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

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12 **Abbr. Running Title:**

13 **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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1 Introduction

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

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

2.1 Sample preparation and myrosinase extraction

Yellow mustard (*Sinapsis alba* L. *subsp maire*), black mustard (*Brassica nigra* L. W.D.J

Koch. *var*. *nigra*) and brown mustard (*Brassica juncea* L. Czern. *var. juncea*) were obtained

from the I.P.K Gene bank (Gatersleben, Germany). All samples were obtained as dried

seeds.

Myrosinase enzyme extraction was done as described by Ghawi *et al.* (2012). The process

involved grinding dry mustard seeds in a coffee grinder. 10mL of buffer (Tris HCl 0.2M, pH

7.5 containing EDTA 0.5mM, dithiothreitol 1.5mM and 0.4g Polyvinylpolypyrrolidone) was

then added to 0.5g powdered mustard and blended on ice (15 minutes) and then

centrifuged (11,738 ×g) for 15 minutes at 4 °C. The supernatant was filtered (0.45µm). The

filtrate was made up to 10mL using the buffer solution and 90% precipitation of protein was

achieved using 6.2g ammonium sulphate with slow blending on ice for 30 minutes. The

mixture was then centrifuged (13,694 ×g) for 15 minutes at 4 °C. The pellet obtained was

suspended in 2mL of 10mM Tris HCl buffer, pH 7.5.

The mix was extensively dialysed at low temperature (4 °C) using cellulose membrane

(Medicell International Ltd, Molecular weight cut-off 12,000-14,000 Da) and 10mM Tris HCl

buffer 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.

2.2 Myrosinase enzyme activity assay

Myrosinase activity was measured according to the coupled enzymatic procedure with

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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3 Results and discussion

3.1 Enzyme activity, protein content and specific enzyme activity of myrosinase enzymes from black, brown and yellow mustard seeds

Table 1 shows the enzyme activity, protein content and specific enzyme activity of myrosinase enzyme from black, brown and yellow mustard. Brown mustard had higher myrosinase activity (2.75 un/mL) than black mustard (1.50 un/mL) and yellow mustard had the least myrosinase activity (0.63 un/mL). The protein content of all studied samples was similar. Myrosinase from brown mustard had the highest proportion of specific activity (2.04un/mg) and yellow mustard the lowest (0.48un/mg). The differences between mustard species in overall enzyme activity were not related to differences in protein content and hence, differences between species in specific activity prevailed. Variations in myrosinase enzyme activity within and between *Brassica* species have been reported previously and have been attributed to genetic and/or environmental factors (agronomic and climatic conditions) (Pocock, Heaney, Wilkinson, Beaumont, Vaughan, & Fenwick, 1987; Wilkinson, Rhodes, & Fenwick, 1984). In addition, Rask *et al*. (2000) had also reported that the difference in thermal stability of myrosinase in *Brassica* plants was probably due to the existence of different isoforms of myrosinase, where some of them interact with myrosinase-binding proteins (a group of proteins found in *Brassica* plants) to form complexes that may improve stability. It is therefore possible that the different species of mustard may have genetic differences, or have adapted to different environmental conditions that have resulted in different isoforms of myrosinase. It has also been suggested that myrosinase activity for similar *Brassica* samples may vary between studies merely due to the different protocols employed (Piekarska, Kusznierewicz, Meller, Dziedziul, Namiesnik, & 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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Figure and table captions

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

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

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

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

Table 3: Effects of combined low pressure and temperature processing on myrosinase activity in black, brown and yellow mustard seeds at 5, 10 and 15 minutes holding time.
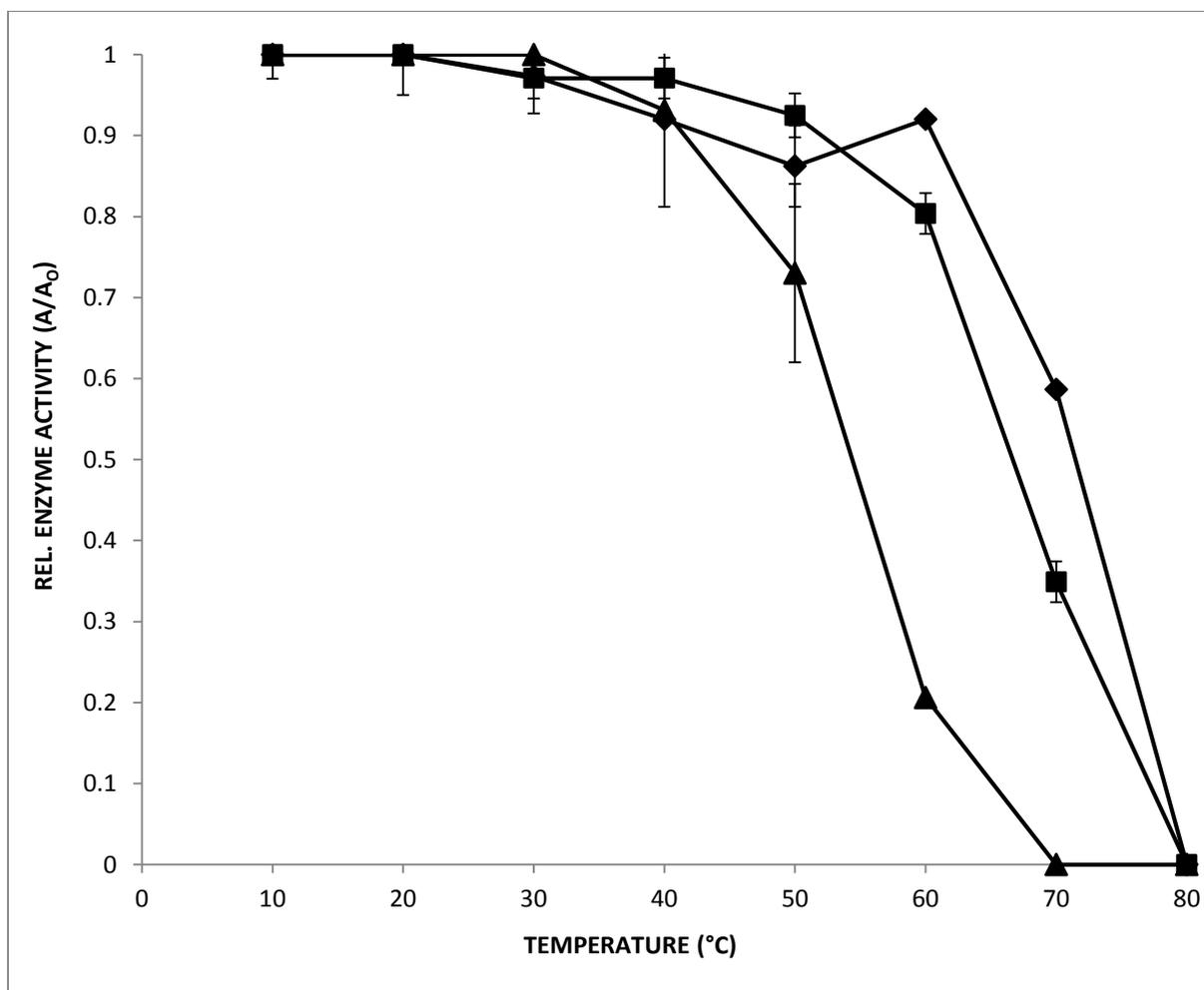
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Error bars represent standard errors of the means

Figure 1

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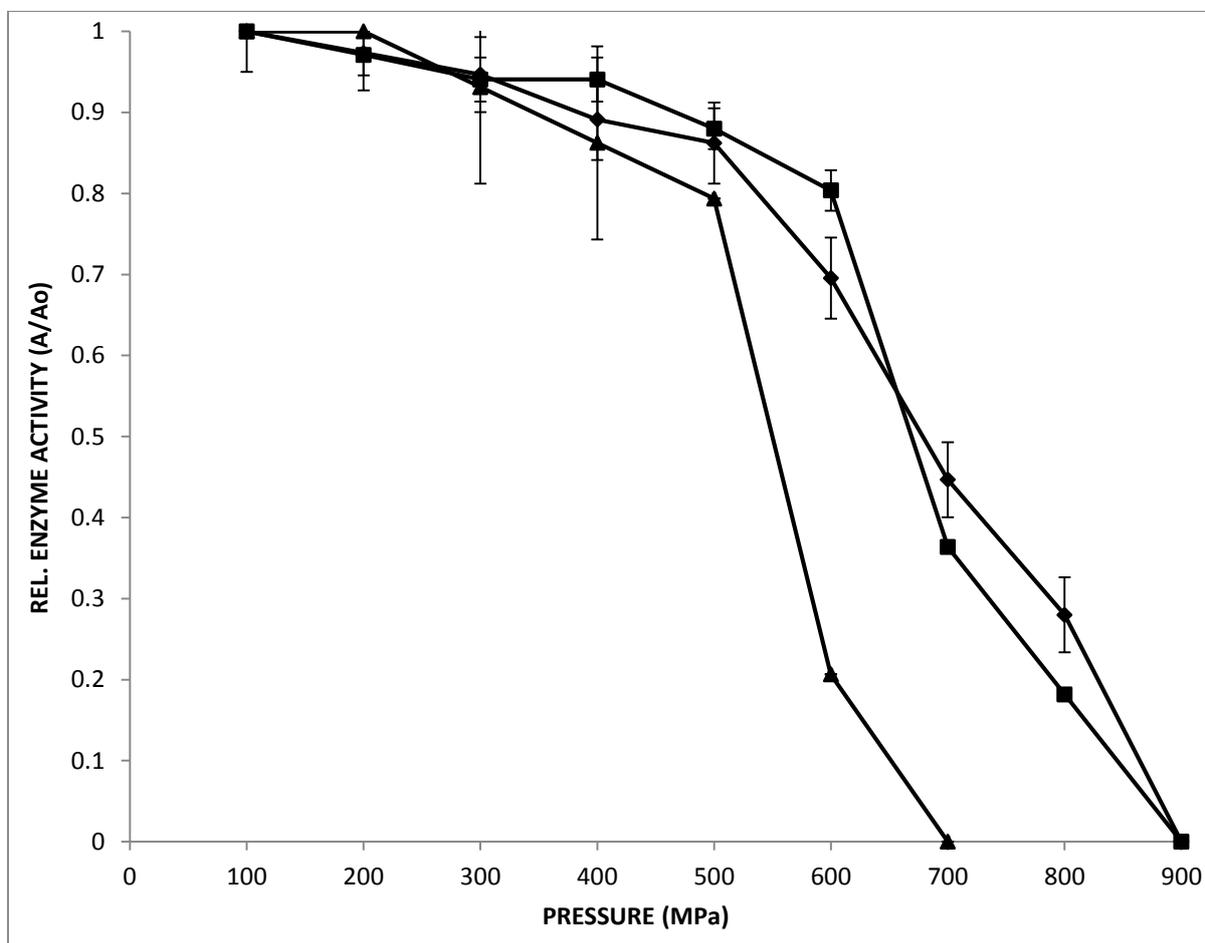520

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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	-	-	-

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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