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Thermal and pressure stability of myrosinase enzymes from black mustard (*Brassica nigra* L. W.D.J Koch. var. *nigra*), brown mustard (*Brassica juncea* L. Czern. var. *juncea*) and yellow mustard (*Sinapsis alba* L. Subsp *Maire*) seeds

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Abbr. Running Title:

Thermal and pressure stability of myrosinase from black, brown and yellow mustard

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28 **Abstract**

29 This study investigates the effects of temperature and pressure on inactivation of
30 myrosinase extracted from black, brown and yellow mustard seeds.

31 Brown mustard had higher myrosinase activity (2.75 un/mL) than black (1.50 un/mL) and
32 yellow mustard (0.63 un/mL).

33 The extent of enzyme inactivation increased with pressure (600-800 MPa) and temperature
34 (30-70 °C) for all the mustard seeds. However, at combinations of lower pressures (200-400
35 MPa) and high temperatures (60-80 °C), there was less inactivation. For example,
36 application of 300 MPa and 70 °C for 10 minutes retained 20%, 80% and 65% activity in
37 yellow, black and brown mustard, respectively, whereas the corresponding activity
38 retentions when applying only heat (70 °C, 10min) were 0%, 59% and 35%. Thus,
39 application of moderate pressures (200-400 MPa) can potentially be used to retain
40 myrosinase activity needed for subsequent glucosinolate hydrolysis.

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44 **Keywords**: Processing, Myrosinase activity, Inactivation, Mustard seed.

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50 # 1 Introduction

51 Mustard plant belongs to the *Brassicaceae* family, the dry seeds being the main part used in
52 food processing (canned mustard leaves are available). Common types of mustard are
53 yellow mustard (*Sinapsis alba*), brown (*Brassica juncea*) and black mustard (*Brassica nigra*).
54 Mustard has a rich chemical composition and its seed flour is widely used in food processing
55 (Abul-Fadl, El-Badry, & Ammar, 2011; Wanasundara, 2008). Mustard is also used for its spicy
56 flavour, produced from the hydrolysis of glucosinolates by myrosinase enzymes
57 (Wanasundara, 2008). Mustard seed is widely used as a condiment, however, its
58 advantageous chemical composition and relatively low price offer wide possibilities for
59 utilization as additives in human food and in animal feeds (Abul-Fadl, El-Badry, & Ammar,
60 2011; Wanasundara, 2008).

61 The glucosinolates (thioglucosides) in mustard seeds are pseudo-thioglucosides containing
62 nitrogen and sulphur. Myrosinase enzymes (thioglucoside) are glucohydrolases (EC 3.2.3.1)
63 (Bones & Rossiter, 1996; Fahey, Zalcmann, & Talalay, 2001; Thangstad & Bones, 1991).
64 According to Thangstad *et al.* (1991) and Bones *et al.* (1996), glucosinolates and myrosinase
65 enzymes coexist in segregated compartments of the plant. After plant tissue damage,
66 glucosinolates are hydrolysed to produce a variety of compounds; some of which are
67 bioactive (isothiocyanates, indoles) (Bongoni, Verkerk, Steenbekkers, Dekker, & Stieger,
68 2014), thiocyanates, oxazolidine-2-thiones and others of which are potentially toxic (nitriles,
69 epithionitriles) by myrosinase enzymes. The nature of the hydrolysis products depends on
70 the structure of glucosinolate and the reaction conditions (Fahey, Zalcmann, & Talalay,
71 2001; Lambrix, Reichelt, Mitchell-Olds, Kliebenstein, & Gershenzon, 2001). These
72 compounds are of immense interest in food processing (taste, aroma, and flavour
73 attributes) and human health (anticarcinogenic and antimicrobial properties) (Drewnowski

74 & Gomez-Carneros, 2000; Fahey, Zhang, & Talalay, 1997; Johnson, Koh, Wang, Yu, & Yuan,
75 2010; Tang & Zhang, 2004; Wanasundara, 2008).

76 Myrosinase enzymes are significantly inactivated at normal cooking temperatures regardless
77 of the method used (Ludikhuyze, Ooms, Weemaes, & Hendrickx, 1999; Van Eylen, Oey,
78 Hendrickx, & Van Loey, 2007; Yen & Wei, 1993), although, the same processing conditions
79 rarely affect glucosinolates if leaching out can be avoided (Oerlemans, Barrett, Suades,
80 Verkerk, & Dekker, 2006). Microflora in the human gut can hydrolyse glucosinolates into
81 bioactive compounds, but the yield is much lower compared to that resulting from plant
82 myrosinases (Conaway, Getahun, Liebes, Pusateri, Topham, Botero-Omary, *et al*., 2001).
83 Hence, the control of myrosinase activity is important to determine the bioavailability of
84 hydrolysis products.

85 Myrosinase enzymes in *Brassica* vegetables are known to exhibit varying degree of thermal
86 stability (Ghawi, Methven, Rastall, & Niranjan, 2012; Ludikhuyze, Ooms, Weemaes, &
87 Hendrickx, 1999; Matusheski, Juvik, & Jeffery, 2003; Van Eylen, Indrawati, Hendrickx, & Van
88 Loey, 2006; Yen & Wei, 1993). Yellow mustard and rapeseed myrosinases are known to have
89 the highest thermal stability of *Brassica* plant species (Kozlowska, Nowak, & Nowak, 1983;
90 Ludikhuyze, Ooms, Weemaes, & Hendrickx, 1999; Pérez, Barrientos, Román, & Mahn, 2014;
91 Van Eylen, Indrawati, Hendrickx, & Van Loey, 2006; Van Eylen, Oey, Hendrickx, & Van Loey,
92 2007; Verkerk & Dekker, 2004; Yen & Wei, 1993). The use of high pressure processing (HPP)
93 as an alternative to thermal processing has been suggested and it has been found to reduce
94 thermal inactivation of certain enzymes, including myrosinase (Hendrickx, Ludikhuyze, Van
95 den Broeck, & Weemaes, 1998; Van Eylen, Oey, Hendrickx, & Van Loey, 2008). Although a
96 narrow range of thermal and pressure stability in *Brassica* vegetables has been reported, no

97 data is yet available for the thermal and pressure inactivation of brown and black mustard
98 seed myrosinase enzymes; and yet, they may be more stable than myrosinase in other
99 *Brasiccas* as they are related to yellow mustard which has already been shown to have
100 higher heat stability (Van Eylen *et al.*, 2006; 2008).

101 In recent studies, it was found that addition of an exogenous source of myrosinase (Daikon
102 radish root and mustard seeds) to processed *Brassica* can reinitiate the hydrolysis of
103 glucosinolates (Dosz & Jeffery, 2013; Ghawi, Methven, & Niranjan, 2013). Hence, evaluating
104 other sources of myrosinase that are more stable under processing conditions is of
105 importance.

106 The hypothesis of this study were that (1) myrosinase from different mustard species differ
107 in thermal and pressure stability and (2) lower pressure processing can be used to decrease
108 thermal inactivation rate of mustard myrosinase. The study aimed to investigate thermal,
109 pressure and combined thermal and pressure inactivation of myrosinase enzymes from
110 black, brown and yellow mustard seeds in order to ascertain the possible extent of
111 degradation that would occur under food processing conditions.

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118 # 2 Materials and methods

119 ## 2.1 Sample preparation and myrosinase extraction

120 Yellow mustard (*Sinapsis alba* L. *subsp maire*), black mustard (*Brassica nigra* L. W.D.J
121 Koch. *var*. *nigra*) and brown mustard (*Brassica juncea* L. Czern. *var. juncea*) were obtained
122 from the I.P.K Gene bank (Gatersleben, Germany). All samples were obtained as dried
123 seeds.

124 Myrosinase enzyme extraction was done as described by Ghawi *et al.* (2012). The process
125 involved grinding dry mustard seeds in a coffee grinder. 10mL of buffer (Tris HCl 0.2M, pH
126 7.5 containing EDTA 0.5mM, dithiothreitol 1.5mM and 0.4g Polyvinylpolypyrrolidone) was
127 then added to 0.5g powdered mustard and blended on ice (15 minutes) and then
128 centrifuged (11,738 ×g) for 15 minutes at 4 °C. The supernatant was filtered (0.45µm). The
129 filtrate was made up to 10mL using the buffer solution and 90% precipitation of protein was
130 achieved using 6.2g ammonium sulphate with slow blending on ice for 30 minutes. The
131 mixture was then centrifuged (13,694 ×g) for 15 minutes at 4 °C. The pellet obtained was
132 suspended in 2mL of 10mM Tris HCl buffer, pH 7.5.

133 The mix was extensively dialysed at low temperature (4 °C) using cellulose membrane
134 (Medicell International Ltd, Molecular weight cut-off 12,000-14,000 Da) and 10mM Tris HCl
135 buffer for 24 hours to remove excess ammonium and sulphate ions and centrifuged (11,738
136 ×g) at 4 °C for 15 minutes to remove insoluble materials. Finally, the supernatant was frozen
137 (-80 °C) and then lyophilised, the resulting powder was stored at -20 °C until further analysis.

138 ## 2.2 Myrosinase enzyme activity assay

139 Myrosinase activity was measured according to the coupled enzymatic procedure with
140 some modifications (Gatfield & Sand, 1983; Ghawi, Methven, Rastall, & Niranjan, 2012;

141 Wilkinson, Rhodes, & Fenwick, 1984). A D-glucose determination kit was used (R-Biopharm
142 Rhone, Heidelberg, Germany). The reaction mixture (1.51mL) included 0.5mL NADP/ATP, 10
143 µL hexokinase/glucose-6-phosphate dehydrogenase, 0.9mL of water containing ascorbic
144 acid 7mM (cofactor) and 50 µL sample. The mix was allowed to equilibrate for 5 minutes
145 and 50 µL sinigrin solution (0.6M) was added. The change in absorbance due to the
146 formation of NADP was measured at 340nm. Myrosinase activity was determined from the
147 initial linear rate of increase in the curve of absorbance against reaction time. A standard
148 myrosinase enzyme (Sigma Aldrich, UK) was used to establish the calibration curve of
149 absorbance against concentration. One unit (un) of myrosinase was defined as the amount
150 of enzyme that produces 1 µmol of glucose per minute at 25 °C and pH 7.5.

151 **2.3 Heat treatment**

152 Thermal inactivation was done under isothermal conditions at different temperatures,
153 between 10-80 °C. 150 µL sample (25mg lyophilised powder/mL de-ionised water) was
154 pipetted into clean durham tube (6.5mm internal diameter, 1mm thickness and 30mm in
155 length, Fischer Scientific, Loughborough, U.K) and sealed. These were carefully placed in
156 heated water bath fitted with a thermometer for pre-set time of 5 and 10 minutes at
157 different temperature (10-80 °C). After each determination, the samples were quickly
158 immersed in an ice bath and the enzyme activity was measured not later than an hour after
159 each heat treatment. Treatments were done in triplicate.

160 **2.4 Pressure treatment**

161 Pressure treatments were performed between 100-900 MPa using a high pressure
162 unit (37mm diameter and 246mm length Food Lab 300 Stansted Fluid Power, Stansted, UK).
163 1, 2-Propanediol (30%) (sigma-Aldrich, Poole, U.K) was used as the pressure transmitting

164 fluid. The processing temperature was controlled by liquid circulation in the outer jacket of
165 the high pressure vessel. 200 µL samples (25mg/mL) were placed in flexible polyethylene
166 bags (LDPE) and air was carefully removed from the bags before sealing. Pressure treatment
167 at different levels for pre-set time of 5 and 10 minutes was applied with temperature
168 controlled at 15°C. Samples were removed from the vessel and rapidly cooled in an ice bath
169 and the enzyme activity was measured not later than an hour after pressure treatment.

170 Combined pressure and temperature treatments were performed using a combination of
171 high pressure (600-800MPa) with moderate temperature (30-70 °C) for 10 minutes and low
172 pressure (200- 400MPa) with slightly higher temperature (60-80 °C) for different pre-set
173 times of 5, 10 and 15 minutes. Pressure build up is usually accompanied with increase in
174 temperature due to adiabatic heating. About 3-5 minutes was needed to reach equilibrium
175 (desired temperature and pressure) and this was added to the holding time. All treatments
176 were done in triplicate.

177 **2.5 Protein assay**

178 Total protein content (unprocessed samples) was determined using the Bradford
179 procedure (Bradford, 1976). This is based on formation of a complex with Brilliant Blue G.
180 The samples (25mg/mL) were mixed with the reagent and the absorbance was measured at
181 595nm after 20 minutes of incubation at 23 °C. Bovine serum albumin BSA (0 - 1.4mg/mL)
182 (Sigma Aldrich, UK) was used in constructing a standard curve.

183 **2.6 Statistical analysis**

184 The statistical differences between the values obtained under different experimental
185 conditions were established by undertaking ANOVA followed by Tukey's HSD multiple

186 pairwise comparison test using SPSS software (PASW Statistics 17.0, IBM, UK). Differences

187 were considered significant at $P<0.05$.

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201 # 3 Results and discussion

202 ## 3.1 Enzyme activity, protein content and specific enzyme activity of myrosinase

203 ## enzymes from black, brown and yellow mustard seeds

204 Table 1 shows the enzyme activity, protein content and specific enzyme activity of

205 myrosinase enzyme from black, brown and yellow mustard. Brown mustard had higher

206 myrosinase activity (2.75 un/mL) than black mustard (1.50 un/mL) and yellow mustard had

207 the least myrosinase activity (0.63 un/mL). The protein content of all studied samples was

208 similar. Myrosinase from brown mustard had the highest proportion of specific activity

209 (2.04un/mg) and yellow mustard the lowest (0.48un/mg). The differences between mustard

210 species in overall enzyme activity were not related to differences in protein content and

211 hence, differences between species in specific activity prevailed. Variations in myrosinase

212 enzyme activity within and between *Brassica* species have been reported previously and

213 have been attributed to genetic and/or environmental factors (agronomic and climatic

214 conditions) (Pocock, Heaney, Wilkinson, Beaumont, Vaughan, & Fenwick, 1987; Wilkinson,

215 Rhodes, & Fenwick, 1984). In addition, Rask *et al*. (2000) had also reported that the

216 difference in thermal stability of myrosinase in *Brassica* plants was probably due to the

217 existence of different isoforms of myrosinase, where some of them interact with

218 myrosinase-binding proteins (a group of proteins found in *Brassica* plants) to form

219 complexes that may improve stability. It is therefore possible that the different species of

220 mustard may have genetic differences, or have adapted to different environmental

221 conditions that have resulted in different isoforms of myrosinase. It has also been suggested

222 that myrosinase activity for similar *Brassica* samples may vary between studies merely due

223 to the different protocols employed (Piekarska, Kusznierewicz, Meller, Dziedziul, Namiesnik,

224 & Bartoszek, 2013). In this study, the observed differences in enzyme activity between the

225 three species cannot be specifically attributed to one or more of the above factors because,
226 even though the seeds were obtained from the same gene bank, they may not have been
227 produced under strictly controlled conditions for drawing such inferences. However, the
228 main purpose of this study is to evaluate the effects of processing on enzyme activity
229 retention, which can robustly be undertaken for a given seed variety by normalising the
230 enzyme activity after processing with the corresponding initial activity.

231 **3.2 Effect of temperature on black, brown and yellow mustard seed myrosinase**

232 Figure 1 shows the effect of temperature and exposure time on myrosinase activity at
233 atmospheric pressure. Exposure time of 10 minutes at temperature ranging between 10-80
234 °C were employed. Recent studies have shown that myrosinase enzymes extracted from
235 broccoli are stable up to 70 °C by blanching intact broccoli florets (Pérez, Barrientos, Román,
236 & Mahn, 2014) whereas the enzyme extracted from yellow mustard is stable up to 60 °C
237 (Ludikhuyze, Ooms, Weemaes, & Hendrickx, 1999; Van Eylen, Indrawati, Hendrickx, & Van
238 Loey, 2006). Results of heating for 5 minutes (data not included) showed no significant
239 inactivation up to 60 °C for black mustard myrosinase whereas the myrosinase from brown
240 and yellow mustard showed activity loss of approximately 28% and 17% respectively.
241 Myrosinase activity decreased significantly at above 60 °C in all the mustards studied.

242 When the exposure time was 10 minutes, the same trend, as observed for 5 minutes
243 processing time, was observed in the case for both black and brown mustard. However,
244 yellow mustard myrosinase was only substantially stable up to 50 °C (27% loss in activity)
245 and lost about 79% of its activity at 60 °C. Heating up to 70 °C led to about 41% loss in
246 activity for black mustard, 65% for brown mustard while there was no myrosinase activity

247 for yellow mustard. At 80 °C, there was no significant myrosinase activity in the case of all
248 three seeds.

249 These results are similar to those in previous studies; Van Eylen *et al.* (2006 & 2008)
250 concluded that the inactivation of myrosinase extracted from yellow mustard occurs at
251 temperatures above 60 °C at an exposure time of 10 minutes. Stoin *et al.* (2009) had earlier
252 reported that myrosinase from black mustard exhibited maximum activity at temperature
253 ranging from 45-50 °C and even at a range of 70-85 °C, a small amount of enzyme activity
254 could be observed. However, the authors suggested that activity at temperature above 85
255 °C may be from other sources for example thermostable desulphatase enzyme using sinigrin
256 as a substrate. The current study has concluded that myrosinases from black and brown
257 mustard are fully inactivated at temperatures above 80 °C and that myrosinase from these
258 mustard sources is more stable than that from yellow mustard.

259 Comparing to heat stability from other *Brassica* sources, studies have shown that in both
260 white and red cabbage, 90% loss in myrosinase activity was observed after heating at 70 °C
261 for 30 minutes (Yen and Wei, 1993), while Matusheski *et al.* (2004) discovered high
262 sulforaphane content in broccoli after treating at 60 °C for 10 minutes, implying that the
263 myrosinase in broccoli was intact at 60 °C. However, in both studies, intact cabbage and
264 broccoli were used, therefore, actual temperatures the myrosinase was exposed to may
265 have been lower. In other studies, only rapeseed has been shown to have a higher
266 inactivation temperature compared to yellow mustard, where inactivation typically occurs
267 above 60 °C at a holding time of 10 minutes (Kozlowska, Nowak, & Nowak, 1983; Stoin,
268 Pirsan, Radu, Poiana, Alexa, & Dogaru, 2009; Van Eylen, Indrawati, Hendrickx, & Van Loey,
269 2006; Van Eylen, Oey, Hendrickx, & Van Loey, 2008). Overall, myrosinase enzyme is

270 temperature sensitive and thermal treatments like blanching or heat processing are known
271 to cause a decrease in myrosinase activity (Ghawi, Methven, Rastall, & Niranjan, 2012;
272 Ludikhuyze, Ooms, Weemaes, & Hendrickx, 1999; Van Eylen, Indrawati, Hendrickx, & Van
273 Loey, 2006; Van Eylen, Oey, Hendrickx, & Van Loey, 2008). Inactivation of myrosinase leads
274 to decrease in formation of beneficial hydrolysis products from glucosinolate-myrosinase
275 hydrolysis, although myrosinase inactivation in *Brassicas* can be used to control sensory
276 characteristics in *Brassica* vegetables.

277 **3.3 Effect of pressure treatment on black, brown and yellow mustard seed**
278 **myrosinase**

279 Figure 2 depicts pressure inactivation of myrosinase from yellow, black and brown
280 mustard seeds. Pressure stability was determined at 10 minutes exposure time at pressures
281 ranging from 100-900 MPa at 15 °C. Myrosinase was substantially stable at 600 MPa with
282 about 19% loss in activity for brown mustard after 5 minutes exposure time (data not
283 included) while loss in activity for black mustard was 31%. Yellow mustard myrosinase was
284 observed to be notably stable up to 500 MPa (14% loss in activity) and there was 79% loss in
285 myrosinase activity at 600 MPa. At 700 MPa, there was 50 and 60% loss in myrosinase
286 activity for black and brown mustard respectively. However, at 700 MPa, myrosinase from
287 yellow mustard was completely inactivated, whilst there was no myrosinase activity at 900
288 MPa for black and brown mustard.

289 Pressure treatment for 10 minutes showed similar trend to those observed for 5 minutes.
290 Myrosinase activity decreased significantly above 600 MPa for both black and brown
291 mustard. At 800 MPa, the loss in enzyme activity was over 70% for both black and brown
292 mustard and there was no enzyme activity at 900 MPa. Yellow mustard myrosinase was

293 however only considerably stable up to 500 MPa (21% loss in activity) and at 600 MPa,
294 about 79% enzyme activity was lost. Van eylen *et al.* (2006) reported that myrosinase from
295 yellow mustard was inactivated at pressures above 600 MPa, however, this is not in
296 agreement with the current study where 79% loss in activity was observed at 600 MPa
297 processing. Compared to some other *Brassicas*, myrosinase from mustard have much higher
298 pressure stability. Pressure stabilities of myrosinase from other *Brassica* types have only
299 been reported for broccoli (300-500 MPa), green cabbage (250-300 MPa) and yellow
300 mustard (above 600 MPa) (Ghawi, Methven, Rastall, & Niranjan, 2012; Ludikhuyze, Ooms,
301 Weemaes, & Hendrickx, 1999; Van Eylen, Oey, Hendrickx, & Van Loey, 2008).

302 **3.4 Effect of combined temperature and pressure treatment on black, brown and**
303 **yellow mustard seed myrosinase**

304 Combined high pressure and temperature stability of myrosinase from brown, black and
305 yellow mustard seed (Table 2) was studied at a temperature of 30-70 °C and pressure of 600
306 -800 MPa for an exposure time of 10 minutes. At low temperatures (30-40 °C) and 600 MPa,
307 it was observed that loss in activity was about 30% for black mustard, 20% for brown and
308 50% for yellow mustard, respectively. Yellow mustard myrosinase showed no activity at 700
309 and 800 MPa. An increase in temperature up to 70 °C led to approximately 60% loss in
310 myrosinase activity at 600 MPa for black mustard and 70% for brown mustard. Overall,
311 there was a gradual loss in myrosinase activity as the temperature and pressure gradually
312 increased**.** This trend is in agreement with previous studies (Van Eylen, Oey, Hendrickx, &
313 Van Loey, 2008), where applying high pressure (over 600 MPa) increased thermal
314 inactivation rate.

315 At a combined pressure of 800 MPa and 70 °C, there was no myrosinase activity in any of
316 the mustard samples studied. This indicates a synergistic effect of high pressure (600-800
317 Mpa) on thermal inactivation of myrosinase in mustard seeds. However, Ghawi *et al.* (2012)
318 reported a synergistic effect at lower pressure level in the case of green cabbage
319 myrosinase. Pressure stability of myrosinase from *Brassica*s is not widely reported. It is clear
320 that myrosinase is inactivated at combined high pressure and temperature, so applying
321 lower pressure and temperature could be more beneficial in retaining myrosinase activity
322 and enabling formation of hydrolysis products.

323 At low pressure (200-300 MPa), it was observed that myrosinase enzyme activity was
324 notably stable at 60 °C for black mustard while significant decrease in activity was observed
325 for brown and yellow mustard (30% and 50%). In earlier studies (Van Eylen, Indrawati,
326 Hendrickx, & Van Loey, 2006) an antagonistic effect of low pressure (200-300 MPa) on
327 thermal inactivation of myrosinase in broccoli juice was reported, while Ghawi *et al.* (2012)
328 reported a synergistic effect of pressure on thermal inactivation of myrosinase in green
329 cabbage. In this study, an antagonistic effect of low pressure on thermal inactivation of
330 mustard seed myrosinase was observed. The loss in myrosinase activity was lower using
331 combined low pressure and temperature than the application of only thermal treatment.
332 For black and brown mustard myrosinase, activity retention at 75 °C and 200-300 MPa for 10
333 minutes processing time was above 70% and 55% respectively . Whereas without pressure,
334 activity retention at 70 °C was approximately 59% for black and 35% for brown mustard
335 myrosinase. Thermal processing of black and brown mustard myrosinase at 80 °C led to full
336 inactivation, however, application of low pressure (200-300 MPa) at 80 °C retained
337 considerable levels of the activity, about 50% for black and 40% for brown mustard. At 400

338 MPa, 80 °C and 10 minutes processing time, myrosinase activity was observed for black
339 (30%) and brown mustard (20%). Similarly, combining low pressure (200-300 MPa) with
340 thermal treatment at 70 °C retained 20% activity of yellow mustard myrosinase, whereas,
341 there was no myrosinase activity under thermal processing for yellow mustard myrosinase
342 at the same temperature. However, at higher temperature levels, there was no protective
343 effect of pressure on thermal inactivation for yellow mustard myrosinase.

344 The differences in initial activity between the mustard species, where brown had the highest
345 activity and yellow the least, led to similar trends in enzyme stability with temperature and
346 pressure, where myrosinase from yellow mustard was the least stable. As discussed earlier,
347 the differences in stability between the mustard species might have resulted from genetic
348 differences or responses to different environmental challenges.

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4 Conclusion

Myrosinase from different mustard species varied in terms of specific enzyme activity as well as temperature and pressure stability. Brown mustard myrosinase had the highest overall myrosinase activity and specific activity. Brown and black mustard myrosinase were more resistant to pressure and thermal treatment than myrosinase from yellow mustard. Combined high pressure-thermal treatment (up to 70 °C and 800 MPa) completely inactivated myrosinase from the mustards studied. However, at low pressure (200-400 MPa), inactivation temperature increased in the mustard samples studied with lower rate of loss in myrosinase activity compared to any of thermal, pressure and combined high pressure-thermal treatment. This difference in myrosinase stability could be utilized to control the hydrolysis level of glucosinolates when mustard seeds are used as a condiment along with cooked *Brassica* vegetables. This could have important health implications through increasing the delivery of bioactive isothiocyanates from the *Brassica*. In addition, controlling enzyme activity can also be used to regulate sensory attributes of *Brassica* vegetables.

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469 vegetables, and some properties of cabbage myrosinase in Taiwan. *Journal of the Science of Food*
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473 **Figure and table captions**

474 Figure 1: Effect of thermal processing on relative myrosinase activity in black, brown and
475 yellow mustard seeds; where temperature exposure time was 10 minutes. (■) brown
476 mustard; (♦) black mustard; (▲) yellow mustard. (A – enzyme activity after thermal
477 treatment, Ao – Initial enzyme activity). Error bars represent standard errors of the means.

478

479 Figure 2: Effect of pressure on relative myrosinase activity in black, brown and yellow
480 mustard seeds. Pressure holding time was 10 minutes and processing temperature was
481 controlled at 15 °C. (■) brown mustard; (♦) black mustard; (▲) yellow mustard. (A – enzyme
482 activity after pressure treatment, Ao – Initial enzyme activity). Error bars represent standard
483 errors of the means.

484

485 Table 1: Myrosinase activity, protein content and specific activity of yellow, brown and black
486 mustard seeds. (*un is activity units defined in section 2.2, lines 150-152).

487

488 Table 2: Combined temperature and high pressure inactivation of myrosinase from black,
489 brown and yellow mustard seeds at 10 minutes holding time.

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491 Table 3: Effects of combined low pressure and temperature processing on myrosinase
492 activity in black, brown and yellow mustard seeds at 5, 10 and 15 minutes holding time.

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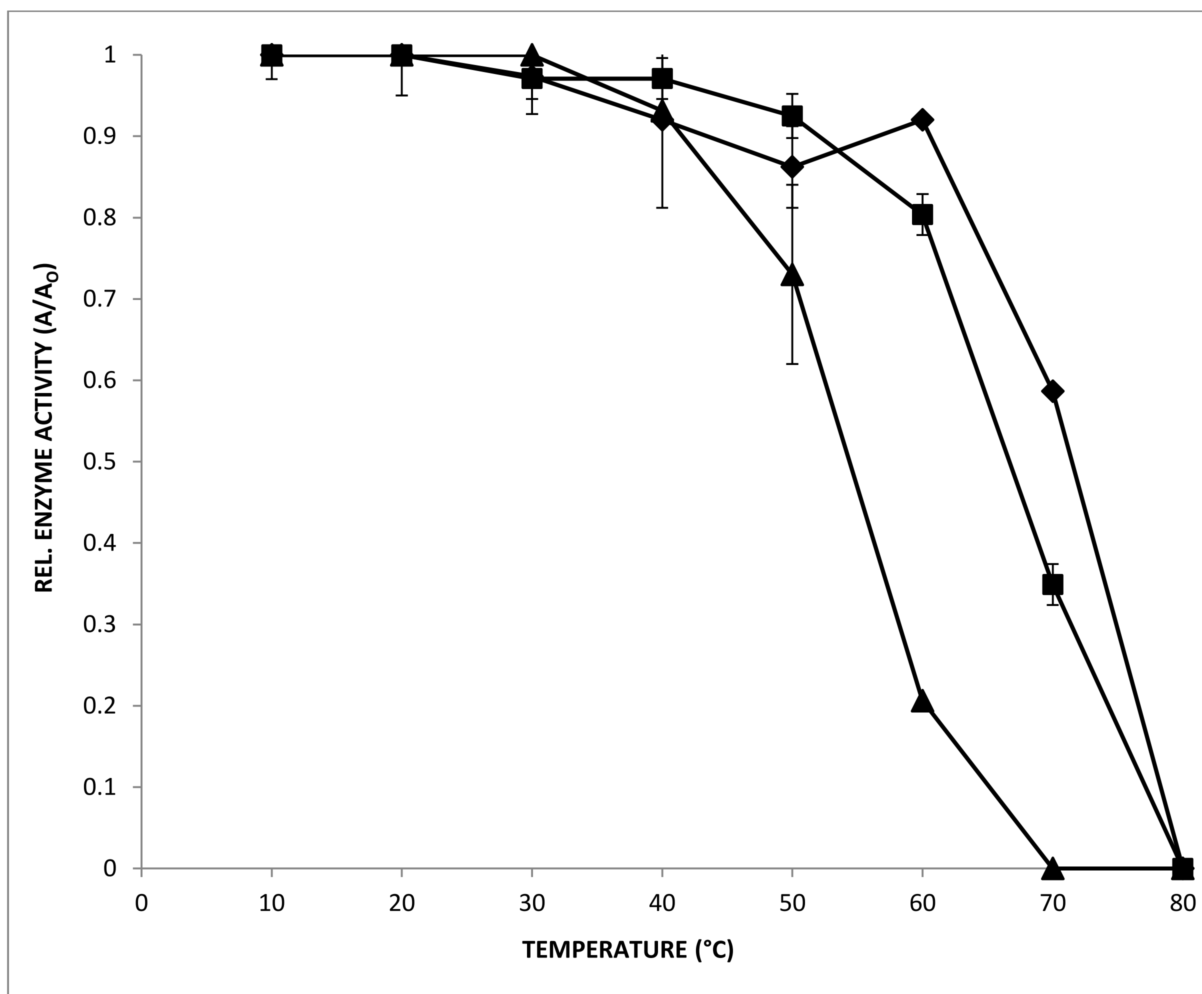

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512 Error bars represent standard errors of the means

513 Figure 1

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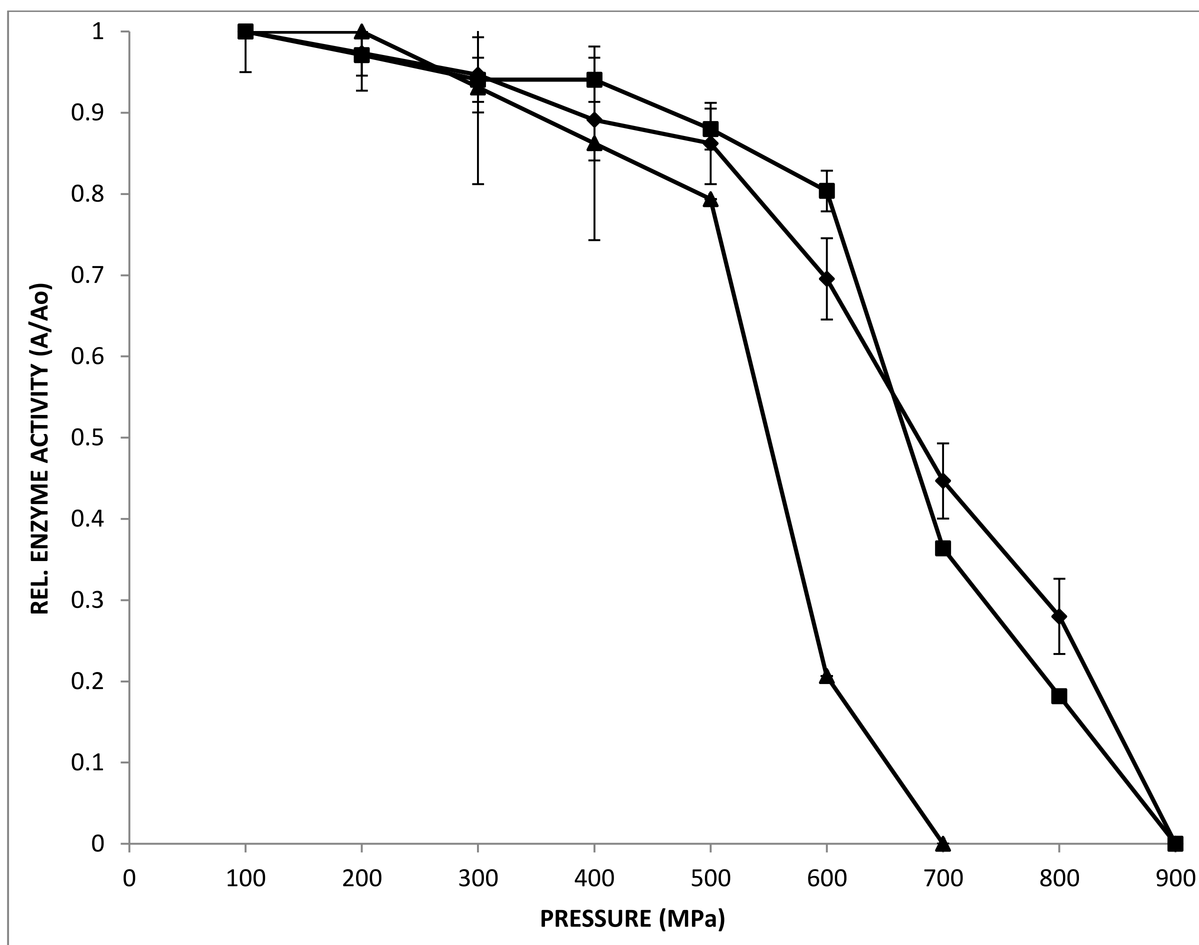

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526 Error bars represent standard errors of the means

527 Figure 2

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Table 1.

Mustard Seed	Myrosinase Activity (un/mL)	Protein Content (mg/mL)	Specific Activity (un/mg)
Black	1.5±0.00	1.21±0.01	1.24
Brown	2.75±0.22	1.34±0.05	2.04
Yellow	0.63±0.00	1.32±0.01	0.48

Table 2

Pressure (MPa)	Temperature (°C)	Relative Enzyme Activity (A/A_o)		
		Black	Brown	Yellow
600	30	0.7 ±0.00[a]	0.8 ±0.00[a]	0.5 ±0.00[a]
	40	0.7 ±0.00[a]	0.8 ±0.00[a]	0.5 ±0.00[a]
	50	0.7 ±0.04[a]	0.5 ±0.00[b]	0.4 ±0.00[b]
	60	0.7 ±0.00[a]	0.4 ±0.00[c]	0.2 ±0.00[c]
	70	0.4 ±0.00[b]	0.3 ±0.02[d]	-
700	30	0.4 ±0.00[a]	0.4 ±0.00[a]	-
	40	0.4 ±0.00[a]	0.4 ±0.00[a]	-
	50	0.3 ±0.04[b]	0.3 ±0.02[b]	-
	60	0.2 ±0.04[c]	0.1 ±0.00[c]	-
	70	0.2 ±0.00[c]	0.1 ±0.02[c]	-
800	30	0.3 ±0.00[a]	0.2 ±0.00[a]	-
	40	0.3 ±0.00[a]	0.2 ±0.00[a]	-
	50	0.2 ±0.00[b]	0.1 ±0.00[b]	-
	60	0.1 ±0.00[c]	0.1 ±0.00[b]	-
	70	-	-	-

540 Values not sharing a common letter are significantly different at $P<0.05$.

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554 Table 3.

P (MPa)	T(°C)	Relative Enzyme Activity (A/A_o)								
		Black			Brown			Yellow		
		Processing time (Minutes)			Processing time (Minutes)			Processing time (Minutes)		
		5	10	15	5	10	15	5	10	15
200	60	0.9±0.07[a]	0.9±0.00[a]	0.9±0.07[a]	0.7±0.07[a]	0.7±0.07[a]	0.7±0.00[a]	0.6±0.00[a]	0.5±0.11[a]	0.5±0.11[a]
	65	0.9±0.05[a]	0.9±0.05[a]	0.9±0.05[a]	0.7±0.03[a]	0.7±0.03[a]	0.7±0.00[a]	0.4±0.00[a]	0.4±0.00[a]	0.4±0.00[a]
	70	0.8±0.00[b]	0.8±0.00[b]	0.8±0.00[b]	0.7±0.00[a,]	0.7±0.00[a]	0.6±0.05[,b]	0.2±0.00[b]	0.2±0.00[b]	0.2±0.00[b]
	75	0.8±0.00[b]	0.8±0.00[b]	0.7±0.05[c]	0.6±0.00[b]	0.6±0.03[b]	0.6±0.03[b]	-	-	-
	80	0.6±0.00[c]	0.6±0.05[c]	0.5±0.08[d]	0.4±0.03[c]	0.4±0.00[c]	0.4±0.03[c]	-	-	-
300	60	0.9±0.07[a]	0.9±0.07[a]	0.9±0.07[a]	0.7±0.07[a]	0.7±0.00[a]	0.7±0.07[a]	0.5±0.11[a]	0.5±0.11[a]	0.5±0.11[a]
	65	0.9±0.05[a]	0.9±0.05[a]	0.9±0.05[a]	0.7±0.03[a]	0.7±0.03[a]	0.7±0.00[a]	0.3±0.11[a]	0.3±0.11[a]	0.3±0.11[a]
	70	0.8±0.05[b]	0.8±0.05[,b]	0.8±0.05[,b]	0.7±0.00[a]	0.6±0.00[b]	0.6±0.03[b]	0.2±0.00[b]	0.2±0.00[b]	0.2±0.00[b]
	75	0.7±0.05[c]	0.7±0.05[c]	0.7±0.05[c]	0.6±0.03[b]	0.6±0.00[b]	0.6±0.03[b]	-	-	-
	80	0.5±0.00[d]	0.5±0.00[d]	0.5±0.00[d]	0.4±0.00[c]	0.4±0.00[c]	0.4±0.07[c]	-	-	-
400	60	0.8±0.00[a]	0.7±0.00[a]	0.7±0.07[a]	0.6±0.07[a]	0.6±0.00[a]	0.6±0.07[a]	0.4±0.00[a]	0.4±0.00[a]	0.3±0.11[a]
	65	0.7±0.00[b]	0.7±0.00[a]	0.7±0.00[a]	0.6±0.00[a]	0.6±0.00[a]	0.6±0.03[a]	0.3±0.11[a]	0.3±0.11[a]	0.2±0.00[a]
	70	0.7±0.00[b]	0.7±0.00[a]	0.7±0.00[a]	0.6±0.03[a]	0.6±0.03[a]	0.6±0.07[a]	0.1±0.11[b]	-	
	75	0.5±0.05[c]	0.5±0.05[b]	0.5±0.05[b]	0.4±0.03[b]	0.4±0.03[b]	0.4±0.05[b]	-	-	
	80	0.3±0.05[d]	0.3±0.00[c]	0.3±0.00[c]	0.2±0.00[c]	0.2±0.03[c]	0.2±0.05[c]	-	-	

555 Values not sharing a common letter are significantly different at $P<0.05$.

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