure; Kinetics

1. Introduction

Lipoxygenase (LOX), an iron-containing dioxygenase which can catalyse the oxidation of polyunsaturated fatty acids containing *cis*,*cis*-1,4-pentadiene units to the corresponding conjugated hydroperoxydiene derivatives by the addition of molecular oxygen (Bhirud & Sosulski, 1993; Indrawati, Loey, Ludikhuyze, & Hendrickx, 2000; Lopez & Burgos, 1995; Shook, Shellhammer, & Schwartz, 2001; Sonati & Appu Rao, 1995), is widely distributed in plant tissues, especially in legumes and vegetables (Ludikhuyze, Indrawati, Broeck, Weemaes, & Hendrickx, 1998a; Zhang, Cavalieri, Powers, & Wu, 1991). Its enzymatic reaction can result in some undesirable effects such as the destruction of essential fatty acids, the development of off-flavours and colour degradation (Ludikhuzye, Indrawati, Broeck, Weemaes, & Hendrickx, 1998b). Therefore, it must be destroyed by blanching before vegetable drying or freezing. The term blanching most often designates heat treatment because of its capacity to inactivate enzymes and destroy microorganisms. However, thermal treatment simultaneously causes some decrease in food quality aspects. These include loss in texture and nutritional quality, formation of a cooked taste, change in colour and loss of soluble solids (Castro, Loey, Saraiva, Smout, & Hendrickx, 2006a, 2006b; Weemaes, Lidikhuyze, Broeck, & Hendrickx, 1998a, Weemaes, Ludikhuyze, Broeck, & Hendrickx, 1998b). Hence, in the context of improving food quality retention, new techniques such as high pressure treatment have drawn much attention in food processing and preservation (Denys, Loey, & Hendrickx, 2000).

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High pressure has been shown to inactivate vegetative microorganisms and some food quality-related enzymes while leaving most quality parameters such as colour, flavour, and nutrient content unaffected (Kowalczyk et al., 2005; Tangwongchai, Ledward, & Ames, 2000; Weemaes et al., 1997). The studied enzymes include polyphenoloxidase (PPO) (Dalmadi, Rapeanu, Loey, Smout, & Hendrickx, 2006; Rapeanu, Loey, Smout, & Hendrickx, 2005a, Rapeanu, Loey, Smout, & Hendrickx, 2005b, 2006), pectin methylesterase (PME) (Castro et al., 2006a, Castro, Loey, Saraiva, Smout, & Hendrickx, 2006b; Sila et al., 2007), polygalacturonase (Fachin, Loey, Indrawati, Ludikhuyze, & Hendrickx, 2002; Rodrigo, Cortes et al., 2006), myrosinase (Eylen, Indrawati, Hendrickx, & Loey, 2006; Ludikhuyze, Ooms, Weemaes, & Hendrickx, 1999), peroxidase (POD) (Seyderhelm, Boguslawski, Michaelis, & Knorr, 1996), LOX and so on. But most kinetic studies regarding the combined high pressure and temperature effect on enzyme inactivation have concentrated on simple model systems, i.e., use of commercial available purified enzymes dissolved in buffer solutions. As a consequence, the intrinsic complexity of food products could not be taken into account and the investigations of simple model systems might have limitations (Indrawati, Ludikhuyze, Loey, & Hendrickx, 2000). As for soybean LOX, it has been previously studied kinetically by Ludikhuyze et al. (1998a) and Indrawati, Loey, Ludikhuyze, and Hendrickx (1999a, 1999b) in buffer solution. However, the kinetic studies in real food systems, such as in soy milk and in crude soybean extract, are completely lacking. Thus, in the present paper, the inactivation of soybean LOX by combined pressure and temperature were investigated in real foodstuffs, namely in soy milk and in crude soybean extract. The purpose of this work was to investigate the kinetic attributes of LOX inactivation in real food systems due to high pressure treatment, and to compare them with the results of the previous research on kinetic inactivation of LOX in simple model systems.

2. Materials and methods

Soybeans (Nannong 99-10) of similar age and freshness, harvested in October 2005, were provided by the National Centre of Soybean Improvement, Ministry of Agriculture, Nanjing Agricultural University. Linoleic acid was obtained from Sigma (Beijing, China) and other chemicals used were of reagent grade.

2.1. Soy milk and crude soybean extract preparation

Soybeans were thoroughly washed and soaked overnight in distilled water at room temperature ($w/v = 1{:}10$). Then the soaked soybeans together with the soaking water were ground in a blender (JYL-360, Joyoung Household Electrical Appliances Company, Jinan, China) for 5 min. To get soy milk the pulp was removed by filtration using a nylon cloth. Crude soybean extract was obtained by centrifuging the soy milk for 30 min at 4 °C and 30,000 × *g* (Beckman Avanti J-26 XPI, Beckman Coulter Inc.). Crude soybean extract and soy milk was kept refrigerated until processing.

2.2. Thermal treatment at ambient pressure

Isothermal inactivation experiments were performed in a thermostatic water bath at constant temperature. To insure direct heating and direct cooling, soy milk or crude soybean extract (about 10 ml) was sealed in polyethylene bags (7 × 18 cm), with no headspace or air bubbles, and the samples were placed in the water bath and the heating time was exactly measured using a stopwatch. Immediately after withdrawal from the water bath, the samples were transferred to an ice bath for rapid cooling and then stored at 4 °C until the residual LOX activity was measured.

2.3. Combined thermal and high pressure treatment

Isobaric–isothermal inactivation treatments were carried out in a high pressure rig (UHPF-800MPa-3L, 52 Institute, Baotou Neimeng, China) with a maximum pressurization capacity of 800 MPa. The pressure-transferring liquid used was an oil, bis(2-ethylhexyl)sebacate (Li-Dong Precision Machinery Company, Shenzhen, China). The pressure chamber (3 l, 12 cm inner diameter), surrounded by a thermostatic jacket connected to a water bath, could be heated or cooled to the desired level of temperature before pressurization.

Soy milk or crude soybean extract (about 10 ml) was sealed in polyethylene bags without air bubbles and then the samples were placed in the pressure chamber for 2 min to equilibrate the sample temperature before high pressure treatment. Pressurization rate was standardized at 125 MPa/min. After achieving the desired pressure, an equilibrium course of 3 min was needed to ensure isobaric–isothermal conditions, then the treatment time was recorded and the chamber was decompressed when the desired treatment time was reached. The pressure release course was less than 3 s. After withdrawal from the chamber, samples were immediately transferred to an ice bath for rapid cooling and then stored at 4 °C until the residual LOX activity was measured. Isobaric–isothermal inactivation of LOX was studied in a pressure range 200–650 MPa and a temperature range from 5 to 60 °C.

2.4. Lipoxygenase activity measurement

Before LOX activity analysis, soy milk samples were centrifuged for 30 min at 4 °C at 30,000 × *g* and the supernatants were used for further testing. Lipoxygenase activity was determined using a continuous spectrophotometric method (UV-3802, Unico Instrument, Shanghai, China), based on the enzymatic oxidation of linoleic acid to the corresponding hydroperoxide. Linoleic acid solution was prepared by adding: 0. 01 ml of linoleic acid and 0.01 ml

of the emulsifier Tween 20 to 4 ml of 0.2 M borate buffer, pH 9.0 at 25 °C. After homogenisation, 0.5 N NaOH was added to clear the solution. Finally, the solution was made up with the same borate buffer to a volume of 60 ml. The solution was stored frozen until used. The reaction was carried out at 25 °C in a quartz cuvette. The assay mixture contained 0.1 ml of enzyme solution, 2.0 ml of substrate solution, and 0.9 ml of 0.2 M borate buffer, pH 9.0 at 25 °C. The absorption at 234 nm was recorded as a function of time (3 min), and the activity was determined from the slope of the linear portion of the curve. The linear relationship between measured activity and enzyme concentration was verified in a preliminary test.

2.5. Data analysis

Literature data (Ludikhuzye et al., 1998b; Park, Kim, & Lee, 1988) showed that both isothermal and isobaric–isothermal inactivation of soybean LOX, either in simple model systems or in real food systems, could be described as a first-order reaction (Eq. (1)). For constant extrinsic (pressure and temperature) and intrinsic factors, the inactivation rate constant (k) is independent of time (t) and Eq. (1) can be integrated to Eq. (2):

$$dA/dt = -kA \quad (1)$$

$$\ln(A/A_0) = -kt \quad (2)$$

On the basis of linear regression analysis (SAS, 2001), 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.

Arrhenius (Eq. (3)) and Eyring (Eq. (4)) relations are frequently applied to estimate the temperature and pressure dependence of the LOX inactivation rate constants, as expressed by E_a (activation energy) and V_a (activation volume) values, respectively. Activation energy (E_a) and activation volume (V_a) values could also be determined based on a linear regression analysis of the natural logarithm of the inactivation rate constant *versus* the reciprocal of absolute temperature, or the pressure, respectively:

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

$$\ln(k) = \ln(k_{refP}) - \frac{V_a}{R_P T}(P - P_{ref}) \quad (4)$$

3. Results and discussion

3.1. Isothermal inactivation kinetics of soybean LOX

Thermal stability of soybean LOX either in soy milk or in crude soybean extract was studied over the temperature range 63–71 °C. Over this temperature range, isothermal inactivation of soybean LOX followed first-order kinetics, allowing inactivation rate constants (k) to be determined from plots of the natural logarithm of relative residual activity, as a function of inactivation time. The estimated k values, together with standard errors and regression coefficients, are summarised in Table 1. Over the entire temperature domain studied, LOX was less thermostable in crude soybean extract than in soy milk and the temperature sensitivity of the rate constants for LOX inactivation in both systems could be estimated using the Arrhenius relation. First-order kinetics for thermal inactivation of soybean LOX have been frequently reported in the literature (Indrawati et al., 1999b; Ludikhuzye et al., 1998b). Ludikhuyze et al. (1998a, 1998b) investigated the thermal inactivation kinetics of commercial soybean LOX in Tris–HCl buffer (0.01 M, pH 9) at two different concentrations (0.4 and 5 mg/ml) over the temperature range 60–70 °C. Compared to their results (Table 2), the LOX in our studies, either in soy milk or in crude soybean extract, exhibited a higher thermal stability with the corresponding smaller inactivation rate constants. The two activation energy values derived from the plots of the natural logarithm of inactivation rate constants, as a function of the reciprocal of the absolute temperature were larger, pointing to higher temperature sensitivity of the k values. Likewise, kinetic inactivation of LOX from many different sources, such as green peas, green beans, potatoes, asparagus, wheat germ, and germinated barley, have also been studied (Bhirud & Sosulski, 1993; Ganthavorn, Nagel, & Powers, 1991; Guenes & Bayindirli, 1993; Hugues et al., 1994; Indrawati et al., 1999a; Park et al., 1988; Svensson & Eriksson, 1974). Indrawati et al. (1999a, 1999b) reported that thermal inactivation of LOX in green bean juice could be described by a two-fraction first-order inactivation model, referring to the existence of two fractions (isozymes) with different thermal stability. However, in our study, this phenomenon was not observed.

Table 1
Estimated inactivation rate constants ($\times 10^{-2}$ min^{-1}) for the isothermal inactivation of LOX in soy milk and in crude soybean extract

T (°C)	Soy milk	Crude soybean extract
63	0.55 ± 0.02[a] $r^2 = 0.993$	0.68 ± 0.03 $r^2 = 0.995$
65	1.35 ± 0.05 $r^2 = 0.995$	1.54 ± 0.05 $r^2 = 0.996$
67	3.57 ± 0.09 $r^2 = 0.998$	4.31 ± 0.12 $r^2 = 0.997$
69	13.25 ± 0.50 $r^2 = 0.994$	14.43 ± 0.42 $r^2 = 0.997$
71	47.72 ± 4.64 $r^2 = 0.972$	53.40 ± 2.74 $r^2 = 0.987$
E_a (kJ/mol)	538.78 ± 29.04 $r^2 = 0.991$	526.94 ± 29.54 $r^2 = 0.991$

[a] Standard error.

Table 2
Inactivation rate constants ($\times 10^{-2}$ min^{-1}) for the isothermal inactivation of commercial soybean LOX in 0.01 M, pH 9 Tris–HCl buffer

T (°C)	Lipoxygenase concentration	
	0.4 mg/ml[b]	5 mg/ml[c]
60		2.09 ± 0.13 $r^2 = 0.978$
62	2.02 ± 0.09[a] $r^2 = 0.987$	4.86 ± 0.21 $r^2 = 0.991$
64	4.94 ± 0.16 $r^2 = 0.993$	10.8 ± 0.61 $r^2 = 0.984$
66	9.18 ± 0.32 $r^2 = 0.992$	29.1 ± 3.37 $r^2 = 0.949$
68	15.5 ± 0.52 $r^2 = 0.992$	
E_a (kJ/mol)	319.8 ± 27.3 $r^2 = 0.986$	408.2 ± 14.7 $r^2 = 0.997$

[a] Standard error.
[b] Ludikhuyze et al. (1998b).
[c] Ludikhuyze et al. (1998a).

3.2. Isobaric–isothermal inactivation kinetics of soybean LOX

Isobaric–isothermal inactivation of LOX either in soy milk or in crude soybean extract was studied over the pressure range 200–650 MPa at temperatures between 5 and 60 °C. To ensure the achievement of isobaric–isothermal conditions, inactivation of LOX in both systems was studied after an equilibration time of 3 min. Under all experimental conditions studied, it was noticed that isobaric–isothermal inactivation of LOX in both systems was irreversible. By analogy with isothermal inactivation, first-order inactivation kinetics were assumed, in order to analyse isobaric–isothermal inactivation of LOX in both systems and verified on semilogarithmic plots of relative residual activity, as a function of inactivation time (Fig. 1). The estimated inactivation rate constants of LOX, together with standard errors, both in soy milk and in crude soybean extract, are summarised in Tables 3 and 4, respectively. Regression coefficients varied between 0.950 and 0.999. From Tables 3 and 4, it could be noticed that at constant temperature, the LOX inactivation rate in both systems was enhanced by increasing pressure, which is consistent with the former reports on the inactivation of green bean LOX (Indrawati, Loey et al., 2000; Indrawati, Ludikhuyze, et al., 2000) and *Bacillus subtilis* α-amylase (Ludikhuyze et al., 1997). In addition at constant pressure, minimal inactivation rate constants were found at about 20 °C in both systems, so that either a temperature increase (above 20 °C) or decrease (below 20 °C) could accelerate the inactivation. Furthermore, the barostability of LOX was somewhat lower in crude soybean extract than in soy milk, i.e., a slightly lower inactivation rate constant of soy milk was obtained in all pressure–temperature combinations tested, which was also confirmed by the result ($P < 0.0001$) of a two sample paired t-test of the means of both data sets. In our opinion, this phenomenon might be attributed to the different food constituents between the two systems, for example, contents of fat, protein, etc. There have been some reports (Castro, Loey, Saraiva, Smout, & Hendrickx, 2005; Indrawati, Loey et al., 2000; Indrawati, Ludikhuyze, et al., 2000; Rapeanu, Loey, Smout, & Hendrickx, 2006; Ven, Matser, & Berg, 2005; Yu-Long, Xing-Rong, & Han-Hu, 2006) about the effect of food constituents on high pressure inactivation of some food quality related enzymes and bacteria. Ven et al. (2005) reported that matrix differences between intact soybeans and soy milk could possibly explain the lower LOX inactivation in whole soybeans compared to soy milk by high pressure treatment. Yu-Long et al. (2006) found that soybean protein and sucrose significantly affected inactivation of *Staphylococcus aureus* in milk by high pressure and mild heat treatment. Indrawati, Loey et al. (2000) and Indrawati, Ludikhuyze, et al. (2000), who kinetically studied the isobaric–isothermal inactivation of LOX both in green bean juice and in intact green beans (*in situ* study), pointed out that LOX was less pressure stable *in situ* than in juice in the pressure–temperature area of 50 up to 650 MPa and −10 up to 70 °C. They also reported that 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, which were significantly influenced by food constituents.

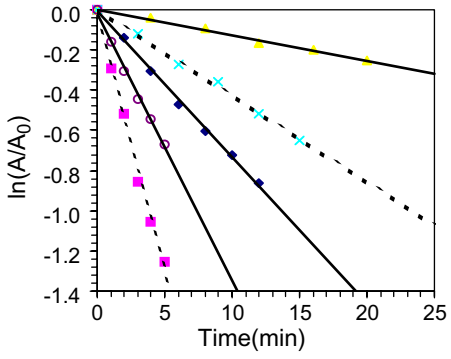

Fig. 1. Inactivation of LOX in soy milk (—) and in crude soybean extract (---) at atmospheric and elevated pressures: (▲) 20 °C and 550 MPa; (×) 67 °C and 0.1 MPa; (♦) 50 °C and 425 MPa; (○) 69 °C and 0.1 MPa; (■) 10 °C and 650 MPa.

Pressure dependence of the inactivation rate constants for both systems could be described accurately by the Eyring equation (Eq. (4)). Activation volumes together with standard errors and regression coefficients are reported in Table 5. For both systems, the activation volumes were negative at all temperatures studied, indicating an acceleration of the LOX inactivation by increasing pressure, and the absolute value of the activation volumes had a maximum at about 30 °C, pointing to the highest sensitivity of

Table 3
Estimated inactivation rate constants ($\times 10^{-2}$ min^{-1}) for the isobaric–isothermal inactivation of LOX in soy milk

P (MPa)	T (°C)								
	5	10	20	30	35	40	45	50	60
225									3.63 ± 0.27
250									6.78 ± 0.72
275									10.78 ± 1.26
300									15.69 ± 0.50
325									26.94 ± 1.36
400								3.18 ± 0.30	
425								7.25 ± 0.18	
450							5.59 ± 0.27	13.90 ± 1.09	
475						2.10 ± 0.19	9.67 ± 0.53	22.63 ± 1.20	
500					1.99 ± 0.06	4.04 ± 0.44	18.21 ± 1.08	43.78 ± 4.51	
525				1.13 ± 0.08	4.67 ± 0.30	10.75 ± 0.61	39.70 ± 1.09		
550	1.56 ± 0.05[a]	1.35 ± 0.13	1.29 ± 0.04	2.32 ± 0.10	9.02 ± 0.43	16.15 ± 1.02	72.38 ± 2.87		
575	2.97 ± 0.15	2.54 ± 0.18	2.42 ± 0.08	5.26 ± 0.31	18.74 ± 0.52	37.79 ± 3.18			
600	5.77 ± 0.33	5.67 ± 0.28	5.30 ± 0.21	16.06 ± 1.91	38.04 ± 1.77				
625	12.33 ± 0.80	10.85 ± 0.64	13.71 ± 0.91	26.72 ± 1.07					
650	26.37 ± 1.33	24.26 ± 1.20	26.64 ± 1.01						

[a] Standard error.

Table 4
Estimated inactivation rate constants ($\times 10^{-2}$ min^{-1}) for the isobaric–isothermal inactivation of LOX in crude soybean extract

P (MPa)	T(°C)								
	5	10	20	30	35	40	45	50	60
225									3.84 ± 0.22
250									7.02 ± 0.25
275									11.27 ± 1.12
300									16.55 ± 1.42
325									28.90 ± 1.06
400								3.57 ± 0.06	
425								8.12 ± 0.59	
450							6.01 ± 0.22	16.08 ± 0.97	
475						2.48 ± 0.30	10.68 ± 0.16	27.81 ± 2.93	
500					2.24 ± 0.17	4.84 ± 0.44	19.33 ± 0.68	52.17 ± 5.07	
525				1.46 ± 0.07	5.21 ± 0.11	11.80 ± 0.42	40.65 ± 2.25		
550	1.71 ± 0.09[a]	1.54 ± 0.12	1.50 ± 0.12	2.78 ± 0.11	9.36 ± 0.53	18.65 ± 1.49	77.92 ± 4.22		
575	3.47 ± 0.14	3.03 ± 0.22	2.59 ± 0.18	6.81 ± 0.48	20.24 ± 0.67	43.25 ± 4.63			
600	7.34 ± 0.37	6.95 ± 0.44	5.41 ± 0.36	18.10 ± 1.00	41.34 ± 1.05				
625	13.89 ± 1.03	11.49 ± 0.45	13.94 ± 1.19	33.68 ± 1.18					
650	28.78 ± 1.71	25.44 ± 1.07	27.56 ± 1.91						

[a] Standard error.

LOX inactivation rate constants to pressure at this temperature. Similar results have been reported for some other food quality related enzymes, e.g., tomato LOX (Rodrigo, Jolie, Loey, & Hendrickx, 2006) and avocado polyphenol oxidase (Weemaes et al., 1998a, 1998b). However, Indrawati, Ludikhuyze, et al. (2000) studied LOX inactivation in green bean juice due to combined pressure and temperature treatment and found that the absolute value of the activation volume first decreased with increasing temperature up to 30 °C and then increased again (Table 5), suggesting enhanced pressure sensitivity at high temperature. From Table 5, we also found that, in our study, the value of the activation volumes in both systems were closer at all temperatures tested. A two sample paired t-test for the means of both data sets proved that the pressure sensitivity of the inactivation rate constants was not influenced by the difference between the two systems, in contrast to the previous study of the LOX inactivation in green beans by Indrawati, Ludikhuyze, et al. (2000).

At elevated pressure, the minimal LOX inactivation rate constants for both systems were at about 20 °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. Therefore, the Arrhenius equation (Eq. (3)) could not be applied directly to both systems to determine the temperature dependence of the inactivation rate constants in the entire temperature domain studied. To apply the Arrhenius equation, the temperature area was divided into two parts, namely, a low temperature area ($\leqslant$20 °C) and a high temperature area ($\geqslant$20 °C). The estimated activation energy values for both systems in the high temperature area are given in Table 6. The activation energy

Table 5
Estimated activation volume (cm^3/mol) of LOX inactivation at various constant temperatures

T (°C)	V_a (cm^3/mol)		
	Green bean juice[b]	Soy milk system[c]	Crude soybean extract[c]
−10	-43.30 ± 5.78[a] $r^2 = 0.966$		
0	-42.59 ± 4.86 $r^2 = 0.975$		
5		-65.49 ± 1.52 $r^2 = 0.998$	-65.07 ± 0.82 $r^2 = 0.999$
10	-37.49 ± 5.03 $r^2 = 0.966$	-68.09 ± 1.58 $r^2 = 0.998$	-65.38 ± 2.42 $r^2 = 0.996$
20	-33.61 ± 4.27 $r^2 = 0.968$	-75.96 ± 2.90 $r^2 = 0.996$	-73.18 ± 3.59 $r^2 = 0.993$
30	-14.75 ± 2.62 $r^2 = 0.941$	-83.30 ± 4.81 $r^2 = 0.990$	-82.19 ± 3.60 $r^2 = 0.994$
35		-74.73 ± 1.69 $r^2 = 0.998$	-73.68 ± 1.98 $r^2 = 0.998$
40	-16.68 ± 2.53 $r^2 = 0.935$	-74.64 ± 4.50 $r^2 = 0.989$	-73.61 ± 3.51 $r^2 = 0.993$
45		-69.15 ± 2.25 $r^2 = 0.997$	-68.38 ± 1.90 $r^2 = 0.998$
50	-38.62 ± 5.44 $r^2 = 0.962$	-68.61 ± 3.44 $r^2 = 0.992$	-70.89 ± 3.09 $r^2 = 0.994$
60		-53.72 ± 2.54 $r^2 = 0.993$	-54.24 ± 2.24 $r^2 = 0.995$

[a] Standard error.
[b] Indrawati, Ludikhuyze, et al. (2000).
[c] This study.

Table 6
Estimated activation energy (kJ/mol) of LOX inactivation in high temperature area

P (MPa)	E_a (kJ/mol)	
	Soy milk system	Crude soybean extract
475	200.29 ± 31.06[a] $r^2 = 0.976$	203.57 ± 22.59 $r^2 = 0.988$
500	178.36 ± 17.83 $r^2 = 0.980$	179.16 ± 14.04 $r^2 = 0.988$
525	184.64 ± 12.02 $r^2 = 0.992$	173.15 ± 9.93 $r^2 = 0.993$
550	122.74 ± 22.32 $r^2 = 0.910$	120.40 ± 21.24 $r^2 = 0.915$
575	106.61 ± 19.78 $r^2 = 0.936$	108.04 ± 14.66 $r^2 = 0.964$
600	96.10 ± 12.59 $r^2 = 0.983$	99.88 ± 9.45 $r^2 = 0.991$

[a] Standard error.

values in the low temperature area could not be estimated, since the number of observations was too low, due to the limited temperature control capacity of the machine. From Table 6, it can be noticed that both in soy milk or in crude soybean extract, there exists a tendency of the temperature sensitivity of LOX inactivation rate constants to decrease with increasing pressure in the high temperature area. Also at some pressure levels (e.g., 550 and 575 MPa), the Arrhenius equation was less appropriate to describe the temperature dependence of the inactivation rate constants, as indicated by a lower correlation coefficient. Therefore, extrapolation of the rate constants to higher or lower temperatures should be avoided, especially at these pressure levels. In addition, temperature sensitivity of the inactivation rate constants for LOX in soy milk is similar to that in crude soybean extract at all temperatures discussed.

4. Formulation of mathematical models

Mathematical models based on different equations have been formulated to describe the pressure–temperature dependence of the inactivation rate constants for several enzymes. Weemaes et al. (1998b) described pressure–temperature inactivation of avocado polyphenol oxidase using the Arrhenius equation as a starting point for kinetic modeling because this equation is valid at all pressures studied, while Ludikhuyze et al. (1998a) selected the Eyring equation as a starting point to describe pressure–temperature inactivation of lipoxygenase because in their studies the Eyring equation, not the Arrhenius, was valid at all pressure–temperature combinations studied. Moreover, some authors (Indrawati et al., 1999b) modified the thermodynamic model proposed by Hawley (1971) into a kinetic version and thus developed a thermodynamic kinetic model (Eq. (5)), which could describe pressure–temperature dependence of the inactivation rate constants when neither of the former equations was valid at all pressure–temperature combinations studied. In our studies, both the kinetic model using the Eyring equation as a starting point and the thermodynamic kinetic model were used to fit the experimentally obtained data on pressure–temperature inactivation of soybean LOX in both systems. For the kinetic model which used the Eyring equation as a starting point, temperature dependence of the inactivation rate constant and activation volume at a reference pressure of 550 MPa could be described by Eqs. (6) and (7), respectively. Implementation of these mathematical expressions into the Eyring equation allows transformation to Eq. (8):

$$\ln(k) = \ln(k_0) - \frac{\Delta V_0^{\neq}}{R_T T}(P - P_0) + \frac{\Delta S_0^{\neq}}{R_T T}(T - T_0) - \frac{1}{2}\frac{\Delta \kappa^{\neq}}{R_T T}(P - P_0)^2 - 2\frac{\Delta \zeta^{\neq}}{R_T T}(P - P_0)(T - T_0) + \frac{\Delta C_P^{\neq}}{R_T T}\left[T\left(\ln\frac{T}{T_0} - 1\right) + T_0\right] \quad (5)$$

$$\ln k_{refP} = a_1(T - 273.15)^2 + b_1(T - 273.15) + c_1 \quad (6)$$

$$V_a = d_1(T - 273.15)\exp[e_1(T - 273.15)] \quad (7)$$

$$\ln k = a_1(T - 273.15)^2 + b_1(T - 273.15) + c_1 - \frac{d_1(T - 273.15)\exp[e_1(T - 273.15)]}{RT}(P - P_{ref}) \quad (8)$$

The parameters of both models were estimated using nonlinear regression analysis, involving an iterative numerical procedure based on the minimal sum of squares. The estimated model parameters are summarised in Table 7. The better quality of fitting of Eq. (5) demonstrated that the thermodynamic kinetic model could describe the pressure–temperature dependence of soybean LOX inactivation in both systems more accurately than the empirical mathematical model. The good agreement between the natural logarithm of experimentally determined inactivation rate constants and the natural logarithm of the rate constants predicted on the basis of Eq. (5) for both systems is depicted in Fig. 2. Weemaes et al. (1998b) found a systematic tendency of residuals *versus* pressure when using Eq. (5) to describe pressure–temperature inactivation of avocado polyphenol oxidase, while no trend in residuals was noticed as a function of temperature, pressure, or experimental inactivation rate constants in our regressions. Indrawati, Ludikhuyze, et al. (2000) used the same thermodynamic kinetic model to describe LOX inactivation in green beans, due to high pressure treatment at subzero and elevated temperature. However, they got very large $\Delta V_0^{\neq}$ (-308.14 ± 24.19 cm^3/mol) values which were about five times those we have obtained and about 10 times the estimated V_a values in their same article. In our opinion, it is due to the misuse of different pressure units in their regressions.

By inserting the corresponding estimated model parameters (Table 7) into Eq. (5), pressure–temperature combinations resulting in a specific preset inactivation rate constant for LOX in soy milk were predicted and are depicted in Fig. 3. The upper line's k value of 0.23 min^{-1} means that 90% of the LOX in soy milk could be inactivated in 10 min under these corresponding pressure–temperature combinations, while the lower line's 0.115 min^{-1} means that inactivation time would be 20 min. From this point of view, the development of mathematical models for high pressure inactivation of LOX in soy milk might be useful for industrial application.

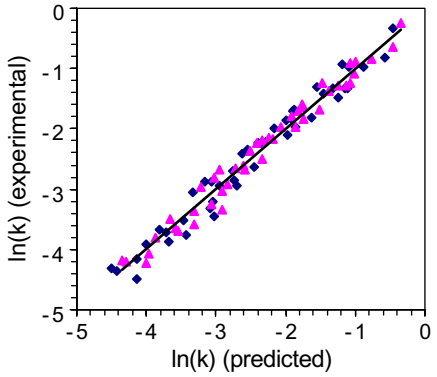

Fig. 2. Relationship between the natural logarithm of experimental determined inactivation rate constants for LOX in soy milk (♦) and in crude soybean extract (▲) and the natural logarithm of inactivation rate constants, predicted according to Eq. (5).

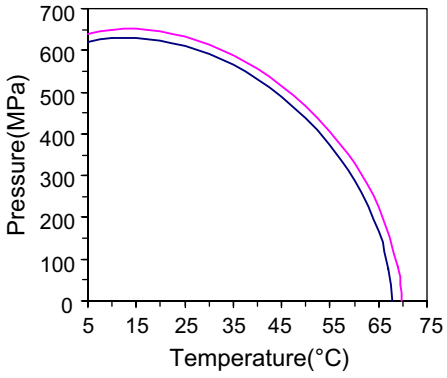

Fig. 3. Combined predicted pressure–temperature combination of the same inactivation rate constant for LOX in soy milk. The upper line indicates a k value of 0.23 min^{-1}, and the lower line indicates a k value of 0.115 min^{-1}.

Table 7
Estimated model parameters for LOX inactivation in soy milk and crude soybean extract based on Eqs. (5)–(8) at reference pressure 55000 N cm^{-2} and reference temperature 293 K

Parameter	Estimated value							
	Soy milk system				Crude soybean extract			
	Eq. (5)	Eq. (6)	Eq. (7)	Eq. (8)	Eq. (5)	Eq. (6)	Eq. (7)	Eq. (8)
k_0 ($\times 10^{-2}$)	1.19 ± 0.08[a]				1.37 ± 0.08			
$\Delta V_0^{\neq}$	-68.07 ± 2.76				-66.69 ± 2.31			
$\Delta S_0^{\neq}$	134.80 ± 10.92				130.80 ± 9.16			
$\Delta\kappa^{\neq}$	-0.16 ± 0.04				-0.15 ± 0.03			
$\Delta\zeta^{\neq}$	-0.22 ± 0.11				-0.24 ± 0.09			
$\Delta C_P^{\neq}$	6867.10 ± 440.00				6893.70 ± 369.30			
a_1 ($\times 10^{-3}$)		3.30 ± 0.36		4.62 ± 0.38		3.29 ± 0.32		4.65 ± 0.36
b_1 ($\times 10^{-2}$)		-7.82 ± 2.30		-15.56 ± 1.94		-7.67 ± 2.09		-15.84 ± 1.83
c_1		-3.94 ± 0.32		-2.86 ± 0.22		-3.83 ± 0.29		-2.68 ± 0.21
d_1			-10.30 ± 1.34	-6.61 ± 0.63			-9.78 ± 1.39	-6.26 ± 0.57
e_1 ($\times 10^{-2}$)			-4.26 ± 0.37	-2.97 ± 0.21			-4.14 ± 0.40	-2.86 ± 0.20
Quality of fitting								
Cor r^2	0.994	0.986	0.978	0.984	0.996	0.989	0.975	0.985

[a] Asymptotic standard error.

5. Conclusion

The advantages of preforming isobaric–isothermal inactivation experiments on enzymes in real foodstuffs is multifold. First, it allows an insight into the effect of intrinsic food complexity on enzyme inactivation, by comparing a real foodstuff with a simple enzymatic model system. Second, the kinetic information obtained in a real foodstuff, which can then be directly applied to the optimisation of the parameters in a high pressure treatment used by the food industry, might be more useful for industrial application than a simple enzymatic model system. Finally, since the chamber has a capacity of 3 l and every sample treated was about 10 ml, the impact of some processing factors, e.g., nonuniformity in the high pressure chamber or in the samples, on the kinetics of enzyme inactivation, could be considered.

Irreversible inactivation of LOX either in soy milk or in crude soybean extract could be realised by a combined thermal and high pressure treatment. Maximal stability of LOX in both systems was observed at around 20 °C at elevated pressures. In the entire pressure–temperature domain studied (250–650 MPa and 5–60 °C), LOX was less pressure-stable in crude soybean extract than in soy milk. On a kinetic basis, neither the reaction order of inactivation nor the pressure and temperature sensitivities of the inactivation rate constants were influenced by the different levels of food complexity between the two systems. Moreover, the pressure–temperature dependence of LOX inactivation rate constants for both systems could be described by either the thermodynamic kinetic model or the empirical mathematical model which used the Eyring equation as a starting point, and the former was more accurate than the latter in our studies.

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