



## A Comparison of Myrosinase Activity and Stability in Fresh Broccoli (*B. oleracea var. Italica*) and Brown Mustard (*B. juncea*) Seeds

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ARTICLE INFO	ABSTRACT
<p>Research Article</p> <p>Received : 24/05/2019 Accepted : 13/09/2019</p> <p>Keywords: Brassica Processing Enzyme Inactivation Human health</p>	<p>The effects of temperature and pressure processing on myrosinase extracted from fresh broccoli and brown mustard seed was studied. Brown mustard seeds had higher myrosinase activity (2.75 un/mL) than fresh broccoli (0.58 un/mL). The extent of enzyme inactivation increased with pressure (200-800 MPa) and temperature (30-80°C) for both brown mustard seeds and fresh broccoli myrosinase. However, at combinations of lower pressures (200-400 MPa) and temperatures (30-80°C), there was less myrosinase inactivation. When processing at a pressure of 300 MPa with a temperature of 70°C for 10 minutes, there was 65% myrosinase activity for brown mustard while at 300 MPa and 60°C, activity retention in fresh broccoli was 30%. Whereas, the corresponding activity retentions when applying only heat (70°C for 10 minutes) was 35% for brown mustard myrosinase, while there was no measurable myrosinase activity for fresh broccoli (60°C, 10 minutes). Thus, application of moderate pressures (200-400 MPa) on brown mustard and fresh broccoli can potentially be used to retain myrosinase activity needed for subsequent glucosinolate hydrolysis.</p>

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### Introduction

*Brassica* vegetables (Broccoli, Brussels sprout, cauliflower, cabbage, kale and condiments like mustard and rapeseed) are globally consumed cruciferous plants from the order *Brassicales* and family *Brassicaceae*. They are rich in phytochemicals particularly glucosinolates, which are beneficial non-nutritive bioactive compounds (Fahey et al., 2001; Girgin and El Nehir, 2015). Glucosinolates are hydrolysed by myrosinase enzymes (which co-exist with glucosinolates in segregated compartments of the plant) to produce a variety of compounds dependent on the reaction condition, nature of glucosinolates and pH. These hydrolysis products have generated a lot of interest due to their toxicological and pharmacological potentials (anticarcinogenic and antimicrobial properties) and impact on sensory attributes of *Brassicaceae* and their products (taste, flavour and aroma) (Drewnowski and Gomez-Carneros, 2000; Johnson et al., 2010).

Sulforaphane is an isothiocyanate, formed from hydrolysis of glucoraphanin (major glucosinolate in broccoli) whose anticarcinogenic potential is well

documented in literature (Brooks et al., 2001; Fahey, Zhang and Talalay, 1997; Zhang, Li and Tang, 2005). However, rapeseeds (*Brassica napus*) form predominantly nitriles from these hydrolysis reaction (Lambrix et al., 2001) implying that different products can be produced from the same glucosinolate, depending on the plant species and reaction conditions. Microflora in the human gut can hydrolyse glucosinolates into bioactive compounds, but the yield is much lower compared to that resulting from plant myrosinases (Conaway et al., 2000).

Mustard, an annual plant has 3 known edible species; yellow mustard (*Sinapis alba* L) (referred to as white mustard in Britain and Europe), brown/oriental mustard (*Brassica juncea*) and black mustard (*Brassica nigra*). Mustard has a characteristic aroma, a hot spicy trait (pungency or the bite) and has been used as an important spice for decades. Mustard has a rich chemical composition. Brown/oriental mustard is the hot mustard and sinigrin is its predominant glucosinolate. Sinigrin releases volatile allyl isothiocyanate (AITC) which possesses a sharp sensation and pungent aroma that

permeates the sinuses (Abul-Fadl, El-Badry and Ammar, 2011; Wanasundara, 2008).

Broccoli (*Brassica oleracea* var. *italica*) is an edible green plant, its flower-head is eaten as a vegetable and is valued globally due to its flavour and its chemo-preventive effects, attributed to the degradation products of its main glucosinolate - glucoraphanin (present in harvested florets of broccoli) (Fahey et al., 2001). It has also been suggested that the hydrolysis products of other glucosinolates in broccoli (glucoiberin, sinigrin and progoitrin) have been identified as having the potential of protecting against human and animal carcinogenesis by either inducing Phase II detoxification enzymes or inhibiting phase I enzymes (Fahey et al., 1998; Farnham et al., 2004).

Irrespective of processing methods used, the phytochemicals present in *Brassicaceae* end up getting affected (Conaway et al., 2000; Jones et al., 2010; Rosa et al., 1997) and this has a huge impact on the production of beneficial hydrolysis products. Blanching, canning, as well as domestic cooking, affect myrosinase in *Brassicaceae* (Ludikhuyze et al., 1999; Van Eylen et al., 2007). It is therefore imperative to optimize processing to ensure that more glucosinolates are converted to nutritionally beneficial isothiocyanates. It was recently suggested that the use of low pressure combined with temperature processing of *Brassicaceae* might be more helpful in the delivery of beneficial glucosinolate-myrosinase hydrolysis products (Okunade et al., 2015). This work studied myrosinase activity and its inactivation in brown mustard seed and fresh broccoli under temperature and pressure processing conditions, with a view to ascertaining the nature of the enzyme under domestic processing and its subsequent ability for re-initiating further glucosinolates hydrolysis.

## Materials and Methods

### Sample Preparation

**Brown mustard:** Brown mustard seeds (*Brassica juncea* L. Czern. var. *juncea*) were obtained from the I.P.K Gene bank (Gatersleben, Germany). 10 g mustard seed was placed in flexible polyethylene bags (low density polyethylene, LDPE) and it was vacuum sealed.

**Broccoli:** Freshly harvested and mature broccoli (*Brassica oleracea* Var. *italica*) was obtained from Produce World Marshalls (Boston, U.K). The broccoli heads were cut approximately 4 cm from the top and thoroughly mixed together. 10 g broccoli portion was placed in flexible polyethylene bags (LDPE) and vacuum sealed. The packaged broccoli florets were stored at 4°C and used no later than 5 hours after storage.

### Myrosinase Extraction and Assay

Myrosinase extraction was done as described by Ghawi et al. (2012) and adapted by Okunade et al. (2015). Buffer solution (10 mL of Tris HCl 0.2 M, pH 7.5 containing EDTA 0.5 mM, dithiothreitol 1.5 mM and 0.15 g (0.4 g for mustard). Polyvinyl pyrrolidone was added to 0.1 g lyophilized powdered broccoli (0.5 g mustard powder), followed by centrifugation at 4°C. The protein was precipitated from the filtered supernatant using ammonium sulfate. The mixture was then re-centrifuged and the pellet obtained was suspended in 2 mL (6.5 mL for mustard) of

10 mM Tris HCl buffer, pH 7.5. The mix was extensively dialysed at low temperature (4°C) for 24 hours to remove excess ammonium and sulphate ions and centrifuged (11,738 × g) at 4°C for 15 minutes to remove insoluble materials. Finally, the supernatant was frozen (-80°C) and then lyophilised, the resulting powder was stored at -20°C until further analysis.

Myrosinase activity was measured according to the coupled enzymatic procedure with some modifications (Ghawi et al., 2012). A D-glucose determination kit was used (R-Biopharm Rhone, Heidelberg, Germany). The reaction mixture and 50 µL sample (25 mg lyophilised powder/mL de-ionised water) was allowed to equilibrate for 5 minutes and 50 µL sinigrin solution (0.6 M) was added. The change in absorbance due to the formation of NADP was measured at 340 nm. Myrosinase activity was determined from a calibration curve for standard myrosinase enzyme (Sigma Aldrich, UK). One unit (un) of myrosinase was defined as the amount of enzyme that produces 1 µmol of glucose per minute at 25°C and pH 7.5.

### Heat Treatment

Thermal inactivation was done under isothermal conditions at different temperatures, between 10-80°C (Ghawi et al., 2012; 2013) for 10 minutes using a water bath fitted with a thermometer. After each temperature treatment, the samples (in the vacuum sealed LDPE bags) were removed and quickly immersed in an ice bath, frozen at -80°C, and lyophilised. The lyophilised powder was finely ground using a coffee grinder and sieved (30 µ mesh, Endecotts Ltd, London). The powdered samples were then stored at -20°C until further analysis. Treatments were done in triplicate.

### Pressure Treatment

Pressure treatments were performed between 100-900 MPa using a high pressure unit (37 mm by 246 mm Food Lab 300 Stansted Fluid Power, Stansted, UK). 1, 2 - Propanediol (30%) (Sigma-Aldrich, Poole, U.K) was used as the pressure transmitting fluid (Ghawi et al, 2012). The processing temperature was controlled by liquid circulation in the outer jacket of the high pressure vessel. The weighed portion of the sample in the LDPE bags was used. Pressure treatment at different levels for pre-set time of 10 minutes was applied with temperature controlled at 15°C. Samples were removed from the vessel and rapidly cooled in an ice bath and the enzyme activity was measured not later than an hour post pressure treatment.

Combined pressure and temperature treatments were performed using low pressure (200- 400 MPa) with temperature (30-80°C) for pre-set time of 10 minutes. About 3-5 minutes was needed to reach equilibrium (desired temperature and pressure) and this was added to the holding time. All treatments were done in triplicate.

### Statistical Analysis

The statistical differences between the values obtained under different experimental conditions were established by undertaking ANOVA followed by Tukey's HSD multiple pairwise comparison test using SPSS software (PASW Statistics 17.0, IBM, UK). Differences were considered significant at P<0.05.

## Results and Discussion

### Effect of Temperature on Myrosinase of Fresh Broccoli Florets and Brown Mustard Seed

Figure 1 depicts relative temperature inactivation of myrosinase extracted from fresh broccoli florets and brown mustard seed. It was observed that the extent of myrosinase inactivation gradually increased with rise in temperature. This same trend had earlier been reported for mustard seeds (Van Eylen et al., 2008; Okunade et al., 2015). At temperatures below 30°C, broccoli myrosinase was stable, while brown mustard myrosinase was predominantly stable up to 50°C. However at 50°C, there was about 40% loss in activity for broccoli myrosinase and at 60°C, there was no measurable activity, implying that the enzyme was completely inactivated at this processing condition and glucosinolates hydrolysis is likely to be greatly inhibited when broccoli is processed at this temperature.

At 60°C, 20% loss in myrosinase activity of brown mustard was observed and at 70°C, 65% loss in myrosinase activity was the case, while there was no measurable enzyme activity at 80°C, indicating complete inactivation at this temperature. Plant myrosinase is essential for the conversion of glucosinolates to beneficial isothiocyanates. Although, human gut microflora can hydrolyse glucosinolates, the yield of hydrolysis products is much more smaller (1:3) compared to that from plant myrosinase (Conaway et al., 2000).

### Pressure Inactivation of Myrosinase From Fresh Broccoli Florets and Brown Mustard Seed

Figure 2 shows the pressure inactivation of fresh broccoli florets and brown mustard seed myrosinase. A similar trend observed for temperature processing of these enzymes was also noticeable during pressure processing. Broccoli and brown mustard myrosinases were stable below 300 and 500 MPa respectively. However, at 500 MPa, there was no measurable myrosinase activity in fresh broccoli florets, while at 800 MPa, brown mustard had minimal enzyme activity (less than 2%). Mustard seed species have been reported as being more pressure stable and more robust compared to some other *Brassicaceae*

(Van Eylen et al., 2009; Ghawi et al., 2012; Okunade et al., 2015). The result of this study is in agreement with these previous studies. The effect of pressure processing on myrosinase from different *Brassica* sources is well documented and varies from one *Brassica* source to another (Kozłowska et al., 1983; Ludikhuyze et al., 1999; Yen & Wei, 1993).

Table 1 shows the relative activity of myrosinase enzymes in fresh broccoli florets and brown mustard seed after pressure processing for 10 minutes at various temperatures. In some previous studies, it was suggested that processing *Brassicaceae* at low pressure with temperature enhanced myrosinase activity and stability (Okunade et al., 2015; Van Eylen et al., 2007; Verkerk & Dekker, 2004; Volden et al., 2008). It was also suggested that combined low pressure with temperature processing may either have a synergistic (in which low pressure has no protective effect on thermal inactivation) (Ghawi et al., 2012) or antagonistic effect (low pressure has a protective effect on thermal inactivation) (Van Eylen et al., 2006; Okunade et al., 2015). There was reduced loss in myrosinase activity when broccoli florets and brown mustard was processed under low pressure with different temperatures implying that low pressure processing combined with temperature has an antagonistic effect on both broccoli and brown mustard seed myrosinase.

Processing fresh broccoli florets and brown mustard seed at 200 MPa and 70°C for 10 minutes, activity retention was 70% respectively, when the temperature was further increased at this pressure, only brown mustard had 40% myrosinase activity retention. Whereas when heat processing broccoli and brown mustard seed at 70°C alone without pressure, there was no measurable myrosinase activity for fresh broccoli florets and only 35% activity retention for brown mustard seed respectively. Even at 200 MPa and 80°C, activity retention in brown mustard seed was 40% (at 80°C alone, there was no measurable myrosinase activity). A similar trend was also observed at 300 MPa with temperature, however at 400 MPa with temperature broccoli had no measurable myrosinase activity whereas brown mustard myrosinase still retained 20% activity at 80°C.

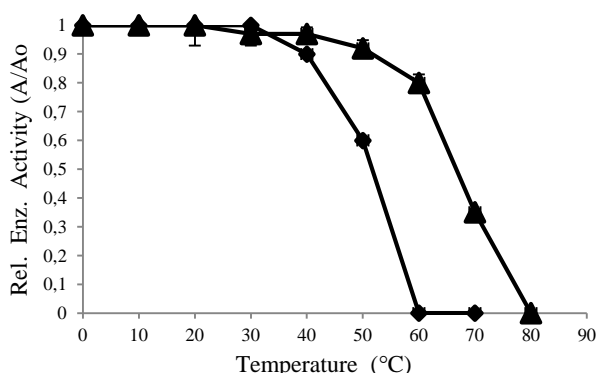


Figure 1 Effect of thermal processing on relative myrosinase activity in fresh broccoli florets and brown mustard seed where temperature exposure time was 10 minutes. (■ - Broccoli florets, ▲ - Brown mustard. A – enzyme activity after thermal treatment, Ao – Initial enzyme activity). Error bars represent standard error of the mean.

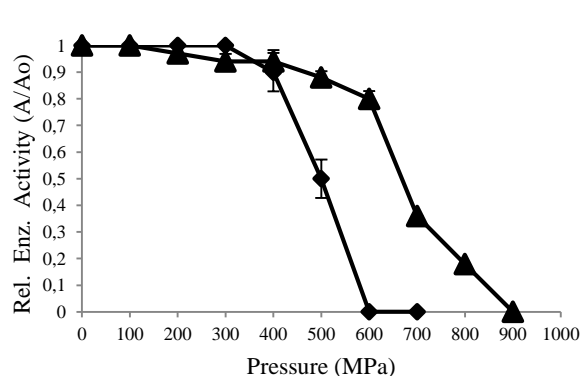


Figure 2 Effect of pressure treatment on relative myrosinase activity in fresh broccoli florets and brown mustard seed. Pressure holding time was 10 minutes and processing temperature was controlled at 15 °C. (■ - Broccoli florets, ▲ - Brown mustard. A – enzyme activity after pressure treatment, Ao – Initial enzyme activity). Error bars represent standard error of the mean.

Table 1 Relative activity of myrosinase enzymes in fresh broccoli florets and brown mustard seed after pressure processing for 10 minutes at various temperatures

Pressure (MPa)	Temperature (°C)	Rel. Enz. Activity (un/ml) Broccoli	Rel. Enz. Activity (un/ml) Brown mustard
200	30	1.0±0.00 <sup>a</sup>	1.0±0.00 <sup>a</sup>
	40	0.9±0.07 <sup>a</sup>	1.0±0.00 <sup>a</sup>
	50	0.9±0.07 <sup>a</sup>	1.0±0.00 <sup>a</sup>
	60	0.7 ±0.24 <sup>b</sup>	0.7±0.07 <sup>a</sup>
	70	-	0.7±0.00 <sup>a</sup>
	80	-	0.4±0.00 <sup>c</sup>
300	30	0.8±0.00 <sup>a</sup>	1.0±0.00 <sup>a</sup>
	40	0.7±0.17 <sup>a</sup>	1.0±0.00 <sup>a</sup>
	50	0.5±0.07 <sup>b</sup>	1.0±0.00 <sup>a</sup>
	60	0.3±0.02 <sup>c</sup>	0.7±0.00 <sup>a</sup>
	70	-	0.6±0.00 <sup>b</sup>
	80	-	0.4±0.00 <sup>c</sup>
400	30	-	1.0±0.00 <sup>a</sup>
	40	-	1.0±0.00 <sup>a</sup>
	50	-	1.0±0.00 <sup>a</sup>
	60	-	0.6±0.00 <sup>a</sup>
	70	-	0.6±0.03 <sup>a</sup>
	80	-	0.2±0.03 <sup>c</sup>

\*Values not sharing a common letter are significantly different at P<0.05.

## Conclusion

Myrosinase isoenzymes in *Brassicaceae* have been reported to vary in terms of activity, temperature and pressure stability. Brown mustard myrosinase had higher myrosinase activity, and was more resistant to thermal and pressure treatment than fresh broccoli florets myrosinase. Processing broccoli florets and brown mustard seed at low pressure (200-400 MPa) with temperature led to higher myrosinase activity retention and increased inactivation temperature compared to thermal or pressure treatment. This difference in myrosinase stability could be utilized to control the hydrolysis level of glucosinolates leading to enhancement in the production of bioactive isothiocyanates from the *Brassica*. Also, the control of enzyme activity may be an important factor in regulating the sensory attributes of *Brassica* vegetable.

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