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Temperature and pressure stability of mustard seed (*Sinapis alba* L.) myrosinase

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Abstract

Myrosinase, an enzyme found in all glucosinolate containing plants, is responsible for the conversion of glucosinolates into products that can be beneficial to our health. In this study, the temperature and pressure stability of partially purified myrosinase from mustard seeds was studied in a model system. Temperature inactivation started at 60 °C and the inactivation kinetics were studied in detail between 65 and 75 °C. Inactivation could be described by the consecutive step or the biphasic model. Mustard seed myrosinase was quite pressure stable, as its activity was retained after pressure treatments up to 600 MPa combined with temperatures up to 60 °C. At low pressures there was an antagonistic effect between pressure and thermal treatment, since myrosinase activity was retained after treatments at 70 °C up to 300 MPa. This pressure stability indicates that pressure treatment may be a valuable alternative for thermal treatment if one wants to retain myrosinase activity.

Keywords: Temperature; Pressure; Myrosinase; Stability; Mustard seed

1. Introduction

Brassicaceae, such as cabbage, broccoli, cauliflower, mustard and Brussels sprouts contain high concentrations of glucosinolates, a group of nitrogen and sulfur containing natural pseudo-glucosides. Glucosinolates belong to a broad group of non nutritive, bioactive phytochemicals that can play an important role in human health and well being (Fahey, Zalcmann, & Talalay, 2001). All glucosinolate-containing-plants also contain myrosinase (thioglucoside glucohydrolase EC 3.2.1.147, formerly EC 3.2.3.1). This enzyme hydrolyzes glucosinolates to form an aglucone and D-glucose. The aglucone is unstable and spontaneously decomposes into nitriles, thiocyanates, isothiocyanates or indoles depending on the side chain, pH, presence of ferrous ions and proteins such as epithiospecifier protein. These compounds give the vegetables not only their typical odor and taste (Fenwick, Griffiths, & Heaney, 1983) but also a possible health promoting effect (Verhoeven, Verhagen, Goldbohm, Van den Brand, & Van Poppel, 1997) since they inhibit tumorigenesis (Smith, Lund, & Johnson, 1998) and play an important role in the prevention of heart diseases (Wu et al., 2004).

Cell disruption, for instance by cutting or cooking, can bring myrosinase in contact with its substrate. During conventional thermal processing, such as blanching, myrosinase will be inactivated and can not transform the glucosinolates into the beneficial products. In the human gut, microorganisms can hydrolyze glucosinolates but the beneficial effects of the reaction products are less pronounced than when the glucosinolates are hydrolyzed by the endogenous plant myrosinase (Smith, Mithen, & Johnson, 2003). Therefore, it may

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be beneficial to control the activity and the stability of myrosinase during processing.

Most research has been focused on the health effects of glucosinolates and their reaction products (Myzak, Karplus, Chung, & Dashwood, 2004; Wang et al., 2004). However, only little information about myrosinase stability during processing is available and most of the research has been done in crude extracts or in vegetable juice. Previous studies (Matusheski, Juvik, & Jeffery, 2004; Wathelet, Mabon, Foucart, & Marlier, 1996; Yen & Wei, 1993) have shown that thermal treatment could inactivate myrosinase. The kinetics of myrosinase inactivation during thermal treatment has been investigated in crude broccoli extract (Ludikhuyze, Ooms, Weemaes, & Hendrickx, 1999) and red cabbage juice (Verkerk, van Boekel, Jongen, & Dekker, 2002). In contrast to the low temperature stability of broccoli myrosinase, myrosinase from other sources seems to be more stable. Also, in broccoli tissue myrosinase seems to be more stable (Matusheski et al., 2004). Broccoli myrosinase in crude extract is relatively labile during high hydrostatic pressure treatments as previously reported by Ludikhuyze and coworkers (1999).

In food processing, new technologies, like high hydrostatic pressure treatment, are investigated. High hydrostatic pressure has the advantage that vegetative microorganisms can be inactivated under conditions that only have a limited effect on quality attributes such as nutritional compounds (San Martìn, Barbosa-Canovas, & Swanson, 2002). Since broccoli myrosinase is more thermolabile then myrosinase from other sources, it can be questioned whether the pressure stability of broccoli myrosinase is a general characteristic of myrosinase or if it also depends on the source.

Therefore, the purpose of this research was to study the effect of temperature and high pressure on the stability of myrosinase partially purified from mustard seed (*Sinapis alba* L.).

2. Materials and methods

2.1. Sample preparation

A single batch of mustard seed (*S. alba* L.) purchased from a local shop was used for this study. A myrosinase crude extract was prepared based on the procedure of water soluble myrosinase described by Eriksson, Ek, Xue, Rask, and Meijer (2001). The seeds (25 g) were washed with distilled water and homogenized in 150 ml imidazol-HCl buffer (20 mM, pH 6.0) containing 150 mM NaCl, 0.1 mM EDTA, 1.6 mM dithioerythritol (DTE) and 6.25 g insoluble PVP. The homogenate was mixed at 4 °C for 15 min using end over end rotation and centrifuged (15,000*g*, 10 min, 4 °C) to remove the insoluble cell material and the PVP. The supernatant was 40% saturated with $(NH_4)_2SO_4$ and stirred at 4 °C for 30 min. The insoluble proteins were removed by centrifugation (18,000*g*, 15 min, 4 °C). Afterwards, the supernatant was 90% saturated with $(NH_4)_2SO_4$, stirred and centrifuged. The resulting pellet was dissolved in Bis–Tris buffer (20 mM, pH 6.5) and extensively dialyzed (for 18 h at 4 °C) against the same buffer. After dialysis, the insoluble material was removed by filtration through a cheese cloth and a sintered glass filter. The remaining light yellow extract was lyophilized (Christ Alpha 2–4 freeze-dryer, Osterode am Harz, Germany) and afterwards stored at −20 °C before further purification.

Myrosinase was further purified using anion-exchange chromatography (AEX). The lyophilized extract was dissolved in water (100 mg/ml) and 5 ml of the extract was applied to a HiPrep 16/10 Q XL column (Amersham Biosciences, Uppsala, Sweden). The column was equilibrated using Bis–Tris buffer (20 mM, pH 6.5) at a flow rate of 4 ml/min and the bound proteins were separated using a linear NaCl gradient (0–0.25 M). The flow rate of the gradient elution was 0.5 ml/min. The fractions (4 ml) containing myrosinase activity were pooled, concentrated and desalted using 10 kDa cut off filters (Centricon Plus-20, Amicon bioseparations, Millipore, Billerica, Massachusetts). The pooled and concentrated fractions containing myrosinase activity were used as sample throughout this study. Prior to temperature and pressure treatments, the concentrated sample was diluted in Bis–Tris buffer (20 mM, pH 6.5). A pH of 6.5 was chosen because this is the pH of broccoli juice.

2.2. Determination of myrosinase activity

Myrosinase activity was determined following a coupled enzymatic procedure described by Gatfield and Sand (1983) in which the glucose formed due to the reaction between myrosinase and sinigrin as a substrate could be used to transform $NADP^+$ to NADPH. In this investigation, the D-Glucose/D-Fructose test kit (Cat no. 10 139 106 035, Boehringer-Mannheim/R-Biopharm, Darmstadt, Germany) was used. The reaction mixture consisted of 0.9 ml of a water solution containing 0.05 g/l $MgCl_2$ and 1 g/l ascorbic acid, 0.5 ml ATP/$NADP^+$ solution (test kit solution 1), 10 µl hexokinase/glucose-6-P-dehydrogenase (test kit solution 2) and 50 µl sample containing myrosinase solution. After homogenization, 50 µl substrate (sinigrin, 0.3 g/ml, Sigma, St. Louis, MO) was added and the formation of NADPH was spectrophotometrically followed at 340 nm and 23 °C for 10 min. The activity was determined based on the slope of the initial linear part of the curve of absorbance versus reaction time. One unit of myrosinase is defined as the amount of enzyme that forms 1 µmol glucose per min at 23 °C and pH 7.6 when sinigrin is used as a substrate.

2.3. Protein determination

Protein concentration was determined using the bicinchoninic acid (BCA) kit for protein determination (Sigma Procedure No. TRPO-562, St. Louis, MO). This method is based on the reduction of Cu^{2+} by proteins in alkali environment. Bicinchonininic acid (BCA) forms a colored complex with the Cu^{+} formed. The absorbance of this complex was measured at 562 nm and 25 °C. The protein content was determined by comparison with a standard curve using bovine serum albumin.

2.4. Molecular mass and pI determination

Under non denaturing conditions, the molecular mass of myrosinase was estimated using gel filtration and gel electrophoresis. For gel filtration analysis, the pooled and concentrated fractions containing myrosinase activity obtained after anion exchange chromatography were applied to a Sephacryl S-200 HR column (Amersham Biosiences, Uppsala, Sweden). The same elution buffer as for anion exchange chromatography (i.e., Bis–Tris, 20 mM, pH 6.5) was used. The elution flow rate was 0.3 ml/min. The molecular mass of myrosinase was compared to the high (HM) and low (LM) molecular mass gel filtration calibration kits (Amersham Biosciences, Uppsala, Sweden). For gel electrophoresis, native-polyacrylamide gel electrophoresis (Native–PAGE) was performed using Phastgel gradient 8-25 gels and the HMW Calibration Native Marker Kit (Amersham Biosciences, Uppsala, Sweden). The pI of myrosinase was determined using Phastgel IEF PAGE (for pH range of 3.0–9.0) and the isoelectric focusing calibration kit broad pI (pH 3–10) (Amersham Biosciences, Uppsala, Sweden) was used as a standard.

To estimate the molecular mass of the myrosinase subunits, sodiumdodecylsulfate (SDS) PAGE was applied, using PhastGel homogeneous 20% gels and Phastgel Tris–tricine SDS buffer strips (Amersham Biosciences, Uppsala, Sweden). The samples were boiled for 5 min in a Tris-buffer (50 mM, pH 8.0) containing EDTA (5 mM), SDS (2.5%) and β-mercaptoethanol (5%). As standards, the low molecular weight calibration kit for SDS electrophoresis (Amersham Biosciences, Uppsala, Sweden) were used.

For all electrophoresis experiments, the Phastsystem (Amersham Biosciences, Uppsala, Sweden) and silver nitrate staining as described by Heukeshoven and Dernick (1985) were used.

2.5. Temperature treatment

To achieve isothermal conditions, the sample was heated in capillaries (100 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 residual enzyme activity measurement (max 1 h).

2.6. Pressure treatment

Pressure treatments were performed in a 6-vessel (6×40 ml) laboratory pilot scale high pressure equipment (Resato, Roden, Netherlands) which allows pressurization up to 800 MPa in combination with temperatures from 10 to 70 °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 pressurization rate of 100 MPa/min. After decompression, the samples were immediately stored in ice water until measurement of the residual enzyme activity (max 1 h).

2.7. Data analysis

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

$$\frac{dA}{dt} = -k.A^n, \tag{1}$$

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

In case of first order reaction kinetics ($n = 1$) and a reaction rate (k) that is independent of time (isothermal/isobaric conditions), the general nth order kinetic model can be simplified, integrated and linearized

$$\ln(A) = \ln(A_0) - kt, \tag{2}$$

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

In the biphasic model, two enzyme fractions are assumed (Eq. (3)), i.e., a thermolabile (N_L) and a more stable fraction (N_S), that both follow a first order kinetic inactivation model. Assuming that the inactivation of both fractions are independent of each other, the mathematical model is given in Eq. (4)

$$N_L \xrightarrow{k_L} I_L \quad \text{and} \quad N_S \xrightarrow{k_S} I_S \tag{3}$$

$$A = A_L \exp(-k_L t) + A_S \exp(-k_S t), \tag{4}$$

where A_L indicates the enzyme activity of the labile form (N_L); A_S the enzyme activity of the stable form (N_S); k_L and k_S are the inactivation rate constants, respectively, to obtain I_L and I_S (min^{-1}).

Ludikhuyze et al. (1999) have reported that myrosinase inactivation in broccoli crude extract could be described by a consecutive step model (Eq. (5)). This model assumes that the enzyme inactivation occurs in

two irreversible consecutive steps. In the first step, an intermediate (I_1) is formed with a different activity than the native form (N) and in the second step, this intermediate (I_1) is turned over to the inactivated form (I_2) (Eq. (6))

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

$$N \xrightarrow{k_1} I_1 \xrightarrow{k_2} I_2, \quad (6)$$

where A_1 indicates the enzyme activity of the native form (N); A_2 the enzyme activity of the intermediate form (I_1); k_1 and k_2 the inactivation rate constants, respectively, to obtain I_1 and I_2 (min^{-1}).

Temperature dependence of the time dependent model parameters (inactivation rate constant, k values) was described using the Arrhenius equation

$$k = k_{\text{ref}} \exp\left(\frac{E_a}{R_t}\left(\frac{1}{T_{\text{ref}}} - \frac{1}{T}\right)\right), \quad (7)$$

where k_{ref} represents the inactivation rate constant at reference temperature (T_{ref}) (min^{-1}) and R_t the universal gas constant (8.314 J mol^{-1} K^{-1}).

To determine the model parameters, the Arrhenius equation can be substituted in the biphasic (Eq. (8)) and consecutive step (Eq. (9)) model

$$A = A_L \exp\left(-k_{L,\text{ref}} \exp\left(\frac{E_a}{R_t}\left(\frac{1}{T_{\text{ref}}} - \frac{1}{T}\right)\right)t\right) + A_S \exp\left(-k_{S,\text{ref}} \exp\left(\frac{E_a}{R_t}\left(\frac{1}{T_{\text{ref}}} - \frac{1}{T}\right)\right)t\right), \quad (8)$$

$$A = (A_1 - A_2 a)\exp\left(-k_{1,\text{ref}} \exp\left(\frac{E_a}{R_t}\left(\frac{1}{T_{\text{ref}}} - \frac{1}{T}\right)\right)t\right) + (A_2 a)\exp\left(-k_{2,\text{ref}} \exp\left(\frac{E_a}{R_t}\left(\frac{1}{T_{\text{ref}}} - \frac{1}{T}\right)\right)t\right) \quad (9)$$

with

$$a = \frac{k_{1,\text{ref}} \exp\left(\frac{E_a}{R_t}\left(\frac{1}{T_{\text{ref}}} - \frac{1}{T}\right)\right)}{k_{1,\text{ref}} \exp\left(\frac{E_a}{R_t}\left(\frac{1}{T_{\text{ref}}} - \frac{1}{T}\right)\right) - k_{2,\text{ref}} \exp\left(\frac{E_a}{R_t}\left(\frac{1}{T_{\text{ref}}} - \frac{1}{T}\right)\right)}.$$

The kinetic parameters were estimated using non linear regression analysis (SAS, 1992).

3. Results and discussion

3.1. Partial purification and characterization of myrosinase from S. alba L. seeds

The chromatogram of myrosinase purification using anion-exchange chromatography is depicted in Fig. 1. The peak at the elution volume between 112 and 120 ml contained the highest activity of myrosinase. The purification factor is given in Table 1. Based on the result of SDS–PAGE Fig. 3(a), five major bands at 21, 25, 29, 36 and 67 kDa and two minor bands at 17 and 47 kDa were observed. In the literature, mustard seed myrosinase is described as a dimer of 140 kDa (Palmieri, Iori, & Leoni, 1986) with two identical subunits (Burmeister, Cottaz, Driguez, Iori, & Palmieri, 1997). It could be that the band at 67 kDa was the myrosinase monomer. It is not clear whether the other bands are impurities or belong to a complex with the myrosinase protein.

To verify whether the other bands are impurities or not, the molecular mass of myrosinase as a native protein was estimated using gel filtration (GF) (Fig. 2). For this purpose, the anion-exchange fractions containing myrosinase activity were pooled, concentrated and applied to a Sephacryl S-200 HR column. The peak that showed myrosinase activity was outside the separation range of the column (i.e., 5–250 kDa) which implies that the molecular mass of myrosinase in this study (>250 kDa) was higher than that described in the literature. Therefore, a Native–PAGE was carried out and indeed, bands at 221 and around 282 were observed. To see which proteins contain myrosinase activity, a Native–PAGE was run but instead of silver staining, the gel was incubated in Bis–Tris buffer (20 mM, pH 6.5) containing ascorbic acid (0.06 mg/ml), $MgCl_2$ (0.03 mg/ml), sinigrin (10 mg/ml) and $BaCl_2$ (0.1 g/ml). After incubation for 1 hour, 3 white bands of $BaSO_4$ were visible on the gel. The clearest precipitation band was located around 221 kDa and the other 2 were located around 282 kDa. When the pooled GF fractions containing myrosinase activity were applied to the SDS–PAGE (Fig. 3(a), lane 5), it was observed that the distribution of the subunit bands from the GF fractions were similar to the anion exchange fractions except for one band at 29 kDa which disappeared after gel filtration. These data indicate the presence of a large protein complex containing myrosinase activity. If one monomer of myrosinase was around 70 kDa (Burmeister et al., 1997), the purified myrosinase in this study could exist as a protein complex containing more than 2 subunits. In the literature, this was also found for myrosinase in rapeseed. Myrosinase can be bound with myrosinase binding proteins (MBPs) or myrosinase associated proteins (Rask et al., 2000). These MBPs have a molecular mass of 30–110 kDa, and can form complexes up to 250–800 kDa. MBPs are also present in *S. alba* (Eriksson et al., 2001). Further research is necessary to get a conclusive identification of the different bands that appear after SDS–PAGE.

On IEF of mustard seed myrosinase purified using AEX and GF (Fig. 3c), 4 bands in the range of pI 5.5–5.2 were found. These are in good agreement with the values found for mustard seed myrosinase by

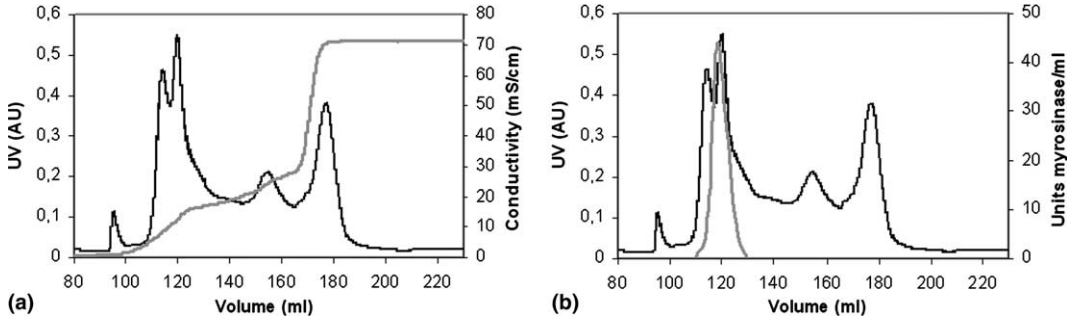

Fig. 1. Chromatogram of myrosinase purification using anion exchange chromatography (Hiprep Q 16/10 XL). (a) UV-spectrum (black line) and conductivity-profile (grey line) during the elution. (b) UV-spectrum (black line) and myrosinase activity (units/ml, grey line) during the elution.

Table 1
Purification of myrosinase from *S. alba* L. seeds using anion exchange chromatography (AEX) and gel filtration (GF)

	Units myrosinase/ml	Protein concentration (mg/ml)	Units myrosinase/mg protein	Purification factor
Crude extract mustard seed	11.8	9.38	1.26	1.0
Pooled AEX fractions	15.37	0.99	15.45	12.3
Pooled GF fractions	13.20	0.38	34.58	27.4

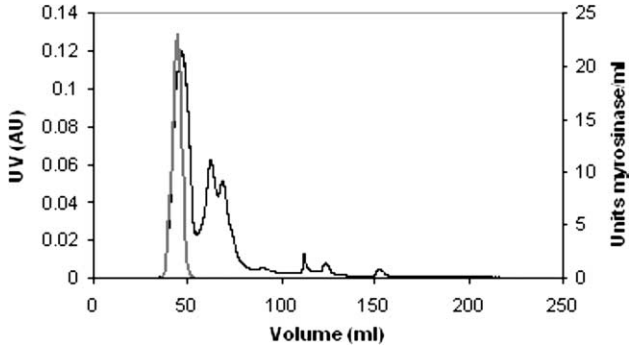

Fig. 2. Chromatogram of myrosinase purification using gel filtration (Sephacryl S-200 HR). UV-spectrum (black line) and myrosinase activity (units/ml, grey line) during the elution.

Pessina, Thomas, Palmieri, and Luigi Luisi (1990). There are also bands visible with a pI below 5. These are probably due to subunit dissociation since it is unlikely that after ion-exchange a mixture of proteins with such a broad pI range is obtained.

3.2. Temperature stability of *S. alba* L. myrosinase

To screen the temperature stability of the partially purified myrosinase, the evolution of the enzyme activity was followed after thermal treatment for 10 min at various temperatures (Fig. 4). Myrosinase was stable until 60 °C, and its activity was reduced by 90% after thermal treatment at 75 °C for 10 min. This is in good agreement with the literature. Bjorkman and Lönnerdal (1973) concluded that the inactivation of myrosinase extracted from *S. alba* L. and *Brassica napus* seeds occurred at temperatures above 60 °C (30 min treatment). The detailed thermal inactivation kinetics of *S. alba* L. myrosinase was studied in the temperature range from 65 to 75 °C.

Both the consecutive step (Fig. 5) and the biphasic (Fig. 6) model were used to describe the time dependence of myrosinase inactivation. A good fitting between the model predictions and the experimentally determined enzyme activity is found for both. The estimated kinetic parameters (Table 2) for both models are very similar

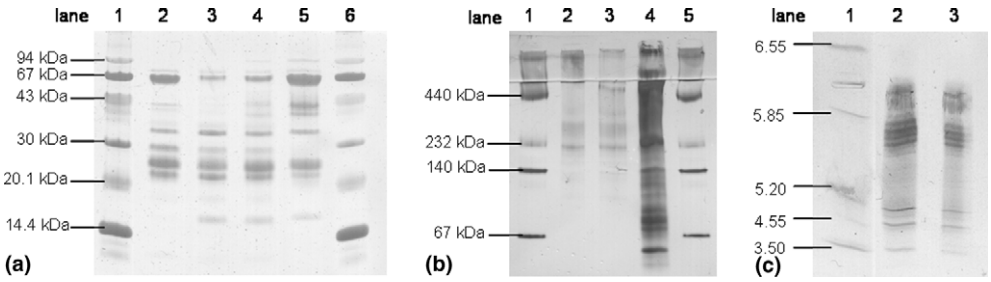

Fig. 3. (a) SDS–PAGE of purified myrosinase. Lane 1: standard; lane 2: AEX elution volume 112–116 ml; (fraction 9); lane 3: AEX elution volume 116–120 ml (fraction 10); lane 4: pooled fractions (8–10) from AEX; lane 5: pooled fractions after GF. (b) Native–PAGE lanes 1 and 5: standard, lane 2: GF fraction with highest myrosinase activity, lane 3: pooled GF fractions, lane 4: concentrated pooled GF fractions. (c) IEF of myrosinase after GF. Lane 1: standard, lane 2: GF fraction with highest myrosinase activity.

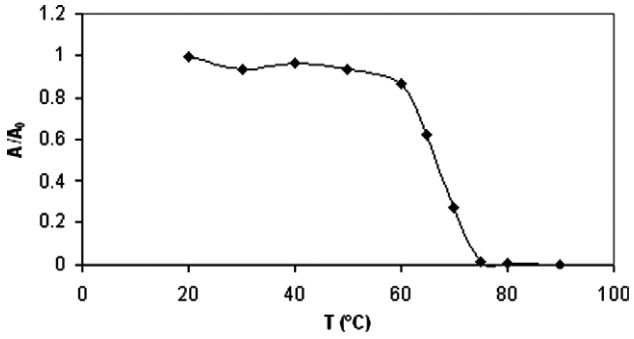

Fig. 4. Activity retention of purified *S. alba* L. myrosinase in Bis–Tris buffer (20 mM; pH 6.5) after 10 min thermal treatments at various temperatures.

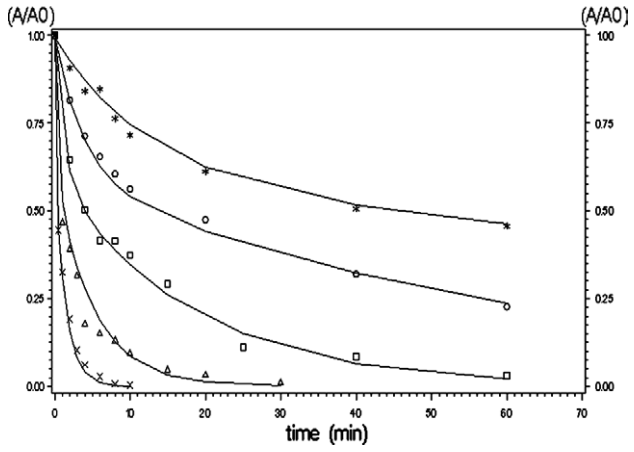

Fig. 5. Thermal inactivation of purified *S. alba* L. myrosinase in Bis–Tris buffer (20 mM; pH 6.5) described by the consecutive step model: (*) 65 °C; (○) 67.5 °C; (□) 70 °C; (△) 72.5 °C; (×) 75 °C.

and there is no statistical evidence for the biphasic or consecutive step model. In favor of the biphasic model (also called distinct isozyme model) is that many myrosinase isozymes have been described in the literature (Bjorkman & Janson, 1972; Xue, Lenman, Falk, & Rask, 1992) and that isozymes may differ in thermal stability. Applying this model, it was estimated that 60% of the myrosinase present belonged to the stable fraction and 40% belonged to the labile fraction. The consecutive step model, on the other hand, was also used by Ludikhuyze et al. (1999) to describe the inactivation of broccoli myrosinase. The inactivation from the native to the intermediate form (k_1 value) of the mustard seed myrosinase occurred faster than from the intermediate to the inactive form. The activity of the intermediate form was estimated to be approximately 55% of the total enzyme activity. Based on the molecular mass estimate of the native myrosinase in the preliminary study, it was concluded that myrosinase in the sample existed as a protein complex of different subunits. Therefore, a hypothesis for the mechanism behind this model could be that in the first step the complex might be (partly) dissociated

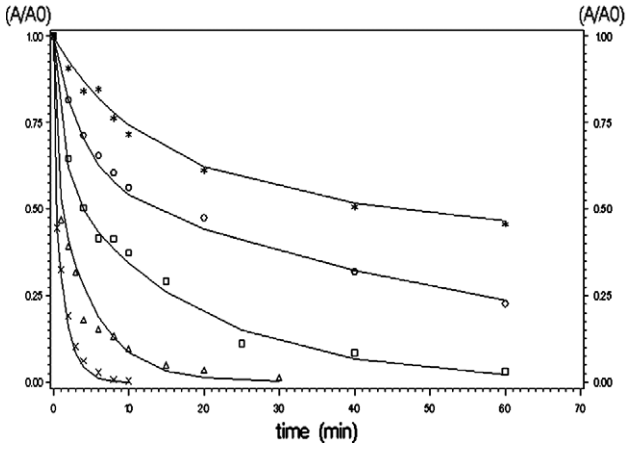

Fig. 6. Thermal inactivation of purified *S. alba* L. myrosinase in Bis–Tris buffer (20 mM; pH 6.5) described by the biphasic model: (*) 65 °C; (○) 67.5 °C; (□) 70 °C; (△) 72.5 °C; (×) 75 °C.

in myrosinase and other subunits and in the second step, the myrosinase is inactivated.

The temperature dependence of the reaction rates was described by the Arrhenius equation. The estimated activation energy values were 425.8 ± 30.4 kJ/mol (E_{a}L) and 493.8 ± 13.7 kJ/mol (E_{a}S) for the biphasic model and 446.4 ± 26.9 kJ/mol (E_{a1}) and 496.0 ± 16.7 kJ/mol (E_{a2}) for the consecutive step model.

In the literature, only few data are present about the thermal inactivation kinetics of myrosinase. Ludikhuyze et al. (1999) have reported thermal inactivation of broccoli myrosinase. Broccoli myrosinase is more thermolabile in comparison to the partially purified *S. alba* L. myrosinase since the inactivation started at 30 °C and the broccoli myrosinase was inactivated for 90% after 3 min at 60 °C. They found activation energy values of 113.5 ± 14.1 and 134.1 ± 28.3 kJ/mol, respectively, for the first and the second inactivation steps. As compared to the results in this study, the inactivation rate constants of broccoli myrosinase had a lower temperature sensitivity than that of *S. alba* L. myrosinase. In contrast, Matusheski and coworkers (2004) found a high myrosinase activity in broccoli after a treatment of 10 min at 60 °C. In red cabbage juice, Verkerk et al.

Table 2
Estimation of the kinetic parameters for thermal inactivation of purified *S. alba* L. myrosinase for the biphasic and the consecutive step model with T_{ref} = 60 °C

Biphasic model	Consecutive step model
A_L = 39.72 ± 2.68%	A_1 = 99.37 ± 1.15%
A_S = 59.76 ± 2.55%	A_2 = 56.20 ± 2.70%
$k_{L,ref}$ = (8.81 ± 2.03)×10^{-3} min^{-1}	$k_{1,ref}$ = (7.53 ± 1.49)×10^{-3} min^{-1}
$k_{S,ref}$ = (3.03 ± 0.61)×10^{-4} min^{-1}	$k_{2,ref}$ = (2.99 ± 0.69)×10^{-4} min^{-1}
E_{a}L = 425.8 ± 30.4 kJ/mol	E_{a1} = 446.4 ± 26.9 kJ/mol
E_{a}S = 493.8 ± 13.7 kJ/mol	E_{a2} = 496.0 ± 16.7 kJ/mol

(2002) found myrosinase inactivation between 25 and 70 °C characterised by an E_a value of 155 kJ/mol.

Other reports on the thermal stability of myrosinase show only qualitative data. Myrosinase in a crude extract from red cabbage was stable up to 60 °C, while myrosinase in a crude white cabbage extract was only stable up to 50 °C (Yen & Wei, 1993). In both white and red cabbage, 90% of myrosinase activity was lost after heating for 30 min at 70 °C. In Brussels sprouts, the effect of blanching on the myrosinase activity was studied (Wathelet et al., 1996). 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 slower, probably due to heat transfer limitations. The Brussels sprout myrosinase in a crude extract was stable up to 50 °C and almost completely inactivated after 30 min at 90 °C (Springett & Adams, 1989). Rapeseed myrosinase in crude extract was stable up to 65 °C (Kozlowska, Nowak, & Nowak, 1983). Thus, the temperature stability of *S. alba* L. myrosinase found in this study was similar to the stability of myrosinase from other sources except for broccoli extract.

3.3. Pressure stability of S. alba L. myrosinase

The pressure stability of purified *S. alba* L. myrosinase was studied at various temperatures, i.e., 10, 25, 40, 55 and 70 °C by pressurizing samples at different pressure levels (up to 800 MPa) for 10 min. In Fig. 7, it can be seen that the enzyme is stable up to 600 MPa and that the enzyme inactivation was enhanced by increasing the pressure levels above 600 MPa. At 70 °C, pressure stability was only determined up to 300 MPa. It is clear that there is an antagonistic effect between temperature and pressure. At atmospheric pressure, almost 60% of the enzyme activity was lost after a 10 min treatment at 70 °C (Fig. 7*), while at this temperature the initial enzyme activity could be maintained by applying pressures up to 300 MPa. This phenomenon was previously also found for other enzymes such as tomato PME (Fachin et al., 2002), avocado polyphenoloxidase (Weemaes, Ludikhuyze, Van den Broeck, & Hendrickx, 1998), orange pectinmethylesterase (Van den Broeck, Ludikhuyze, Van Loey, & Hendrickx, 2000) and α-chymotrypsin (Mozhaev, Lange, Kudryashova, & Balny, 1996). For broccoli myrosinase, this antagonistic effect was also found and the maximum protective effect at 35 °C occurred at 350 MPa (Ludikhuyze et al., 1999).

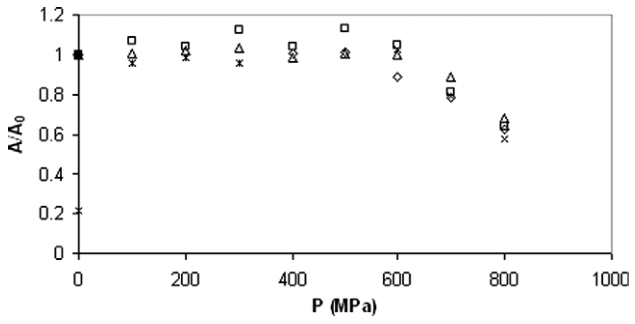

Fig. 7. Activity retention after 10 min pressure treatments at different temperatures: (a) 10 °C (◇); (b) 25 °C (□); (c) 40 °C (△); (d) 55 °C (×) and (e) 70 °C (∗).

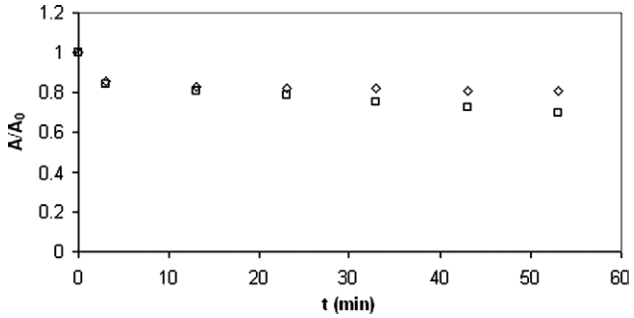

Fig. 8. Activity retention after treatments of 700 (◇) and 750 (□) MPa at 50 °C in function of the time. In the first 3 min dynamic conditions exist, from $t = 3$ min isothermal/isobaric conditions exist in the pressure vessels.

To study the time dependency of the enzyme inactivation during pressure treatment, the evolution of myrosinase activity was followed at 700 and 750 MPa for different times at 50 °C (Fig. 8). About 15% of the initial activity was lost during pressure build-up and adiabatic heating (first 3 min). When adiabatic heating effects have vanished (3 min after pressurization) and isothermal/isobaric conditions appear, the inactivation went much slower. Pressure treatment at 700 MPa/50 °C/50 min resulted in approximately 5% of enzyme inactivation while pressure treatment at 750 MPa/50 °C/50 min resulted in approximately 20% of enzyme inactivation during the isothermal/isobaric conditions. This implies that the reaction rate increases with increasing pressure. However, the inactivation is insufficient to determine an adequate inactivation model.

In the literature, only the pressure stability of broccoli myrosinase in crude extract has been studied on a kinetic basis (Ludikhuyze et al., 1999). As compared to *S. alba* L. myrosinase, broccoli myrosinase was more pressure labile since the inactivation started at 250 MPa and 20 °C.

4. Conclusion

In this study the effect of temperature and pressure on the stability of myrosinase partially purified from mustard seeds (*S. alba* L.) was determined. The enzyme was temperature sensitive and consequently, a thermal treatment such as blanching can result in a decrease of myrosinase activity which indirectly re-

duces the health promoting effect of the food product because less glucosinolate degradation products are available. However myrosinase was very pressure stable, which implies that a high myrosinase activity can still be retained after pressure treatment. With regard to blanching, pressure blanching can be a good alternative for thermal blanching because undesired quality related enzymes such as lipoxygenase (i.e., blanching indicator) can be inactivated while desired nutrition related enzymes such as myrosinase can be maintained. Since pressure treatment can accelerate the enzyme-substrate reaction, pressure effects on myrosinase activity and the pressure/temperature stability of glucosinolates and their degradation products must be further studied.

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