Kinetic Study of the Irreversible Thermal and Pressure Inactivation of Myrosinase from Broccoli (*Brassica oleracea* L. Cv. Italica)

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Thermal and pressure inactivation of myrosinase from broccoli was kinetically investigated. Thermal inactivation proceeded in the temperature range 30−60 °C. These results indicate that myrosinase is rather thermolabile, as compared to other food quality related enzymes such as polyphenol oxidase, lipoxygenase, pectinmethylesterase, and peroxidase. In addition, a consecutive step model was shown to be efficient in modeling the inactivation curves. Two possible inactivation mechanisms corresponding to the consecutive step model were postulated. Pressure inactivation at 20 °C occurred at pressures between 200 and 450 MPa. In addition to its thermal sensitivity, the enzyme likewise is rather pressure sensitive as compared to the above-mentioned food quality related enzymes. By analogy with thermal inactivation, a consecutive step model could adequately describe pressure inactivation curves. At 35 °C, pressure inactivation was studied in the range between 0.1 and 450 MPa. Application of low pressure (<350 MPa) resulted in retardation of thermal inactivation, indicating an antagonistic or protective effect of low pressure.

Keywords: *Thermal inactivation; pressure inactivation; myrosinase; broccoli*

INTRODUCTION

Myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) is found in all glucosinolate-containing plants, especially in Cruciferae. Many of these species are frequently used in the human diet either as vegetables or condiments (cabbage, Brussels sprouts, radish, turnip, watercress, mustard). Myrosinase catalyzes the hydrolysis of glucosinolates, a group of sulfur-containing pseudo-glycosides. This hydrolysis results in the formation of sulfate, D-glucose, and a series of sulfur- or nitrogen-containing compounds such as isothiocyanates, thiocyanates, nitriles, and thiones. Isothiocyanates are responsible for the specific flavor and aroma of several cruciferous vegetables (Gatfield and Sand, 1983; Wilkinson et al., 1984). More recently, hydrolysis products of indole glucosinolates have received special attention due to their anticarcinogenic functions (Dunford and Temelli, 1996; Wathelet et al., 1996). On the other hand, these hydrolysis products can also have undesirable effects owing to their pungency, bitterness, and toxic or goitrogenic activity. Hence, control of myrosinase activity in cruciferous vegetables is highly desirable.

Until a decade ago, thermal treatments were preeminently used to reduce or control enzyme activity in foods. However, they can result in considerable losses of sensorial (taste, flavor, color) or nutritional (vitamins/nutrients) quality attributes (Lund, 1977). Therefore, new methods for controlling enzyme activity are being investigated, including high hydrostatic pressure (Ogawa et al., 1990; Cheftel, 1991; Knorr, 1993; Anese et al., 1995; Seyderhelm et al., 1996; Hendrickx et al., 1998). High pressure clearly has potential for the inactivation of vegetative microorganisms and spoilage enzymes while affecting quality attributes to only a limited extent. Fresh flavor and vitamin C content are only slightly affected by high-pressure processing (Cheftel, 1991; Van den Broeck et al., 1998). Likewise, the fresh green color of broccoli juice was retained after pressure treatment (Van Loey et al., 1998; Weemaes et al., 1998b).

The objective of this work was to investigate on a kinetic basis thermal and pressure inactivation of myrosinase from broccoli. To date, the number of publications concerning thermal inactivation of myrosinase is small, and they contain merely qualitative data (Yen and Wei, 1993; Dunford and Temelli, 1996; Wathelet et al., 1996); no information is available on the effects of high pressure on this enzyme.

MATERIALS AND METHODS

Enzyme Source. Broccoli at commercial maturity was purchased from the local supermarket. From this broccoli, the myrosinase was extracted, partially purified, and lyophilized. The lyophilized powder contained 40% proteins (micro protein determination kit, Sigma Diagnostics, product A610) and ∼1.5 units/mg of protein. One unit corresponds to the amount of enzyme that produces 1 μmol of glucose from sinigrin per minute at pH 6.55 and 25 °C in a total volume of 1 mL. The lyophilized powder was dissolved at a concentration of 0.015 g/mL in a 0.1 mol/L phosphate buffer at pH 6.55, which corresponds to the pH of the fresh broccoli juice.

Extraction and Partial Purification of Myrosinase from Broccoli. The procedure to extract myrosinase from broccoli was based on the method proposed by Wilkinson et al. (1984) and adjusted in the following way. The edible part of the broccoli crop was cut into small pieces, divided in 50 g portions, wrapped in tin foil, and frozen in liquid nitrogen. The frozen broccoli was then suspended in 150 mL of Tris-HCl (0.2 mol/L, pH 7.5) containing 0.1 mmol/L EDTA and 1.6 mmol/L DTE, together with 50 g of insoluble PVP, and mixed during 60 s at 4 °C in a metal Waring commercial blender (New Hartford, CT). The homogenate was successively filtered

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Table 1. Kinetic Equations for Overall Inactivation of Enzymes

model	mathematical expression	eq
first-order		
$N \xrightarrow{k} I$	$A_t = A_0 \exp(-kt)$	(7)
distinct isozyme		
$N_l \xrightarrow{k_l} I_l \quad N_s \xrightarrow{k_s} I_s$	$A(t) = A_l \exp(-k_l t) + A_s \exp(-k_s t)$	(8)
consecutive step		
$N \xrightarrow{k_1} X \xrightarrow{k_2} I$	$A(t) = \left[A_1 - A_2\left(\frac{k_1}{k_1 - k_2}\right)\right] \exp(-k_1 t) + \left[A_2\left(\frac{k_1}{k_1 - k_2}\right)\right] \exp(-k_2 t)$	(9)
fractional conversion		
$N + RF \xrightarrow{k} I + RF$	$A(t) = A_{rf} + (A_0 - A_{rf}) \exp(-kt)$	(10)

through a plastic filter and cheesecloth. Subsequently, the filtrate was centrifuged (Beckman, J2-HS) at 12400*g* for 15 min at 0 °C. $(NH_4)_2SO_4$ was added to the supernatant to 90% saturation, and the solution was incubated 1 h at 4 °C. After centrifugation (39200*g*, 15 min, 0 °C), the pellet was dissolved in 25 mL of 0.005 mol/L Tris-HCl at pH 7.5. The extract was then dialyzed (cellulose membrane, Medicell International Ltd., 6-27/32) for 15 h against the same buffer to remove the excess of ammonium and sulfate ions. Finally, the extract was frozen in liquid nitrogen and lyophilized during 18 h (Christ Alpha 2-4 freeze-dryer). The lyophilized powder was stored in the freezer until use.

Determination of Myrosinase Activity. Myrosinase activity was determined according to a coupled enzymatic procedure previously described by Wilkinson et al. (1984) and Gatfield and Sand (1983), with the distinction that a Boehringer Mannheim test kit for glucose determination was used.

NADP (2.45 mg/mL)/ATP (5.75 mg/mL) reagent (0.5 mL), 0.01 mL of hexokinase (290 units/mL)/glucose-6-phosphate dehydrogenase (145 units/mL) reagent, 0.9 mL of a distilled H_2O solution containing 0.1 g/L $MgCl_2$ and 2 g/L ascorbic acid, and 50 μL of enzyme solution (0.015 g/mL in 0.1 mol/L phosphate buffer at pH 6.55) were placed in each of two microcuvettes (1.5 mL, 1 cm light path), one of which was used as reference sample. In a preliminary experiment, concentrations of 0.1 g/L $MgCl_2$ and 2 g/L ascorbic acid were verified to give rise to optimal myrosinase activity. After equilibration for 3 min at 23 °C, 50 μL of a sinigrin solution (0.3 g/mL) was added to the sample cuvette and the absorbency increase at 340 nm due to the formation of NADPH was measured during 10 min (LKB Biochrom, Ultrospec K, Amersham Pharmacia Biotech). The activity was then determined from the slope of the straight portion of the curve. After the activity assay had been finalized, some experiments were carried out to determine its accuracy and reproducibility. A standard curve was constructed and showed a linear relationship ($r^2 = 0.993$) between the measured enzyme activity and the concentration of lyophilized powder in the range of 0.0025−0.04 g/mL. It was verified that the assay was carried out under substrate saturation conditions because further increase in sinigrin concentration above 0.3 g/mL did not result in a significant increase in enzyme activity. Finally, reproducibility tests at different enzyme concentrations showed the mean standard errors on the activity to range between 4 and 7%, indicating a sufficient reproducibility of the assay method.

Heat Inactivation Study. Isothermal inactivation experiments were performed in a thermostatic water bath at a constant temperature between 30 and 60 °C. To avoid heating and cooling lags, the enzyme solution was filled in capillary tubes (Hirschmann labogeräte, 15 mm diameter, 150 mm length). After filling, the tubes were placed in the water bath and heated for different preset times at one constant temperature. Immediately after withdrawal from the water bath, the samples were quickly cooled on ice and stored here until activity measurement (<120 min). The remaining enzyme activity reached a constant value immediately after cooling on ice and maintained this activity for at least 2 h.

Pressure Inactivation Study. Treatments at constant pressure were performed in a laboratory pilot scale, multivessel high-pressure equipment (HPIU-10000, 95/1994, Resato). The equipment consists of eight individual vessels (8 mL volume, 10 mm diameter, 100 mm length), which are connected to the central pressure circuit using T-joints and valves. Each of the vessels is surrounded by a thermostatic mantle, which is linked to a cryostat, allowing experiments to be performed at controlled, constant temperature. High pressure (up to 1000 MPa) is generated using a pressure intensifier in the central pressure circuit. The pressure-transferring liquid is a glycol−oil mixture (TR15, Resato).

After the samples are enclosed in the vessels (filled in 0.25 mL microtubes, Elkay), only the central valve is closed and pressure is built up slowly (100 MPa/min) until the desired value is reached. Once the maximum pressure is reached, the individual vessels are isolated from the central pressure tubing and the central valve is opened. As a function of time, pressure is then released in the individual vessels. To ensure treatment of the samples at constant pressure and temperature, the activity of the sample from the first vessel, where pressure is released the moment the central valve is opened (after 4 min), is considered as the reference (A_0, initial activity). Pressure inactivation of myrosinase from broccoli was studied both at ambient temperature (20 °C; 350−500 MPa) and at a temperature at which inactivation at atmospheric pressure occurs (35 °C; 0.1−450 MPa).

Data Analysis. It was endeavored to set forth a mathematical model describing adequately the course of myrosinase inactivation as a function of time. The models proposed (Table 1) were critically considered with regard to their phenomenological meaning. Remaining relevant models were subsequently analyzed in terms of residual distribution and statistical parameters (Schokker, 1997; Ludikhuyze et al., 1998b), namely the corrected regression coefficient (corrected r^2, eq 1), the residual variance (s^2, eq 2), Akaike's optimization criterion (AOC, eq 3), and Schwarz's optimization criterion (SOC, eq 4). From a statistical point of view, the best model is that with random residual distribution, the highest corrected r^2, and the smallest s^2, AOC, and SOC values.

$$\text{corrected } r^2 = 1 - \left(\frac{(m - i)(1 - r^2)}{m - p}\right) \quad (1)$$

$$s^2 = \left(\frac{\text{SSE}}{m - p}\right) \quad (2)$$

$$\text{AOC} = m \ln\left(\frac{\text{SSE}}{m}\right) + \frac{m(m + p)}{(m - p - 2)} \quad (3)$$

$$\text{SOC} = m \ln\left(\frac{\text{SSE}}{m}\right) + p \ln(m) \quad (4)$$

Temperature and pressure dependence of the time-dependent model parameters (inactivation rate constants) was described using the Arrhenius (eq 5) and Eyring (eq 6) equations, respectively. The validity of these equations was evaluated by plotting $\ln(k)$ versus reciprocal temperature or pressure, respectively, and determining the goodness of the fit by means of the regression coefficient and residual analysis.

Table 2. Two-Isozyme Model: Estimation of the Kinetic Parameters for Thermal Inactivation of Myrosinase from Broccoli (0.015 g of Lyophilized Powder/mL in 0.1 mol/L Phosphate Buffer at pH 6.55)

T (°C)	A_l[a]	k_l (min^{-1})	A_s[a]	k_s (min^{-1})
30	NC[b]	NC	NC	NC
35	21.0 ± 3.9[c]	(1.2 ± 0.4[c]) × 10^{-1}	80.2 ± 4.1[b]	(3.2 ± 1.6[b]) × 10^{-4}
37.5	28.5 ± 3.6	(3.3 ± 0.9) × 10^{-1}	71.2 ± 2.7	(1.8 ± 1.0) × 10^{-3}
40	43.8 ± 5.6	(2.4 ± 0.3) × 10^{-1}	56.1 ± 4.8	(4.9 ± 0.7) × 10^{-3}
42.5	45.4 ± 3.2	(4.1 ± 0.7) × 10^{-1}	53.3 ± 2.6	(6.1 ± 1.2) × 10^{-3}
45	49.5 ± 4.5	(9.8 ± 2.3) × 10^{-1}	50.1 ± 2.7	(1.2 ± 0.3) × 10^{-2}
50	65.2 ± 3.2	(9.3 ± 1.5) × 10^{-1}	34.6 ± 2.2	(1.5 ± 0.3) × 10^{-2}
60	91.9 ± 1.1	(27.9 ± 1.7) × 10^{-1}	8.1 ± 0.7	(3.5 ± 0.7) × 10^{-2}

[a] Given in percent of overall initial activity. [b] NC, no convergence. [c] Standard error.

$$\ln(k) = \ln(k_{refT}) - \left[\frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right] \quad (5)$$

$$\ln(k) = \ln(k_{refP}) - \left[\frac{V_a}{RT}(P - P_{ref})\right] \quad (6)$$

RESULTS AND DISCUSSION

Thermal Inactivation of Myrosinase from Broccoli. Thermal inactivation of myrosinase from broccoli (0.015 g lyophilized powder/mL in 0.1 mol/L phosphate buffer at pH 6.55) was studied at temperatures ranging from 30 to 60 °C. The activity remained rather constant at 30 °C, and significant inactivation occurred at 40 °C and higher. A treatment of 3 min at 60 °C was sufficient to reduce enzyme activity by 90%. These results show myrosinase from broccoli to be rather thermolabile. Only a few studies on the thermal stability of myrosinase from other Cruciferae species were found in the literature. Dunford and Temelli (1996) reported inactivation of myrosinase in crude rapeseed extract to proceed at temperatures >65 °C, whereas inactivation in flaked seeds required temperatures in the range of 90−100 °C. For swede and turnip, soaking in boiling water for 1.5 and 1 min, respectively, resulted in complete myrosinase inactivation (Dunford and Temelli, 1996). Wathelet et al. (1996) investigated inactivation of myrosinase from Brussels sprouts by blanching. Clearly inactivation proceeded more readily in the exterior of the crop than in the interior part. In the latter, treatments of 10 min at 90 °C, 5 min at 95 °C, and <5 min at 105 °C (steam blanching) resulted in >90% activity loss. For both red and white cabbages, myrosinase is inactivated >90% after a treatment at 70 °C for 30 min (Yen and Wei, 1993). The higher temperatures reported for myrosinase inactivation in these cases may be due to limitations in heat transfer involved in the heat treatment of whole vegetables as compared to vegetable enzyme extracts.

First, the validity of the first-order kinetic model (Table 1), which is frequently reported in the literature to describe enzyme inactivation (Suzuki, 1960; Lopez and Burgos, 1995; Ludikhuyze et al., 1997, 1998a), was evaluated. Because regression coefficients varied between 0.413 and 0.845 and residuals were not randomly distributed, it was concluded that a first-order kinetic model could not describe the irreversible thermal inactivation of myrosinase. Hence, the appropriateness of three other mathematical models (Table 1), likewise suggested in the literature for modeling enzyme inactivation (Robert et al., 1995; Weemaes, 1998), has been evaluated. A two-isozyme model refers to the existence of two isozymes, behaving differently when subjected to heat. This results in an initial fast inactivation period, followed by a much slower decay, eventually leading to a plateau phase. A consecutive step model can be used to describe two different cases of enzyme inactivation. (i) The consecutive step model can describe an inactivation process proceeding as a succession of two irreversible reaction steps:

$$N \xrightarrow{k_1} I \xrightarrow{k_2} D$$

In the first step, the native enzyme converts to an intermediate form, the activity of which may be dependent on the inactivation temperature applied. On its turn, this intermediate form is converted to the inactive form. (ii) The consecutive step model can likewise be used to describe the inactivation of enzymes that exist as two different forms (e.g., free enzyme and enzyme interacting with impurities or substrates) which are in equilibrium with each other.

$$\begin{array}{ccc} N_1 & \overset{K}{\longleftrightarrow} & N_2 \\ \downarrow k_1 & & \downarrow k_2 \\ D_1 & & D_2 \end{array}$$

The transformation of each enzyme form to the inactive form is characterized by a distinct inactivation rate constant. Clearly the position of the equilibrium between the two forms can be dependent on temperature. The fractional conversion model, on its turn, refers to a first-order inactivation process in which a resistant enzyme fraction, not affected after prolonged heating at the preset temperature, is taken into account. Calculation of the kinetic parameters corresponding to the mathematical equations proposed was accomplished by fitting the data to eqs 8, 9, and 10, respectively, using a nonlinear regression procedure (SAS Institute, 1982).

Concerning the two-isozyme model, activity estimates of both the native thermostable (A_s) and thermolabile (A_l) enzyme forms are presented in Table 2, showing both A_s and A_l to vary with temperature. Activity of the native thermostable form decreased with increasing

Table 3. Fractional Conversion Model: Estimation of the Kinetic Parameters for Thermal Inactivation of Myrosinase from Broccoli (0.015 g of Lyophilized Powder/mL in 0.1 mol/L Phosphate Buffer at pH 6.55)

T (°C)	A_f[a]	k (min^{-1})
30	89.5 ± 1.5[b]	(3.9 ± 1.8[b]) × 10^{-2}
35	80.7 ± 1.3	(1.2 ± 0.2) × 10^{-1}
37.5	53.4 ± 3.1	(1.3 ± 0.3) × 10^{-1}
40	51.7 ± 1.7	(2.1 ± 0.3) × 10^{-1}
42.5	41.3 ± 2.5	(2.6 ± 0.4) × 10^{-1}
45	35.8 ± 3.5	(4.2 ± 1.0) × 10^{-1}
50	23.5 ± 2.7	(5.5 ± 1.1) × 10^{-1}
60	4.5 ± 0.9	(24.0 ± 3.0) × 10^{-1}

[a] Given in percent of the initial overall activity. [b] Standard error.

Table 4. Consecutive Step Model: Estimation of the Kinetic Parameters for Thermal Inactivation of Myrosinase from Broccoli (0.015 g of Lyophilized Powder/mL in 0.1 mol/L Phosphate Buffer at pH 6.55)

T (°C)	A_1[a]	k_1 (min^{-1})	A_2[a]	k_2 (min^{-1})
30	NC[b]	NC	NC	NC
35	101.2 ± 1.6[c]	(1.4 ± 0.4[c]) × 10^{-1}	81.6 ± 3.3[b]	(4.0 ± 0.4[c]) × 10^{-4}
37.5	99.7 ± 2.7	(3.3 ± 0.9) × 10^{-1}	70.2 ± 2.7	(1.8 ± 0.2) × 10^{-3}
40	99.9 ± 3.6	(2.4 ± 0.7) × 10^{-1}	55.7 ± 4.6	(4.9 ± 0.7) × 10^{-3}
42.5	98.6 ± 2.4	(4.2 ± 0.7) × 10^{-1}	52.5 ± 2.6	(6.1 ± 1.2) × 10^{-3}
45	99.6 ± 3.8	(9.8 ± 0.2) × 10^{-1}	49.5 ± 1.3	(1.2 ± 0.2) × 10^{-2}
50	99.9 ± 2.5	(9.3 ± 0.1) × 10^{-1}	34.1 ± 2.1	(1.5 ± 0.2) × 10^{-2}
60	99.9 ± 0.7	(27.9 ± 1.7) × 10^{-1}	7.9 ± 0.7	(3.5 ± 0.7) × 10^{-2}

[a] Given in percent of overall initial activity. [b] NC, no convergence. [c] Standard error.

Table 5. Statistical Analysis of the Fractional Conversion Model (FCM) and the Consecutive Step Model (CSM) To Select the Model Most Adequately Describing Thermal Inactivation of Myrosinase from Broccoli

T (°C)	model	corrected r^2	s^2 (×10^4)	AOC	SOC
30	FCM	0.9997	2.77	−38.7	−55.8
	CSM				
35	FCM	0.9997	2.37	−49.1	−64.9
	CSM	0.9997	3.16	−22.0	−61.7
37.5	FCM	0.9927	39.2	−37.7	−53.1
	CSM	0.9986	7.51	−42.0	−67.8
40	FCM	0.9976	12.4	−42.7	−58.1
	CSM	0.9971	14.5	−25.1	−55.3
42.5	FCM	0.9945	23.2	−37.0	−52.4
	CSM	0.998	6.49	−32.3	−62.6
45	FCM	0.9847	51.5	−29.9	−45.3
	CSM	0.9957	14.2	−25.3	−55.5
50	FCM	0.9558	34.2	−27.7	−43.6
	CSM	0.9973	6.24	−16.6	−56.3
60	FCM	0.996	4.95	−43.2	−59.0
	CSM	0.9996	0.55	−35.9	−75.7

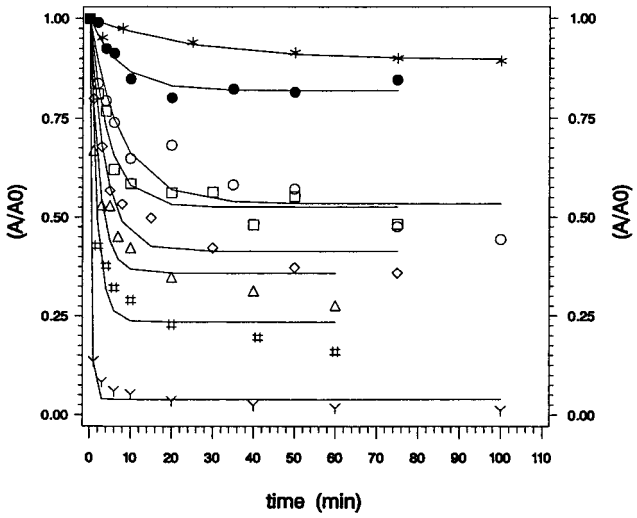

Figure 1. Thermal inactivation of myrosinase from broccoli (0.015 g of lyophilized powder/mL in 0.1 mol/L phosphate buffer at pH 6.55) modeled using the fractional conversion model: (*) 30 °C; (●) 35 °C; (○) 37.5 °C; (□) 40 °C; (◇) 42.5 °C; (△) 45 °C; (#) 50 °C; (Y) 60 °C.

temperature while that of the native thermolabile enzyme increased. This inconsistency of the initial activity estimates indicated a lack of evidence for the presence of two fractions differing in thermostability. Indeed, in this case the activity estimates should be independent of temperature because they are the initial activities of two native isozymes. Consequently, the two-isozyme model was rejected. Subsequently, the remaining models, that is, the fractional conversion model (Table 3) and the consecutive step model (Table 4), were evaluated and statistically compared by calculating s^2, AOC, and SOC (Table 5). Table 3 indicates the activity estimates of the hypothetical intermediate (A_2) to vary significantly with temperature. However, exclusion of the consecutive step model based on this observation is not justified because the activity of the intermediate or the position of the equilibrium between two enzyme forms may depend on the inactivation temperature (Robert et al., 1995). From a statistical point of view, the fractional conversion model is preferred at temperatures <40 °C, whereas the consecutive step model gives the best results at higher temperature. Because of this temperature dependency, statistical analysis did not allow a definite model selection. However, visual inspection of the inactivation curves modeled using fractional conversion (Figure 1) showed a significant underestimation of the extent of inactivation at high temperature and long treatment time; that is, at high temperature no indication for a fraction of the enzyme to be resistant was noted. The consecutive step model, on the other hand, seemed to describe satisfactorily the course of inactivation, at both high and low temperature. Hence, the consecutive step model was selected as the best model to describe thermal inactivation of myrosinase from broccoli. Although the exact mechanism of inactivation could not be determined by these kinds of kinetic experiments, the second hypothesis seemed to be the most likely one because of two reasons. First, all experiments are carried out with a crude enzyme extract, containing a lot of impurities. Such impurities can easily interact with the myrosinase enzyme, thereby altering its thermostability. Second, activation energy values are similar (113.5 ± 14.1 and 134.1 ± 28.3 kJ/mol, respectively), indicating a similar temperature sensitivity of the respective inactivation rate constants. Indeed, interaction of enzymes with other substances has often been reported to alter the thermostability of enzymes without affecting the temperature sensitivity of the inactivation rate constants (Gray, 1988; De Cordt et al., 1992). Otherwise, the fact that two completely independent steps in an inactivation reaction would be characterized by the same temperature dependence of the inactivation rate constants is less obvious.

Pressure Inactivation of Myrosinase from Broccoli. At first, pressure inactivation of myrosinase from broccoli (0.015 g of lyophilized powder/mL in 0.1 mol/L phosphate buffer at pH 6.55) was studied at 20 °C in the pressure range 250−500 MPa. At pressures <250 MPa, the activity remained constant and no postprocess activation of the enzyme was noted (data not shown). Significant inactivation was observed between 300 and 500 MPa. These results show myrosinase to be rather pressure sensitive as compared to other food quality related enzymes such as peroxidase, polyphenol oxidase, lipoxygenase, and pectinmethylesterase (Hendrickx et al., 1998).

Table 6. Two-Isozyme Model: Estimation of the Kinetic Parameters for Pressure Inactivation of Myrosinase from Broccoli (0.015 g of Lyophilized Powder/mL in 0.1 mol/L Phosphate buffer at pH 6.55) at 20 °C

P (MPa)	A_l[a]	k_l (min^{-1})	A_s[a]	k_s (min^{-1})
250	10.3 ± 3.6[b]	(2.6 ± 0.6[b]) × 10^{-2}	89.7 ± 3.7[b]	(1.0 ± 0.4[b]) × 10^{-3}
350	22.1 ± 3.7	(5.9 ± 1.2) × 10^{-2}	78.2 ± 3.8	(3.1 ± 0.6) × 10^{-3}
400	25.8 ± 9.8	(12.3 ± 7.4) × 10^{-2}	74.6 ± 9.8	(5.1 ± 2.3) × 10^{-3}
450	37.4 ± 1.1	(45.1 ± 6.9) × 10^{-2}	62.8 ± 18.2	(10.5 ± 1.1) × 10^{-3}
500	24.2 ± 4.8	(76.7 ± 40.4) × 10^{-2}	75.6 ± 3.7	(25.2 ± 3.8) × 10^{-3}

[a] Given in percent of overall initial activity. [b] Standard error.

Table 7. Fractional Conversion Model: Estimation of the Kinetic Parameters for Pressure Inactivation of Myrosinase from Broccoli (0.015 g of Lyophilized Powder/mL in 0.1 mol/L Phosphate Buffer at pH 6.55) at 20 °C

P (MPa)	A_f[a]	k (min^{-1})
250	78.7 ± 0.4[b]	(1.6 ± 0.04[b]) × 10^{-2}
350	58.1 ± 1.4	(3.1 ± 0.3) × 10^{-2}
400	53.1 ± 3.5	(5.8 ± 1.3) × 10^{-2}
450	43.1 ± 4.4	(17.9 ± 4.3) × 10^{-2}
500	33.7 ± 5.2	(11.4 ± 2.4) × 10^{-2}

[a] Given in percent of the initial overall activity. [b] Standard error.

In agreement with thermal inactivation, a first-order model could not be applied to analyze pressure inactivation of broccoli myrosinase. Therefore, the three models proposed in the previous paragraph were again evaluated for their ability to describe pressure inactivation of myrosinase. Table 6 clearly indicates the two-isozyme model to be deficient in describing adequately pressure inactivation of broccoli myrosinase at 20 °C, because estimates for the initial activity of the native thermostable and thermolabile enzyme form significantly varied with pressure. There seems to be a tendency for the A_s value to decrease and for the A_l value to increase with increasing pressure. However, the reverse should be expected. When pressure is increased, inactivation of the pressure sensitive enzyme form during pressure buildup would become more pronounced, thereby causing the contribution of the pressure resistant enzyme form to the total myrosinase population at time zero to increase. On the basis of the above stated observations, the two-isozyme model was excluded. Subsequently, the fractional conversion model (Table 7) and the consecutive step model (Table 8) were evaluated for their ability to describe pressure inactivation of broccoli myrosinase and statistically compared (Table 9). Except for AOC, all statistical parameters revealed the consecutive step model to be more adequate in describing pressure inactivation of broccoli myrosinase at 20 °C. The same conclusion could be drawn based on visual inspection of the inactivation curves modeled using fractional conversion (Figure 2), which showed a consistent underestimation of the inactivation extent at high pressure and long treatment time. This modeling deficiency was not observed when using the consecutive step model. Consequently, in agreement with thermal inactivation, the consecutive step model was selected for further

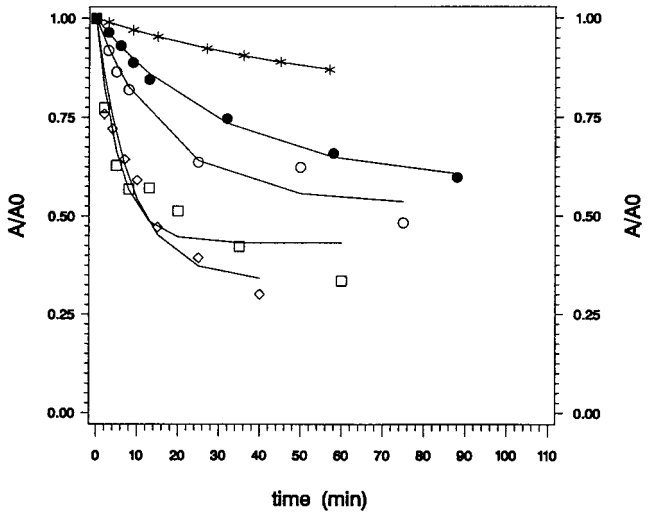

Figure 2. Pressure inactivation of myrosinase from broccoli (0.015 g of lyophilized powder/mL in 0.1 mol/L phosphate buffer at pH 6.55) at 20 °C modeled using the fractional conversion model: (*) 250 MPa; (●) 350 MPa; (○) 400 MPa; (□) 450 MPa; (◇) 500 MPa.

modeling purposes. Pressure dependence of the distinguished inactivation rate constants was calculated according to the Eyring equation as −34.5 ± 4.4 and −30.8 ± 1.9 cm^3/mol, respectively, pointing out a similar pressure dependence.

In a second phase, pressure inactivation of broccoli myrosinase was studied at 35 °C, a temperature at which inactivation at atmospheric pressure occurs slowly, to check whether (low) pressure has an antagonistic (or protective) effect on thermal inactivation. Antagonistic effects of pressure have been frequently reported in the literature, both for reversible and for irreversible enzyme denaturation. Pressures in the range of 40−80 MPa counteracted the reversible thermal denaturation of egg albumin at temperatures between 65 and 70 °C, the effect being most pronounced at the highest pressure (Suzuki et al., 1958). Also, for chymotrypsinogen (Hawley, 1971), ribonuclease A (Brandts et al., 1970), metmyoglobin (Zipp and Kauzman, 1973), and ovalbumin and carbonylhemoglobin (Suzuki, 1960; Suzuki and Kitamura, 1960), application of low pressure seemed to retard reversible thermal denaturation. More recently, antagonistic effects were also observed for irreversible inactivation of food quality

Table 8. Consecutive Step Model: Estimation of the Kinetic Parameters for Pressure Inactivation of Myrosinase from Broccoli (0.015 g of Lyophilized Powder/mL in 0.1 mol/L Phosphate Buffer at pH 6.55) at 20 °C

P (MPa)	A_1[a]	k_1 (min^{-1})	A_2[a]	k_2 (min^{-1})
250	100.0 ± 0.04[b]	(2.6 ± 0.6[b]) × 10^{-2}	86.3 ± 3.1[b]	(1.0 ± 0.3[b]) × 10^{-3}
350	100.3 ± 0.6	(5.9 ± 1.2) × 10^{-2}	74.1 ± 3.6	(3.1 ± 0.6) × 10^{-3}
400	100.4 ± 3.3	(12.3 ± 4.7) × 10^{-2}	71.4 ± 9.7	(5.2 ± 2.1) × 10^{-3}
450	100.1 ± 1.6	(45.1 ± 6.9) × 10^{-2}	61.3 ± 15.3	(10.5 ± 1.1) × 10^{-3}
500	99.8 ± 3.1	(76.7 ± 10.1) × 10^{-2}	73.1 ± 4.2	(25.3 ± 3.1) × 10^{-3}

[a] Given in percent of overall initial activity. [b] Standard error.

Table 9. Statistical Analysis of the Fractional Conversion Model (FCM) and the Consecutive Step Model (CSM) To Select the Model Most Adequately Describing Pressure Inactivation of Myrosinase from Broccoli

P (MPa)	model	corrected r^2	s^2 ($\times 10^4$)	AOC	SOC
250	FCM	1.0000	0.002	−104.5	−120.4
	CSM	1.0000	0.002	−80.8	−120.5
350	FCM	0.9998	1.01	−55.8	−71.7
	CSM	0.9999	0.48	−37.1	−76.8
400	FCM	0.9978	15.6	−26.6	−43.7
	CSM	0.9983	12.7	−24.4	−44.8
450	FCM	0.9909	41.4	−26.2	−42.0
	CSM	0.9991	2.82	−22.9	−62.6
500	FCM	0.9942	27.7	−29.4	−45.3
	CSM	0.9979	9.56	−13.1	−52.9

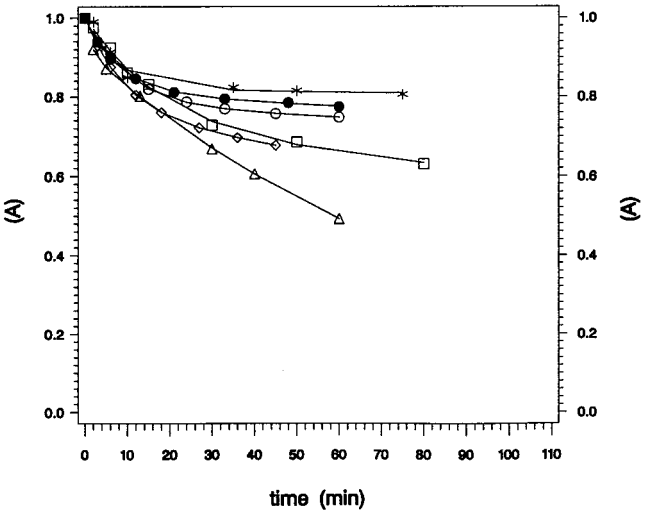

Figure 3. Pressure inactivation of myrosinase from broccoli (0.015 g of lyophilized powder/mL in 0.1 mol/L phosphate buffer at pH 6.55) at 35 °C modeled using the consecutive step model: (*) 0.1 MPa; (●) 150 MPa; (○) 250 MPa; (□) 350 MPa; (◇) 400 MPa; (△) 450 MPa.

related enzymes. Weemaes et al. (1998a) reported thermal inactivation of avocado polyphenol oxidase at temperatures >62.5 °C to be counteracted by pressures up to 250 MPa. Likewise, thermal inactivation of pectinmethylesterase from oranges (Hendrickx et al., 1998) and lipoxygenase from soybean (Heinisch et al., 1995) was found to be retarded by application of low pressure. The latter results, however, were somewhat contradicted by Ludikhuyze et al. (1998a), who observed an antagonistic effect on the inactivation of soybean lipoxygenase at low temperature and high pressure.

As mentioned above, a consecutive step model was used to analyze the data of pressure inactivation of myrosinase from broccoli at 35 °C (Figure 3). The concomitant kinetic parameters are presented in Table 10. From this table it becomes clear that application of low pressure retards the inactivation of the first myrosinase enzyme form, because inactivation rate constants first decreased with pressure increasing up to 350 MPa and then decreased with further increase in pressure. The maximal protective effect was noted at a pressure around 350 MPa. Calculation of the activation volumes in distinct low- and high-pressure areas was statistically insignificant because of the few data points available for linear regression.

Such an antagonistic effect was not observed for the second myrosinase enzyme form. In this case, inactivation rate constants showed a consistent increase with increasing pressure, characterized by an activation volume of -15.7 ± 3.8 cm^3/mol. Hence, the pressure sensitivity of the inactivation rate of the second enzyme form is significantly lower at 35 °C than at 20 °C. Similar findings were previously reported for egg albumin (Suzuki et al., 1958), carbonylhemoglobin (Suzuki and Kitamura, 1960), chymotrypsin (Taniguchi and Suzuki, 1983), avocado polyphenol oxidase (Weemaes et al., 1998a), and soybean lipoxygenase (Ludikhuyze et al., 1998a).

Conclusion. Both elevated temperature and elevated pressure can be used to control the activity of myrosinase from broccoli. At atmospheric pressure inactivation proceeds in the temperature range 30−60 °C, whereas inactivation at 20 °C occurs in the pressure range 250−500 MPa. These results indicate myrosinase from broccoli to be both thermal and pressure sensitive. At 20 °C, no activation of the enzyme was observed, whereas at 35 °C, thermal inactivation could be retarded by application of a low pressure, indicating an antagonistic effect between low pressure and temperature, at which inactivation at atmospheric pressure proceeds.

ABBREVIATIONS USED

A_t, enzyme activity at time t; A_0, enzyme activity at time 0; A_l, specific activity of the labile enzyme form; A_s, specific activity of the stable enzyme form; A_1, specific activity of the first enzyme form; A_2, specific activity of the second enzyme form; A_∞, enzyme activity at equilibrium; AOC, Akaike's optimization criterion; ATP, adenosine triphosphate; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetate; E_a, activation energy (kJ/mol); I, inactivated enzyme; k, inactivation rate constant (min^{-1}); k_{refT}, inactivation rate constant (min^{-1}) at reference temperature; k_{refP}, inactivation rate constant (min^{-1}) at reference pressure; m, number of observations; N, native enzyme; NADP, nicotinamide adenine dinucleotide phosphate; p, number of parameters; P, pressure (MPa); P_{ref}, reference pressure (0.1 MPa); PVP, poly(vinylpyrrolidone); R, universal gas constant (8.314 J/mol·K); RF, resistant fraction; T, temperature (K or °C); T_{ref}, reference temperature (273 K or 0 °C); r^2, regression coefficient; s^2, residual variance; SSE, residual sum of squares; t, time (min); Tris,

Table 10. Consecutive Step Model: Estimation of the Kinetic Parameters for Pressure Inactivation of Myrosinase from Broccoli (0.015 g of Lyophilized Powder/mL in 0.1 mol/L Phosphate Buffer at pH 6.55) at 35 °C

P (MPa)	A_1[a]	k_1 (min^{-1})	A_2[a]	k_2 (min^{-1})
0.1	100.2 ± 1.6	(13.8 ± 4.1) × 10^{-2}	81.6 ± 3.3	(4.0 ± 0.4) × 10^{-4}
150	100.0 ± 0.2	(12.4 ± 0.6) × 10^{-2}	80.6 ± 0.5	(7.3 ± 0.8) × 10^{-4}
250	99.9 ± 0.1	(10.4 ± 0.1) × 10^{-2}	77.6 ± 0.2	(7.6 ± 0.4) × 10^{-4}
350	100.4 ± 0.8	(5.5 ± 0.1) × 10^{-2}	69.5 ± 5.2	(16.3 ± 1.6) × 10^{-4}
400	100.0 ± 0.2	(10.5 ± 0.1) × 10^{-2}	73.9 ± 0.2	(25.5 ± 0.1) × 10^{-4}
450	100.1 ± 0.2	(64.0 ± 6.1) × 10^{-2}	88.9 ± 0.5	(103.3 ± 0.8) × 10^{-4}

[a] Given in percent of overall initial activity. [b] Standard error.

tris(hydroxymethyl)aminomethane; X, intermediate form of the enzyme.

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