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Kinetics of the Stability of Broccoli (*Brassica oleracea* Cv. Italica) Myrosinase and Isothiocyanates in Broccoli Juice during Pressure/Temperature Treatments

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The *Brassicaceae* plant family contains high concentrations of glucosinolates, which can be hydrolyzed by myrosinase yielding products having an anticarcinogenic activity. The pressure and temperature stabilities of endogenous broccoli myrosinase, as well as of the synthetic isothiocyanates sulforaphane and phenylethyl isothiocyanate, were studied in broccoli juice on a kinetic basis. At atmospheric pressure, kinetics of thermal (45−60 °C) myrosinase inactivation could be described by a consecutive step model. In contrast, only one phase of myrosinase inactivation was observed at elevated pressure (100−600 MPa) combined with temperatures from 10 up to 60 °C, indicating inactivation according to first-order kinetics. An antagonistic effect of pressure (up to 200 MPa) on thermal inactivation (50 °C and above) of myrosinase was observed indicating that pressure retarded the thermal inactivation. The kinetic parameters of myrosinase inactivation were described as inactivation rate constants (k values), activation energy (E_a values), and activation volume (V_a values). On the basis of the kinetic data, a mathematical model describing the pressure and temperature dependence of myrosinase inactivation rate constants was constructed. The stability of isothiocyanates was studied at atmospheric pressure in the temperature range from 60 to 90 °C and at elevated pressures in the combined pressure−temperature range from 600 to 800 MPa and from 30 to 60 °C. It was found that isothiocyanates were relatively thermolabile and pressure stable. The kinetics of HP/T isothiocyanate degradation could be adequately described by a first-order kinetic model. The obtained kinetic information can be used for process evaluation and optimization to increase the health effect of *Brassicaceae*.

KEYWORDS: Pressure; temperature; myrosinase; isothiocyanates; stability; kinetics

INTRODUCTION

Glucosinolates are natural pseudo-glucosides that can be found in considerable amounts in *Brassicaceae* (*1*). As such, these phytochemicals are inactive or can even have a negative effect on our health, e.g., glucoraphanin (*2*), but after hydrolysis by myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147), active compounds such as isothiocyanates (ITCs) or indoles can be formed, depending on pH, Fe^{2+} ions, and the presence of epithiospecifier protein (ESP), which have a positive effect on our health. For example, broccoli contains aliphatic glucosinolates, e.g., glucoraphanin and gluconapin, a phenyl glucosinolate, gluconasturtiin, and indole glucosinolates, e.g., glucobrassicin (*3*−*5*). Myrosinase hydrolysis of glucoraphanin, most abundant in broccoli, can result in sulforaphane and sulforaphane nitrile. The ITC, sulforaphane, has been shown to have anticarcinogenic properties, since it is a very potent inducer of phase II detoxifying enzymes while it inhibits phase I enzymes (*6*, *7*), whereas the nitrile does not (*8*). An important factor in broccoli that determines whether the ITC or the nitrile is formed is ESP. As it is relatively heat sensitive as compared to myrosinase, a short heat treatment (i.e., 5 or 10 min at 60 °C) inactivates ESP but leaves the myrosinase active (*9*, *10*). To achieve high amounts of the active sulforaphane, it is necessary to inactivate the ESP while myrosinase is still active (*10*). Therefore, it is important to know the optimal processing conditions. Also, other broccoli glucosinolate hydrolysis products, like indoles and ascorbigen, can have a beneficial health effect (*11*, *12*).

Myrosinase exists as different isozymes and can form complexes with myrosinase binding proteins and myrosinase-associated proteins (*13*). Structural analysis by X-ray has shown that *Sinapis alba* L. myrosinase is a dimer linked by a zinc atom and has a characteristic $(\beta/\alpha)_8$-barrel structure (*14*). In intact vegetables, glucosinolates and myrosinase are located in different cell compartments so tissue disruption is necessary for glucosinolate hydrolysis to occur. In the human gut, microorganisms can hydrolyze glucosinolates but the beneficial effects

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are less pronounced than when the glucosinolates are hydrolyzed by the plant myrosinase (*15*). The glucosinolates and its degradation compounds also give the brassica vegetables, including broccoli, a typical odor and taste (*16*, *17*). This taste is often bitter or pungent and not appreciated by consumers (*18*).

Most research has been focused on the health effects of glucosinolates and their hydrolysis products (*19*, *20*), and little information, especially kinetic data, about the effect of processing on myrosinase and ITC stability is available. Of the research on myrosinase thermal stability, most has been performed in buffers with crude extract (*21*, *22*), whereas studies in vegetable juice, e.g., red cabbage juice (*23*), or in food systems (*24*) are still limited. For sulforaphane, there has been a thermal degradation study in aqueous solution (*25*). These authors found that increasing the temperature to 50 or 100 °C for 1 h caused degradation of sulforaphane. However, no kinetic data are reported.

In the past two decades, novel technologies for food processing and preservation have been explored, e.g., a high hydrostatic pressure (HP) treatment, because it can inactivate vegetative microorganisms under conditions that result in only a limited effect on food quality attributes such as nutritional value (*26*, *27*). The pressure−temperature stability of myrosinase has been reported for broccoli myrosinase (*21*) and mustard seed myrosinase (*28*) in a buffer system. Because HP/T stability of enzymes in buffer systems can be different from food matrices (*29*, *30*), it is important to validate the available data toward food systems. Therefore, the purpose of this research was to study the effect of temperature and high pressure on the stability of the endogenous myrosinase in broccoli (*Brassica oleracea* cv. Italica) juice. As a high retention of ITCs during processing is required to benefit from the health-promoting properties of *Brassicaceae*, the thermal and HP/T stability of two ITCs, namely, sulforaphane and phenylethyl ITC, was determined on a kinetic basis.

MATERIALS AND METHODS

Study on Myrosinase Stability. *Preparation of Broccoli Juice.* Throughout this study, a single batch of broccoli juice was used. The broccoli juice was obtained by homogenizing the edible parts of broccoli in a juice centrifuge. The resulting juice was centrifuged for 10 min at 10000*g* (4 °C) and filtered through a sintered glass filter to remove the insoluble cell components. The juice was frozen in liquid nitrogen and stored at −80 °C for further use. Prior to treatments, it was thawed in a water bath at 20 °C.

Determination of Myrosinase Activity. The myrosinase activity was determined following a coupled enzymatic procedure as described by Van Eylen et al. (*28*). In this reaction, the glucose formed by myrosinase using sinigrin (Fluka, Buchs, Switzerland) as a substrate was used to convert $NADP^+$ to NADPH, which was followed spectrophotometrically at $\lambda = 340$ nm. The enzyme activity was determined based on the slope of the initial linear part of the curve of absorbance vs reaction time.

Because the original glucose concentration present in the broccoli juice caused a starting absorbance ($\lambda = 340$ nm) that was too high to use this coupled enzymatic procedure directly, myrosinase was isolated from the broccoli juice after the treatments.

The juice was centrifuged at 17000*g* and 4 °C for 5 min, and the myrosinase activity was detected in the pellet. The pellet was washed with 1 mL of phosphate buffer (pH 7.5, 50 mM), and myrosinase was precipitated a second time under the same conditions. Prior to the enzyme activity measurement, the pellet was dissolved in 250 μL of phosphate buffer (pH 7.5, 50 mM). It was preliminarily confirmed that the centrifugation step had no significant effect on the enzymatic activity.

Data Analysis. A general scheme for thermal and pressure inactivation of enzymes was proposed to be partial enzyme unfolding, followed by an irreversible reaction step (eq 1) (*31*).

$$\mathrm{N} \overset{K}{\leftrightarrow} \mathrm{U} \xrightarrow{k_{ir}} \mathrm{I} \tag{1}$$

with N the native, U the partially unfolded, and I the irreversibly inactivated enzyme form.

The observed, experimentally measured rate constant (k_{obs}) can be mathematically expressed as $k_{obs} = k_{ir}K(1 + K)$ with $K = \mathrm{U/N}$ (*32*). At conditions (high temperature and high pressure) where the concentration of the native form is much smaller than that of the reversibly unfolded form ($K \gg 1$), $k_{obs} = k_{ir}$.

In general, the decrease of enzyme activity (A) as a function of processing time (t) can be described by an n-th-order kinetic model (eq 2)

$$\frac{dA}{dt} = -kA^n \tag{2}$$

where k is the inactivation rate constant and n represents the reaction order.

Although enzyme inactivation is a complex chain of events, often a first-order reaction kinetic model ($n = 1$) can be applied to describe its inactivation kinetics. If the reaction rate (k) is independent of time (isothermal/isobaric conditions), the first-order kinetic model can be integrated and linearized (eq 3).

$$\ln(A) = \ln(A_0) - kt \tag{3}$$

where A_0 represents the initial enzyme activity at time = 0.

The consecutive step model (eq 4) was used before to describe the thermal inactivation of broccoli myrosinase (*21*) and can be applied to describe enzyme inactivation that occurs in two irreversible consecutive steps. In the first step, an intermediate (U_2) is formed that still has an active form (N_2) when returned to standard conditions. However, this activity can be different than that of the native form (N_1). In the second step, this intermediate (U_2) is turned over to the inactivated form (I). This model can also be applied when it is assumed that N_1 and N_2 are two enzyme forms, e.g., free enzyme and enzyme interacting with impurities or substrate, both having an activity and where one form can be turned over in the other. The amount of N_2 can be dependent on temperature. The mathematical equation describing the consecutive step model is given in eq 5.

$$\begin{array}{c} N_1 \leftrightarrow U_1 \xrightarrow{k_1} U_2 \xrightarrow{k_2} I \\ \updownarrow \\ N_2 \end{array} \tag{4}$$

$$A = \left[A_1 - A_2\left(\frac{k_1}{k_1 - k_2}\right)\right]\exp(-k_1 t) + \left[A_2\left(\frac{k_1}{k_1 - k_2}\right)\right]\exp(-k_2 t) \tag{5}$$

where A_1 is the relative enzyme activity at $t = 0$, A_2 is the relative enzyme activity of the second enzyme form (N_2), and k_1 and k_2 (min^{-1}) are the inactivation rate constants, respectively, to obtain U_2 and I (min^{-1}).

Temperature dependence of the time-dependent model parameter (inactivation rate constant, k) was described using the Arrhenius equation (eq 6) while its pressure dependence was described using the Eyring equation (eq 7).

$$k = k_{ref}\exp\left[\frac{E_a}{R_t}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \quad (R_t = 8.314\ \mathrm{J\ mol^{-1}\ K^{-1}}) \tag{6}$$

where k_{ref} is the inactivation rate constant at the reference temperature T_{ref} and E_a is the activation energy.

$$\ln(k) = \ln(k_{ref}) - \left[\frac{V_a}{R_p T}(P - P_{ref})\right] \quad (R_p = 8.314\ \mathrm{cm^3\ MPa\ K^{-1}\ mol^{-1}}) \tag{7}$$

where k_{ref} is the inactivation rate constant at the reference pressure P_{ref} and V_a is the activation energy.

To determine the model parameters for thermal inactivation at atmospheric pressure, the Arrhenius equation (eq 6) can be inserted in the consecutive step model (eq 4) yielding eq 8.

$$A = \left[A_1 - A_2\left(\frac{a}{a-b}\right)\right]\exp(-at) + \left[A_2\left(\frac{a}{a-b}\right)\right]\exp(-bt)$$

with

$$a = k_{1,ref}\exp\left[\frac{E_{a1}}{R_t}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right]$$

and

$$b = k_{2,ref}\exp\left[\frac{E_{a2}}{R_t}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \quad (8)$$

Combined Pressure/Temperature Model for the Inactivation Rate Constant. The thermodynamic model previously described by Hawley (*33*) can be converted (eq 9) to describe the combined HP/T dependence of inactivation rate constants. This model has been successfully applied to describe several enzyme inactivation data (*31*).

$$\ln(k) = \ln(k_0) + a(P-P_0)^2 + b(P-P_0)(T-T_0) + c(P-P_0) + d(T-T_0) + e\left[T\left(\ln\frac{T}{T_0} - 1\right) + T_0\right] \quad (9)$$

where $a = -(\Delta\beta^{\ddagger}/2RT)$, $b = -(\Delta\alpha^{\ddagger}/RT)$, $c = -(\Delta V_0^{\ddagger}/RT)$, $d = \Delta S_0^{\ddagger}/RT$, and $e = \Delta C_p^{\ddagger}/RT$ and where $\Delta\alpha^{\ddagger}$ is the thermal expansivity factor (cm^3/mol), $\Delta\beta^{\ddagger}$ is the compressibility factor (cm^6/J mol), $\Delta V_0^{\ddagger}$ is the volume change between native and denatured states at T_0 and P_0 (cm^3/mol), $\Delta S_0^{\ddagger}$ is the entropy change between native and denatured states at T_0 and P_0 (J/mol K), T_0 is the reference temperature (K) and P_0 is the reference pressure (MPa), and $\Delta C_p^{\ddagger}$ is the heat capacity (J/mol K).

In cases where $\Delta C_p^{\ddagger}$, $\Delta\alpha^{\ddagger}$, or $\Delta\beta^{\ddagger}$ are temperature- or pressure-dependent (*34*), an extended analysis of ln(k) is necessary, where higher order terms are also involved. In this study, the model was extended with one-third-order term, i.e., $\Delta\beta_2^{\ddagger}/2RT\,(P-P_0)^3$ (third-order model, eq 10).

$$\ln(k) = \ln(k_0) + a(P-P_0)^2 + c(P-P_0) + d(T-T_0) + e\left[T\left(\ln\frac{T}{T_0} - 1\right) + T_0\right] + f(P-P_0)^3 \quad (10)$$

with a, c, d, and e as described in eq 9 and $f = \Delta\beta_2^{\ddagger}/2RT$.

In this study, the reference temperature and pressure were 40 °C and 400 MPa, respectively. The kinetic parameters of the model were estimated using nonlinear regression analysis (*35*).

The quality of the model fitting was determined by several statistical parameters and criteria: (i) corrected r^2 (eq 11), which measures how well the model fits to the data and depends on the number of observations (m) and parameters (j), (ii) the standard deviation of the model (eq 12), (iii) visual inspection of the relation between experimental and predicted k values, (iv) standard deviation of the estimated parameters, and (v) the random distribution of the residuals.

$$\text{corrected } r^2 = \left[1 - \frac{(m-1)\left(1 - \frac{SSQ_{regression}}{SSQ_{total}}\right)}{(m-j)}\right] \quad (11)$$

and

$$\text{standard deviation of the model (SD)} = \sqrt{\frac{SSQ_{residual}}{(m-j)}} \quad (12)$$

where SSQ is the sum of squares.

Study on ITC Stability. *Sample Preparation.* Because of the presence of ESP in broccoli, very little ITCs are formed after myrosinase hydrolysis of the endogenous glucosinolates (*9*). To perform an adequate kinetic study, synthetic DL-sulforaphane (Sigma, St. Louis, MO) and 2-phenylethyl ITC (Fluka, Buchs, Switzerland) were used in this study. The stock solutions of these ITCs were prepared by dissolving 7.5 μL of each ITC in 500 μL of methanol. Prior to treatments, the sulforaphane stock solution (1%) was mixed with the centrifuged (15000*g*, 5 min, 4 °C) broccoli juice, and the phenylethyl ITC stock solution (1%) was mixed with a centrifuged (15000*g*, 5 min, 4 °C) broccoli juice/methanol (1:1) solution, because of an insolubility problem.

Determination of ITC Concentration. To determine the concentration of ITC present in the samples, the method described by Zhang et al. (*6*) was applied. After treatments, 100 μL of the sample was mixed with 900 μL of phosphate buffer (pH 8.5, 0.1 M) and 1 mL of methanol containing 80 mM benzene-1,2-dithiol (Fluka). This mixture was incubated for 90 min at 60 °C. Afterward, it was cooled at room temperature and the absorbance was measured spectrophotometrically at $\lambda = 365$ nm. In the case of sulforaphane in pure broccoli juice, an additional centrifugation step (5 min, 5000*g*, 4 °C) was necessary before absorption measurement to remove the precipitated proteins. This was not necessary for the phenylethyl ITC in the broccoli juice/methanol (1:1) mixture, probably because many proteins already precipitated in the first centrifugation step.

A standard curve was generated for both phenylethyl ITC and sulforaphane with r^2 values of 0.9958 and 0.9964, respectively. When a blank was temperature-treated (80 °C) or HP/T-treated (700 MPa and 50 °C) for different time intervals, there was no change in absorbance after the reaction with benzenedithiol, indicating that no products were formed that interfered with the assay.

Data Analysis. Analogous to the decrease of enzyme activity (A) as a function of processing time (t), a general n-th-order kinetic model can be used to describe the degradation of chemical compounds (eq 13).

$$\frac{dC}{dt} = -kC^n \quad (13)$$

where k is the inactivation rate constant, n represents the reaction order, and C is the concentration.

If the reaction rate (k) is independent of time (isothermal/isobaric conditions), a first-order reaction kinetic model ($n = 1$) can be integrated and linearized (eq 14), with C_o as the initial concentration at time = 0.

$$\ln(C) = \ln(C_0) - kt \quad (14)$$

Temperature dependence of the time-dependent model parameter (inactivation rate constant, k) can be described using the Arrhenius equation (eq 6).

Temperature Treatment. To achieve isothermal conditions, samples were filled in glass capillaries (250 μL, Blaubrand, Wertheim, Germany). The capillaries were placed in a water bath and heated for different preset time intervals at various constant temperatures. To stop the heating effect, the samples were immediately cooled in ice water after their withdrawal and stored in ice water until the measurement of the residual enzyme activity or ITC concentrations.

Pressure−Temperature Treatment. Pressure−temperature treatments were performed in an eight-vessel (8 × 8 mL) laboratory pilot-scale high-pressure equipment (Resato, Roden, Netherlands), which allows pressurization up to 800 MPa in combination with temperatures from −20 to 60 °C. The pressure medium was a glycol−oil mixture (TR 15, Resato, Roden, Netherlands). The enzyme solution was enclosed in 0.3 mL flexible microtubes (Elkay, Leuven, Belgium). Pressure was built up using a standardized pressurization rate of 100 MPa/min. Following a temperature equilibration time of 2 min after pressure build-up, a blank was taken. After decompression, the samples were withdrawn from the vessel and immediately stored in ice water until the measurement of the residual enzyme activity or ITC concentrations.

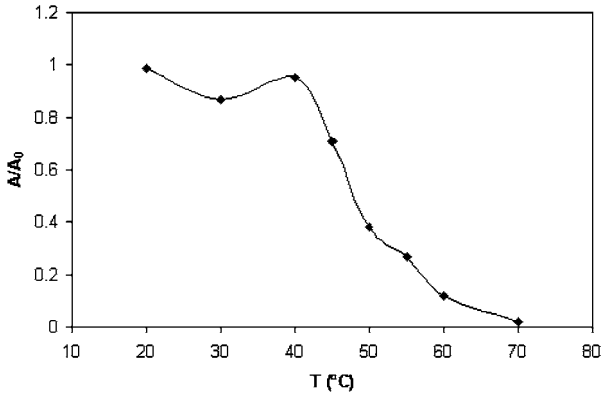

Figure 1. Activity retention of endogenous myrosinase in broccoli juice after 10 min thermal treatments at various temperatures.

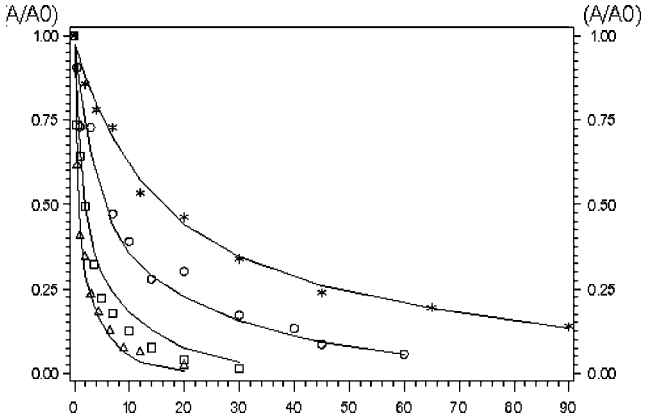

Figure 2. Thermal inactivation of broccoli myrosinase in broccoli juice described by the consecutive step model with inserted Arrhenius equation (eq 8). Key: *, 45 °C; °, 50 °C; ◇, 55 °C; and △, 60 °C.

RESULTS AND DISCUSSION

Thermal Stability of Endogenous Myrosinase in Broccoli Juice. To determine the thermal stability of endogenous myrosinase in broccoli juice, the juice was treated for 10 min at various temperatures. **Figure 1** shows that broccoli myrosinase was stable up to 40 °C, and its activity was reduced by 90% after a 10 min thermal treatment at 60 °C. This is in good agreement with literature, i.e., myrosinase in crude broccoli extract in phosphate buffer (pH 6.55) is stable until 35 °C (*21*). The detailed thermal inactivation kinetics of endogenous myrosinase in broccoli juice was investigated in a temperature range from 45 to 60 °C.

Endogenous myrosinase inactivation in broccoli juice could not be described by a first-order kinetic model (eq 3). A consecutive step model was applied to model the thermal inactivation data of endogenous myrosinase (**Figure 2**) in broccoli juice. The estimated kinetic parameters are presented in **Table 1**. This model was also used to describe the thermal inactivation of broccoli myrosinase in buffer (*21*). As compared to the results of this study, broccoli myrosinase seems to be more stable in broccoli juice than in buffer solution (e.g., the *k* value of the labile phase at 40 °C in juice is 10 times smaller than in the buffer system) and the inactivation rate constants of broccoli myrosinase in buffer had a lower temperature sensitivity (113.5 ± 14.1 and 134.1 ± 28.3 kJ/mol, respectively, for the first and the second inactivation step) than that of endogenous myrosinase in broccoli juice.

Matusheski and co-workers (*9*) still found a high glucosinolate conversion in homogenized 5 cm long broccoli florets, which were treated for 5 or 10 min at 60 °C. This could be due to phenomena of heat transfer and the long (8 h) incubation time. In Brussels sprouts, the effect of blanching on the myrosinase stability has been reported (*24*). A treatment at 90 °C for 2 min destroyed 74.2% of the myrosinase in the outer zone while the inactivation in the inner part was lower, probably due to heat transfer limitations. In literature, the inactivation kinetics of myrosinase in a food matrix, i.e., red cabbage juice, are reported by Verkerk and Dekker (*23*). The red cabbage myrosinase inactivation studied at temperatures between 25 and 70 °C is described by a first-order model, and the estimated E_a value is 155 kJ/mol (comparable to this study).

Table 1. Estimated Kinetic Parameters for Thermal Inactivation of Endogenous Myrosinase in Broccoli Juice Based on a Consecutive Step Model (Eq 8) with T_{ref} = 52.5 °C

parameter	estimated value
A_1 (%)	97.48 ± 1.77
A_2 (%)	38.60 ± 5.50
$k_{1,ref}$ ($\times 10^{-1}$ min^{-1})	4.35 ± 0.77
$k_{2,ref}$ ($\times 10^{-1}$ min^{-1})	0.558 ± 0.091
E_{a1} (kJ/mol)	185.36 ± 9.72
E_{a2} (kJ/mol)	161.83 ± 13.37

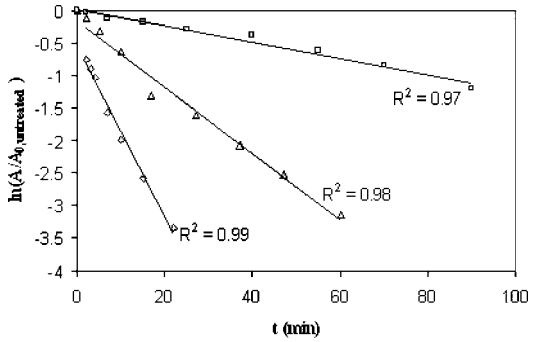

Figure 3. Inactivation of broccoli myrosinase at 10 °C in combination with different pressures: □, 400 MPa; △, 500 MPa; and ◇, 600 MPa. An untreated sample is considered as blank.

Combined Pressure/Temperature Stability of Endogenous Myrosinase in Broccoli Juice. The inactivation kinetics of myrosinase in broccoli juice was studied in the temperature range from 10 to 60 °C and the pressure range from 100 to 600 MPa. During pressure build up and after an equilibration time of 2 min (after the achievement of the desired pressure), a 3.4−86.8% decrease in myrosinase activity was observed, depending on the pressure and temperature applied. It was clearly observed that during isobaric/isothermal conditions the decrease of endogenous myrosinase activity in broccoli juice as function of time followed a first-order reaction (only one fraction was observed; **Figure 3**). For example, there was only a 12% decrease in enzyme activity during pressure buildup for pressure treatment at 10 °C and 500 MPa and the inactivation could be well-described by a first-order model until a residual activity of 4%. It seems that one of the phases observed during the thermostability study disappears in the pressure stability. The consecutive step model can be used to describe enzyme inactivation where it is assumed that two forms of the enzyme are present, e.g., free enzyme and enzyme interacting with impurities or substrate, where one can be turned over in the other (*21*). It is possible that the interaction is broken during elevated pressure, leaving only one inactivation phase.

The estimated *k* values are summarized in **Table 2** (r^2 values between 0.911 and 0.994). On the basis of these estimated *k*

Table 2. k Values ($\times 10^3$ Min^{-1}) for Combined Pressure–Temperature Inactivation of Endogenous Myrosinase in Broccoli Juice[a]

	P (MPa)							V_a values
T (°C)	100	150	200	300	400	500	600	(cm^3/mol)
10					12.60 ± 0.73[b]	52.00 ± 2.38	130.58 ± 5.38	−27.52 ± 3.38
20					16.81 ± 1.20	76.92 ± 2.81	180.77 ± 9.61	−28.95 ± 4.69
30				4.37 ± 0.60	31.12 ± 1.77	134.93 ± 9.02	287.05 ± 17.82	−35.29 ± 4.80
40				9.46 ± 0.89	47.77 ± 1.98	250.18 ± 11.08		−42.64 ± 0.27
50	23.48 ± 1.52[b]	16.64 ± 1.38	14.00 ± 1.27	38.90 ± 2.03	103.11 ± 4.72			13.92 ± 2.67[c]
								−26.82 ± 0.37[d]
55	37.60 ± 2.39	33.02 ± 2.27	29.62 ± 1.70	47.88 ± 2.82	129.80 ± 7.23			6.53 ± 0.34[c]
								−20.16 ± 4.06[d]
60	66.89 ± 2.21	56.40 ± 5.86	51.25 ± 3.73	84.35 ± 2.89	158.71 ± 8.35			6.95 ± 0.92[c]
								−15.67 ± 1.05[d]
E_a values (kJ mol^{-1})	92.11 ± 5.44	109.32 ± 6.72	116.14 ± 9.35	84.81 ± 6.31	42.03 ± 2.55	38.75 ± 3.52	28.03 ± 3.36	

[a] V_a, activation volume; E_a, activation energy. [b] Standard error of regression. [c] Value for the activation volume ≤ 200 MPa. [d] Value for the activation volume ≥ 200 MPa.

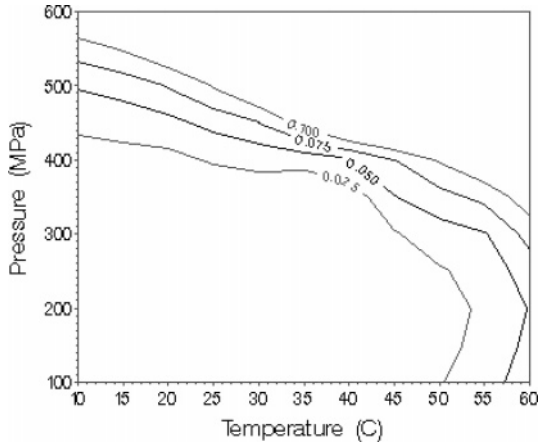

Figure 4. Isorate contour plot of the combined pressure–temperature inactivation of endogenous myrosinase in broccoli juice.

values, an antagonistic effect of pressure on thermal inactivation was observed since the inactivation was slower by increasing pressure for instance at 50, 55, or 60 °C from 100 to 200 MPa. When the inactivation at atmospheric pressure was compared to the inactivation at 100 MPa, it was noticed that a 20 min treatment at 60 °C and atmospheric pressure reduced the activity to 3%, while during isobaric/isothermal treatment for the same treatment time at 60 °C and 100 MPa 25% of the activity was retained (pressure build-up had already resulted in 77% of the myrosinase inactivation). For broccoli myrosinase, this antagonistic effect was also reported by Ludikhuyze et al. (*21*) for the first enzyme form and the highest protective effect occurred at 350 MPa and 35 °C. This phenomenon was also previously reported for other enzymes such as α-chymotrypsin (*36*), avocado polyphenoloxidase (*37*), tomato (*29*), and orange pectinmethylesterase (*38*).

The activation energy of myrosinase inactivation was the highest at 200 MPa, the pressure at which the antagonistic effect was most outspoken. In the HP/T area where the antagonistic effect occurred, the V_a values are positive. The most negative V_a value was at 40 °C (**Table 2**). At 20 °C, the activation volume of endogenous myrosinase in broccoli juice was approximately the same as for the second phase of the broccoli myrosinase inactivation in phosphate buffer (*21*). The V_a value of broccoli myrosinase inactivation in buffer at 35 °C was higher than at 20 °C, while in juice V_a seems to decrease until 40 °C.

On the basis of the estimated kinetic parameters from combined high-pressure/thermal inactivation data, an isorate contour plot can be drawn (**Figure 4**). The lines as interpolated from the experimental data represent combinations of pressure and temperature resulting in the same inactivation rate constant (k) for myrosinase inactivation in broccoli juice.

Table 3. Estimated Parameters of Myrosinase Inactivation in Broccoli Juice as a Function of Pressure and Temperature Using the Modified Hawley Model (Eq 9) and the Third-Order Model (Eq 10), Both without the Term $b(P - P_0)(T - T_0)$ (P_0 = 400 MPa and T_0 = 40 °C)

	estimated values	
parameter	modified Hawley model	third-order model
$\Delta\beta^{\neq}$ (cm^6/J mol)	−0.0869 ± 0.0159	−0.0237 ± 0.0134
$\Delta V_0^{\neq}$ (cm^3/mol)	−26.63 ± 1.99	37.77 ± 2.05
$\Delta S_0^{\neq}$ (J/mol K)	175.2 ± 18.3	185.9 ± 10.9
$\Delta C_p^{\neq}$ (J/mol K)	1410.4 ± 504.3	1460.3 ± 298.1
k_0 (min^{-1})	0.0413 ± 0.0052	0.0485 ± 0.0038
$\Delta\beta_2^{\neq}$ (cm^9/J^2 mol)		(4.90 ± 0.74) × 10^{-4}
r^2	0.87	0.95
SD	0.41	0.25

The Hawley model (eq 9) was applied to describe the combined pressure/temperature dependence of the inactivation rate constant (k) of myrosinase in broccoli juice. On the basis of the estimated model parameters, it was noticed that a higher r^2 and a lower SD were obtained when the term $b(P - P_0)(T - T_0)$ was omitted. The estimated parameters of eq 9 without this term are summarized in **Table 3**.

By inserting the estimated parameters (**Table 3**) in eq 9, it was found that the modified Hawley model was not able to describe the antagonistic effect of pressure and temperature in the low pressure/high temperature area (**Figure 5a**). Therefore, the third-order model (eq 10) was used to describe the experimental data (**Figure 5b**), again without $b(P - P_0)(T - T_0)$. This model gives a better representation of the experimental data and clearly indicates the antagonistic effect. A third-order pressure model was also used to describe the inactivation of plasmin (*39*) and pectinmethylesterase of orange (*38*), pepper (*40*), banana, and carrot (*41*, *42*).

When the experimental k values are compared to the predicted k values (**Figure 6**), it is clear that the correlation resulting from the third-order model (eq 10) is better than the second-order model (eq 9) that is represented in the corrected r^2 and the SD (**Table 3**).

Stability of Sulforaphane and Phenylethyl ITC. The thermal stability of sulforaphane and phenylethyl ITC was screened by heating the sample for 20 min at various temperatures (**Figure 7**). These two ITCs were chosen because they can occur naturally in broccoli. Sulforaphane can be formed

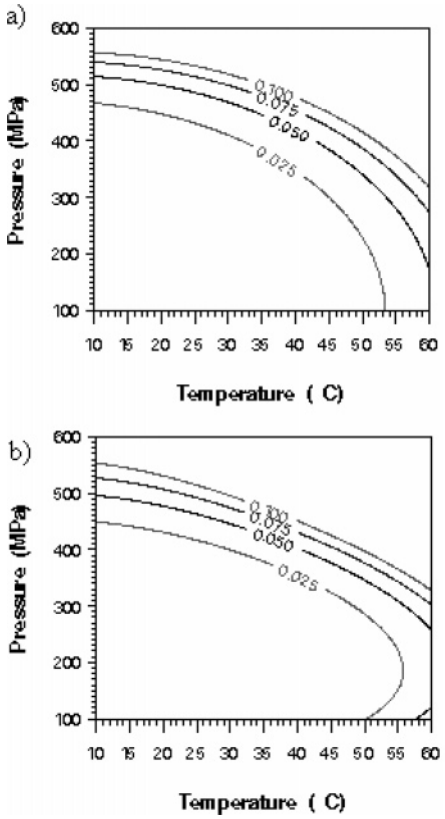

Figure 5. Simulated isorate contour plot of myrosinase inactivation in broccoli juice as function of pressure and temperature using (**a**) the modified Hawley model (eq 9) and (**b**) the third-order model (eq 10), both without $b(P - P_0)(T - T_0)$ (P_0 = 400 MPa and T_0 = 40 °C).

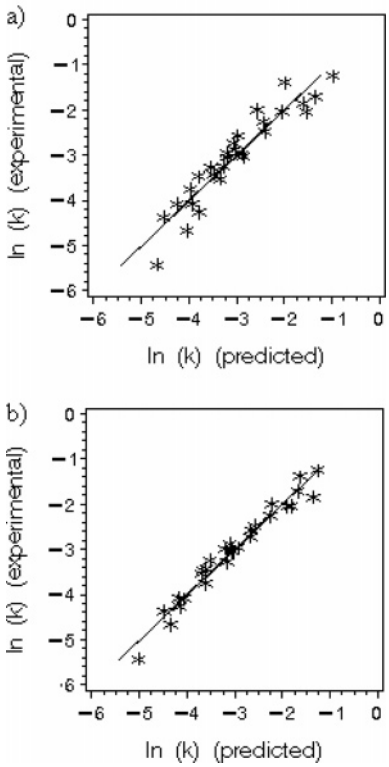

Figure 6. Correlation between experimental and predicted *k* values based on (**a**) a modified Hawley model and (**b**) an extended modified Hawley model.

after hydrolysis of glucoraphanin, while phenylethyl ITC can be formed after hydrolysis of gluconasturtiin, both present in broccoli (*3*, *4*). The ITCs were stable until 60 °C and were degraded for more than 90% after a 20 min treatment at 90 °C.

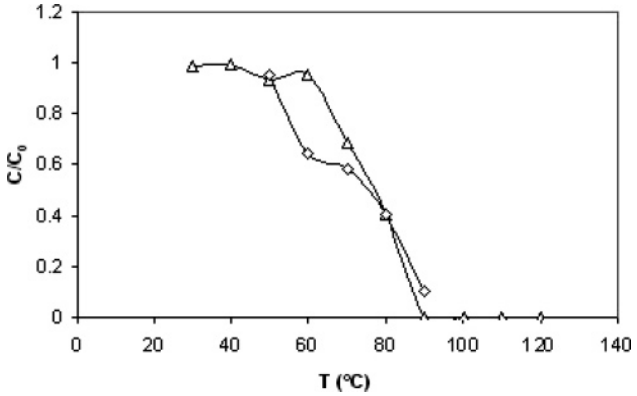

Figure 7. Residual concentrations of sulforaphane (◊) and phenylethyl ITC (△) after 20 min thermal treatments at various temperatures.

Table 4. Estimated Kinetic Parameters ($\times 10^2$ Min^{-1}) for the Thermal Degradation of Sulforaphane and Phenylethyl ITC in Broccoli Juice

T (°C)	sulforaphane	r^2	phenylethyl ITC	r^2
60	0.752 ± 0.065[a]	0.96	0.625 ± 0.087	0.88
70	1.418 ± 0.194	0.91	1.397 ± 0.061	0.99
80	4.115 ± 0.507	0.94	2.731 ± 0.123	0.99
90	10.145 ± 0.860	0.97	5.233 ± 0.661	0.93
E_a (kJ/mol)	89.03 ± 7.26		70.92 ± 1.40	

[a] Standard error of regression.

Table 5. Estimated Kinetic Parameters ($\times 10^2$ Min^{-1}) for Combined Pressure–Temperature Degradation of Sulforaphane in Broccoli Juice

	P (MPa)		
T (°C)	600	700	800
30			0.142 ± 0.010[a]
40	0.213 ± 0.018[a]	0.213 ± 0.070	0.332 ± 0.046
50	0.546 ± 0.052	0.438 ± 0.027	1.467 ± 0.123
60	1.467 ± 0.188	1.372 ± 0.144	2.114 ± 0.175
E_a (kJ/mol)	83.64 ± 2.67	80.57 ± 11.98	80.70 ± 1.15

[a] Standard error of regression.

Table 6. Estimated Kinetic Parameters ($\times 10^2$ Min^{-1}) for Combined Pressure–Temperature Degradation of Phenylethyl ITC in a Broccoli Juice/Methanol (1:1) Mixture

	P (MPa)		
T (°C)	600	700	800
30		0.086 ± 0.018	0.051 ± 0.020[a]
40	0.322 ± 0.020[a]	0.313 ± 0.015	0.258 ± 0.054
50	0.515 ± 0.042	0.561 ± 0.033	0.564 ± 0.063
60	0.814 ± 0.065	1.200 ± 0.094	1.346 ± 0.072
E_a (kJ/mol)	40.21 ± 0.42	71.69 ± 7.76	89.21 ± 9.77

[a] Standard error of regression.

The detailed thermal degradation kinetics of both ITCs were studied in the temperature range from 60 to 90 °C. A first-order kinetic model was applied to describe the degradation of both ITCs (**Table 4**). The sulforaphane degradation rate was more temperature sensitive than that of phenylethyl ITC, as shown by the E_a values.

Under pressure (**Tables 5** and **6**), the degradation rate constants of both ITCs are situated in the same order of magnitude. Combined HP/T processing seems to enhance the

ITC degradation, but the effect is much smaller than during thermal treatment at atmospheric pressure; for example, degradation at 60 °C and 800 MPa is only half as fast as at 80 °C at atmospheric pressure.

These data indicate that during the traditional boiling of broccoli, the formed sulforaphane is rapidly degraded. In contrast, a high-pressure treatment in combination with mild temperatures could be an alternative to the thermal process in which the health beneficial ITCs, in particular sulforaphane, are still maintained.

In conclusion, in this work, the stability of endogenous myrosinase in broccoli juice during combined temperature−pressure treatments was studied. The thermal inactivation of the myrosinase could be described by the consecutive step model, while during combined isobaric−isothermal treatment, broccoli myrosinase inactivation followed first-order kinetics. In the high temperature−low pressure domain, an antagonistic effect was observed between temperature and pressure on the myrosinase inactivation. Because glucosinolate hydrolysis products in broccoli, such as sulforaphane, are believed to have beneficial health effects, a mild pressure treatment where the myrosinase is stabilized could be advantageous. On the basis of the obtained kinetic results, it is clear that ITC had a higher HP/T stability than myrosinase, indicating that these glucosinolate breakdown products are stable during HP/T treatments where myrosinase is still active. Furthermore, to optimize the health-promoting properties of *Brassicaceae*, beneficial compounds should be retained during food processing. On the basis of this work, it was found that sulforaphane and phenylethyl ITC were temperature labile but pressure stable. Glucosinolate hydrolysis products can give *Brassicaceae* a specific taste and odor, often not appreciated by consumers. The obtained kinetic information about the pressure/temperature effect on myrosinase and ITC stability can be further used for process evaluation and optimization. The application of mild pressure in intact vegetable is also interesting because pressure processing has simultaneous effects on cell disruption and enzyme activation. However, further investigation is still needed to study the myrosinase activity and stability in intact vegetables.

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