



## Studies regarding the myrosinase enzymatic activity from black mustard (*Brassica nigra*) seeds

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### Abstract

The aim of this study consists in the presentation of a simple and fast method for myrosinase activity quantification towards sinigrin from the aqueous extracts obtained from black mustard (*Brassica nigra*) seeds as raw material (SM) or crushed down (CM). The myrosinase activity was determined under different pH (5-8.5), temperature ( $T = 25-85^{\circ}\text{C}$ ) and reaction time ( $\tau = 30-390$  minutes) conditions, according to the spectrophotometric method and was expressed depending on the concentration of the glucose resulted from enzymatic reaction. The optimum parameters corresponding to the maximum myrosinase activity in the aqueous extracts of black mustard were the following: pH 7,  $T 55^{\circ}\text{C}$  and  $\tau 210$  minutes for the crushed down mustard samples and  $\tau 240$  minutes for the mustard seeds samples. The myrosinase activity is significantly influenced by the physical state of the vegetable material (raw or crushed down), this values being of  $116.3510 \mu\text{g glucose/g.min}$  for CM in comparison to the  $85.0660 \mu\text{g glucose/g.min}$  for SM. The enzymatic hydrolysis reaction of the sinigrin takes place with heat releasing.

**Key words:** *Brassica nigra*, glucosinolates (GLS), sinigrin (SIN), myrosinase (MYR), enzymatic activity.

### Introduction

Glucosinolates (GLS) are natural compounds belonging to S-glycosides class, being sulphated esters of the S-( $\beta$ -D-glucopyranosyl)-metanethoxyhydroxamic, C-substituted. GLS are formed in plants from amino acid precursors upon some biochemical reactions. In vegetable products, these compounds are found as glycosylated form and passed/transformed into active pharmaceutical-dynamic form as a result of the enzymatic degradations<sup>2,8,16,19</sup>. Until now, GLS have been identified only in dicotyledonous plants from the Brassicaceae, Capperaceae, Resedaceae, Moringoaceae and Tovaraceae families, rarely appearing in some species of the Limantroceae, Caricaceae, Gyrostemonaceae, Salvodoraceae and Euphorbiaceae families<sup>5,6,8</sup>. GLS are intravacuolarly located in the cells, and upon grinding, breaking or cutting tissues they get in touch with the myrosinase (MYR), enzyme that determines the division of the glucose (GLU) rest. A variety of volatile compounds (isothiocyanates, thiocyanates, nitriles, hydroxynitriles, epithyonitriles) with antimicrobial and phytotoxic properties are released upon enzymatic hydrolysis along with GLU<sup>3,4,13,14</sup>.

Among GLS degradation products, the isothiocyanates are responsible for the "spicy" and hot taste. These compounds are found in mustard, horseradish, cabbage and broccoli. Unlike their precursors, characterized by a neutral or weak activator effect towards microorganisms development, the hydrolysis products have proved to be powerful inhibitors of the microbial flora<sup>15</sup>. The GLS content is higher in black mustard seeds and horseradish roots (over 10% by dry weight) than in the other constituent parts of the Brassicaceae<sup>11,14,15</sup>.

As a result of their high variety and bioactivity, GLS are suited to be used in the pharmaceutical industry, but they also have a series of applications in the food industry. The interest in GLS has started from several observations: these compounds are involved in the plants defensive mechanism, have a protective role against carcinogen chemical agents and they interfere with the sulphur metabolism and regulation of plant development. Also, the researches performed reflect the chemopreventive effect of GLS on different human cancer types<sup>12,18</sup>. The GLS known as sinigrin (SIN) (thio- $\beta$ -glucopyranosyl-1-N-sulphate-2-propenylimidate) is in significant content in mustard (*Armoracia rusticana*) seeds<sup>3,4</sup>.

MYR enzymes (thiogluco-side-GLS) are a group of isoenzymes that catalyses the GLS hydrolysis (naturally occurring thiogluco-sides). Usually MYR enzyme physically occurs from GLS, but upon wounding of plant (e.g. during cooking, mastication or damage of the insects) it catalyses their hydrolysis to obtain mainly isothiocyanates, GLU and bisulphate. The reaction involves an initial hydrolysis of the breakdown products:  $\beta$ -D-glucose and thiohydroximate-O-sulphonate. MYR enzyme catalyzed hydrolysis of GLS initially involves the cleavage of the thiogluco-side linkage yielding D-glucose and thiohydroximate-O-sulphonate. This intermediation rapidly rearranges resulting in the production of sulphate and thiocyanate, isothiocyanate or nitrite depending on different factors such as substrate, pH, temperature and availability of ferrous ions<sup>9,17</sup>. The enzymatic hydrolysis of the SYN is influenced by a series of factors like reaction medium pH, reaction temperature, the necessary speed for reaching the

equilibrium and the presence of some inhibitor/promoter substances<sup>1,9,10,17</sup>.

Starting from these observations, our study aims to describe a simple and fast method for the MYR quantification on SIN present in black mustard (*Brassica nigra*) seeds as raw material (SM) or crushed down (CM). The study was performed under different pH, temperature and reaction time conditions. The MYR enzymatic activity was expressed depending on the concentration of GLU released upon enzymatic reaction. The o-toluidine spectrophotometrical method was used for determination of GLU<sup>1,10</sup>.

### Materials and Methods

**Samples:** The black mustard, *Brassica nigra* (L.), was harvested from Didactic Experiment Station of Banat's University of Agricultural Sciences and Veterinary Medicine, Timisoara. The seeds were harvested and conditioned (dust and metal impurities removal, liquid nitrogen freezing and storage at -66°C until analysis).

**Determination of the pH influence on the MYR activity:** The pH influence on enzymatic reaction was established by suspending the vegetable material (1 g from CM and SM) in 10 ml solution of phosphate buffer for pH 5-7.5 and borate buffer for pH 8 and 8.5, respectively. The samples were put into shaker at 35°C and continuously shaken for 30 minutes. Afterwards, in order to stop the MYR activity, 2 ml of 20% trichloroacetic acid solution (TCA) was added in each sample. The resulted reaction mixture was centrifuged at 6000 rot/min for 10 minutes. From each supernatant, 5 ml sample was taken and passed through filtrating membranes, and the clear samples were stored in test tubes with stoppers and stored in refrigeration conditions (4-6°C) until analysis. For each pH value, the experiments were performed three times each.

**Determination of the reaction temperature effect on the MYR activity:** The temperature effect on MYR activity was studied through this parameter variation in the range of 25-85°C, the reaction time being constantly maintained at 30 minutes. Vegetable material (1 g from CM and SM) was suspended in 10 ml phosphate buffer pH 7. The samples were put into a shaker at the corresponding temperatures and continuously stirred for 30 minutes. Afterwards, the samples were submitted to the same operations described above. For each reaction temperature, the experiments were performed three times each.

**The kinetics study of the SIN enzymatic hydrolyze reaction:** The enzymatic hydrolysis process was studied *in vitro* using as a substrate the SIN from the vegetable material taken into study, and not the SIN purified<sup>1</sup>. The SIN enzymatic hydrolysis reaction kinetics was performed under the following experimental conditions: pH 7, reaction time  $\tau$  30-390 minutes and reaction temperature T 25-75°C. The concentration of glucose resulted from the reaction was taken into sight parameter. The reaction order and the speed constant were experimentally determined from the graphical representations  $\ln(\text{conc GLU}) = f(\text{time})$ . The activation energy was determined according to Arrhenius equation<sup>19</sup>. One g vegetable material (SM and CM) was weighed and 10 ml phosphate buffer pH 7 was added. The samples were hermetically closed and put into a shaker at the corresponding reaction

temperature. Every 30 minutes, a sample was taken from each lot. The enzymatic hydrolysis reaction was stopped by adding 2 ml 20% TCA solution each. The samples were passed through PTFE 0.45  $\mu\text{m}$  filtrating membranes, stored in test tubes with stoppers and maintained in refrigerating conditions until analysis. The experiments were performed three times each.

**Spectrophotometric determination of the MYR activity:** The MYR (*EC 3.2.3.1*) enzymatic activity was determined by the method of Al-Turky and Dick<sup>1</sup>. According to this method, "one unit of enzymatic activity represents the enzyme amount that hydrolyses the sinigrin, releasing 1  $\mu\text{g}$  glucose/g.min under the presented analysis conditions". MYR activity determination is based on the reaction between GLU and o-toluidine that forms a blue-greenish complex that exhibits maximum absorbance at  $\lambda = 630 \text{ nm}$ . In order to prepare the standard curve  $A=f(\text{conc GLU})$  from the GLU stock solution (5 mg/ml), standard solutions with the concentrations in the range of 0.1-1.5 mg/ml were prepared. From these solutions, 100  $\mu\text{l}$  were taken and passed in graduated test tubes of 10 ml. One ml of 3% TCA and 5 ml of o-toluidine reagent solution were added. After shaking, the samples were put into a thermostatic water bath at boiling point and maintained for 13 minutes until a blue-greenish coloration appeared. The samples were cooled to the room temperature and the absorbance was read against the blank sample containing o-toluidine and 3% TCA solution prepared under the same conditions. For each standard solution three determinations were made. The standard curve equation is  $Y = 0.6368 + 0.0129X$  and the regression coefficient  $R^2 = 0.9983$ .

### Results and Discussion

**The influence of pH on MYR activity from Brassica nigra:** The pH effect on MYR activity is shown in Fig. 1 and the results are according to the literature<sup>1,9,10,17</sup>. The MYR activity is affected by the reaction medium pH especially due to the enzyme protein nature. The corresponding optimum pH to MYR activity in white mustard is in the range of 4.5-4.9<sup>17</sup>. The study of the pH influence on enzyme activity was done in the range of 5-8.5 because at higher pH there is interference of desulphatase enzyme that generally exhibits optimum activity at an alkaline pH, the action substrate being common<sup>7</sup>.

From the experimental data shown in Fig. 1, it can be seen that MYR is active in a wide pH range, the enzymatic activity continuously increasing from pH 5 (SM 38.90%; CM 46.56%) to pH 7 (SM 73.11%; CM 100%), and after that it's activity starts decreasing, remaining practically constant at pH above 8 (SM 36.81%; CM 46.84%). The MYR activity is also significantly

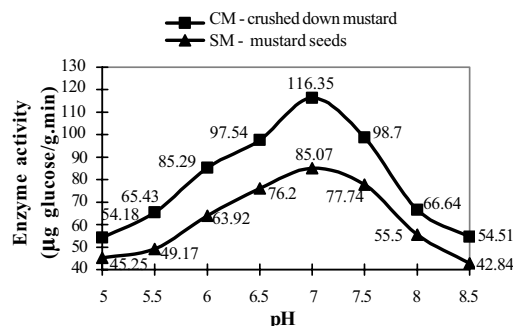


Figure 1. Phosphate buffer pH effect on MYR activity.

influenced by the physical state of the vegetable material (raw or crushed down), this values being of 116.3510  $\mu\text{g}$  glucose/g.min for CM, in comparison to 85.0660  $\mu\text{g}$  glucose/g.min for SM.

**The influence of temperature on MYR activity from *Brassica nigra*:** The temperature effect on MYR activity is shown in Fig.2. The temperature directly influences the speed of the hydrolysis enzymatic reaction, that is, along with the increasing of the temperature, the MYR activity increases also, but this increase is limited by the thermal stability of the molecule of protein nature of that enzyme<sup>20</sup>. From the experimental data, it can be appreciated that MYR exhibits maximum activity at temperatures in the range of 45-55°C. Also, it can be seen that at high temperatures 75-85°C too, MYR still exhibits activity even if very weak (for SM 27.21%), results that are contrary to the data obtained by Ludikhuyze and van Doom<sup>9, 17</sup>, who mentioned that MYR is inhibited at temperatures higher than 75°C. A possible explanation for the obtained results could be that in this case the experiments took place by using as a substrate the SIN from the aqueous extracts and not the SIN isolated and purified, thus being possible that activity is a result of the desulphatase enzyme, thermostable to temperatures of 90-95°C, enzyme that uses SIN as a substrate, too. The data presented in Fig. 2 have led to obtaining some isotherm curves resembling those obtained by Sharma and Garg<sup>13</sup>, who used purified MYR from *Brassica juncea*. It can be appreciated that the enzymatic hydrolysis reaction of the SIN takes place with heat releasing, in all situations the reaction enthalpy exhibiting negative values (at 55°C SM 1,214,771 J/mol.K; CM 1,333,768 J/mol.K). Also, the variation in inverse proportion of the reaction enthalpy with the reaction temperature confirm the above mentioned assumptions that MYR exhibits optimum activity at moderate temperature.

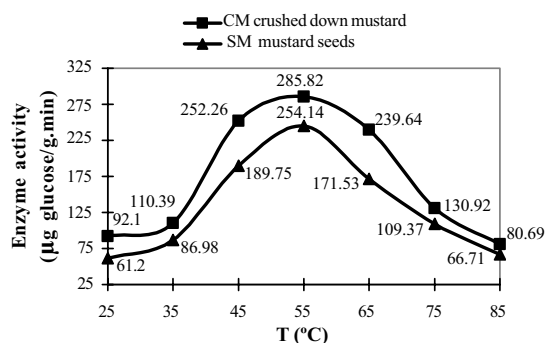


Figure 2. Temperature effect on MYR activity.

**The influence of reaction time on MYR activity from *Brassica nigra*:** The reaction time effect on MYR activity is shown in Fig. 3. The study of the reaction time influence on MYR activity was performed in temperature range of 25-75°C, but significant results were obtained especially in 45-55°C. From Fig. 3 it can be seen that the curves form remains the same for the entire studied time range. For example, the MYR activity in the case of the mustard not submitted to grinding operations, increases almost linearly with the incubation time until 128-180 minutes, reaches a maximum in the range of 210-240 minutes and after that slowly decreases to 270 minutes, and after this time the enzymatic hydrolysis reaction

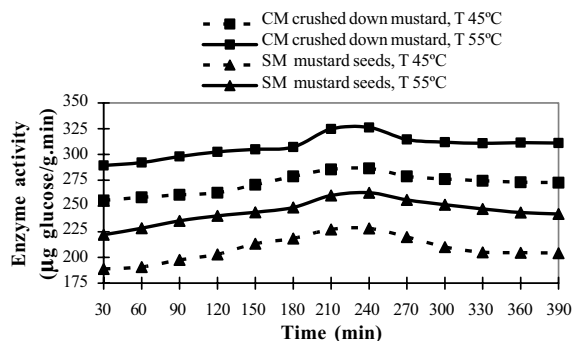


Figure 3. Incubation time influence on MYR activity.

reaches the equilibrium, fact proved by almost constantly maintaining of the MYR activity value.

It can be noticed that in the case of the mustard, the linear variation of the enzyme activity with the reaction time is significantly influenced by the preparing step of vegetable material but also by the reaction temperature, that is, the increasing of the quantity of the released GLU in the reaction. A possible explanation for this behavior can be given if we take into account Bones<sup>2</sup> researches regarding the localization of the substrate and enzyme in the subcellular compartments of the same vegetable cell, but also of the morphological forms of the enzyme<sup>9</sup>. Thus, if the assumption is that the MYR is generally located in the cell wall, the mechanical disruption of the cell membrane will determine the shortening of the time for reaction initiation that will determine the starting of the hydrolysis almost in a snapshot and thus, the quantity of GLU produced is almost double (124.43 g glucose/g.min for CM in comparison with 76.2 g/g.min in the case of SM). In exchange, the MYR activity initially increases no matter the reaction temperature value, and once the value of 45°C is exceeded, the MYR activity is very slow (124.43 g glucose/g.min at 25°C and 228.50 g glucose/g.min at 45°C, respectively, only 278.10 g glucose/g.min at 55°C) at the same reaction time. The function linear form of enzymatic activity/reaction time in the range of 180 minutes indicates that the process of GLU formation is a first order reaction, at least for this time range.

Incubation time higher than 210 minutes caused a deviation of the reaction speed from the linearity, suggesting that the hydrolysis reaction of the SIN became limited, the reaction products (GLU, allyl-isothiocyanate and possibly thiocyanate and nitriles) becoming feeding source for the possible microorganisms present in the aqueous extracts or accumulating. From the allure of the presented curves in Fig. 3, it may be concluded that a reaction time of maximum 180-200 minutes is enough for testing the sinigrin-myrosinase system potential. From the mathematical expression of the enzymatic hydrolysis reaction speed, it was ascertained that except for the values obtained at 25°C,  $R^2 < 0.95$  for SM, the step determined by the speed represents a first order reaction.

### Conclusions

The study of the myrosinase activity from *Brassica nigra* has led to the conclusion that in general, the sinigrin-myrosinase system is influenced by a variety of medium factors, among the most important being the pH, temperature and reaction time. Upon enzymatic hydrolysis of the sinigrin at optimum parameters (pH 7; reaction temperature T 55°C; incubation time  $\tau$  210-240 minutes)

results a significant quantity of D-glucose that may be spectrophotometrically determined and used as a measure for the enzymatic activity of the myrosinase from *Brassica nigra*. The presented method is fast, accurate and can be used in almost all laboratories.

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