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Myrosinase activity in different plant samples; optimisation of measurement conditions for spectrophotometric and pH-stat methods

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a b s t r a c t

Myrosinase (EC 3.2.3.1) found in Brassicaceae plants, is the enzyme responsible for hydrolysis of glu-cosinolates. As a result a variety of biologically active metabolites are liberated, whose importance in crop protection and especially in cancer chemoprevention is rapidly gaining recognition. The growing practical application of glucosinolate degradation products requires that sensitive and reliable meth-ods of myrosinase activity determination in different types of plant samples are established. With the use of commercial myrosinase prep, we systematically optimised conditions of measurement of this enzyme activity by spectrophotometric and pH-stat methods. The parameters evaluated included: sample preparation, choice of substrate, its concentration, reaction temperature and detection wavelength.

Two substrates with different spectral properties were chosen: sinigrin (SIN) and glucotropaeolin (GTL). For both substrates, the best reliability was achieved at reaction temperature of 37 °C and substrate con-centration of 0.2 mM and 5 mM for spectrophotometric and pH-stat methods, respectively. GTL exhibiting higher absorption at the recommended detection wavelength of 230 nm ensured greater sensitivity of spectrophotometric determination of myrosinase activity in the case of transparent plant samples. GTL seemed to increase also the sensitivity of pH-stat method, however, in this case homogenisation of plant samples turned out to be most important. The optimised conditions were then verified for a range of plant samples. Based on these results, the optimised protocols of myrosinase activity determination for both methods are proposed.

Keywords: Myrosinase, Brassica plants, Enzymatic activity determination

1. Introduction

The enzyme myrosinase (β-thioglucosidase glucohydrolase, EC 3.2.3.1) is found mostly in *Brassicaceae* plants, which also contain glucosinolates (GLS), a class of organosulphur secondary metabolites. The GLS – myrosinase system provides brassicas with the natural defence against attack by herbivores as the products of enzymatic GLS hydrolysis, mainly isothiocyanates (ITC), show broad biocidal activity including insecticidal, nematicidal and fungicidal effects (Burow et al., 2007; Larkin and Griffin, 2007; Manici et al., 1997). The common structure of GLS comprises of a β-D-thioglucose group linked to a sulphonated aldoxime moiety and a variable side chain derived from amino acids. Several different myrosinase isoenzymes have been characterised in seeds, seedlings and vegetative tissues of *Brassicaceae*. These isoenzymes are glycoproteins with different degrees of glycosylation, variable isoelectric points and seem to be both plant organ and species specific (Bones and Iversen, 1985; Thangstad et al., 1990). Myrosinase has traditionally been reported to be composed by two identical subunits (Björkman and Janson, 1972), although some studies have shown, that it can form complexes with so called myrosinase binding or associated proteins which also may play a role in the GLS – myrosinase system (Burow et al., 2007; Eriksson et al., 2002; Zhang et al., 2006).

In plant tissue, myrosinase and GLS are located in separate cellular compartments; the enzyme is found in the myrosin cells (Andreasson et al., 2001; Hoglund et al., 1991), whereas GLS are situated in vacuoles of various types of cells (Mithen, 2001). Therefore, the enzymatic conversion of GLS into antibiological compounds occurs only after cell disruption, *e.g.* as a result of pathogen attack or upon processing during food preparation or mastication. Myrosinase catalyses the hydrolysis of the thioglucosidic bond in GLS

Abbreviations: GLS, glucosinolate; SIN, sinigrin; GTL, glucotropaeolin; ITC, isothiocyanates.

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releasing thiohydroximate-O-sulfonate, an intermediate that can be further converted to a variety of products, whose structures depend on the parent GLS, the hydrolysis conditions, presence of ferrous ions and additional protein factors (Finiguerra et al., 2001; Mithen, 2001).

Products of GLS enzymatic degradation, especially ITC, have found application in the ecological method of crop protection, so called biofumigation. This term refers to the agricultural use of natural compounds, like ITC, by *Brassica* rotation or green manure crops to suppress soil-borne pests and pathogens. Interest in biofumigation has increased recently in horticultural industries due to the prohibition of several synthetic pesticides and soil fumigants (*e.g.* methyl bromide, ethylene dibromide) (Gimsing and Kirkegaard, 2009; Kirkegaard and Sarwar, 1998). However, ITC are perceived nowadays not only as promising natural pesticides. The greater expectations are associated with their ability to trigger a number of health promoting effects (Smith et al., 2003), most importantly inhibition of tumorigenesis, anti-inflammatory properties and prevention of heart diseases (Wu et al., 2004). Several mechanisms have been proposed to underlie anticarcinogenic benefits of ITC. Those most frequently quoted involve the inhibition of phase I enzymes, such as certain isoforms of cytochrome P450, induction of phase II enzymes (*e.g.* quinone reductase, glutathione S-transferses, UDP-glucuronosyl transferases) responsible for detoxification of potential carcinogens (Fahey et al., 1997; Singh et al., 2007), decreasing the rate of mitosis and stimulation of apoptosis in human tumour cells (Johnson, 2002).

To fully exploit the potential of the GLS – myrosinase system in health beneficial or pathogen defensive applications, the reliable and reproducible methods of determination of myrosinase activity are needed. The aim of this study was to compare and optimise two frequently used methods of determination of this enzyme activity: spectrophotometric and pH-stat assays. Among many different methods proposed, these two are not only suitable for kinetic analyses, but also seem simple and versatile enough to be employed for a variety of experimental or practical applications. The basis of the first method is a spectrophotometric monitoring of the decrease of substrate absorbance at 227 nm during hydrolysis (Palmieri et al., 1987). pH-stat method relies on alkali titration of H^+ ions released during myrosinase catalysed reaction (Finiguerra et al., 2001; Palmieri et al., 1987). However, in literature different protocols are described and the published values of myrosinase activity vary substantially for apparently similar samples. Consequently, it is difficult to assess the reliability of available data, hence their practical application is problematic.

Another popular method of determination of myrosinase activity involves measuring the amount of glucose released during GLS hydrolysis (Kleinwächter and Selmar, 2004; Palmieri et al., 1987; Shikita et al., 1999; Wilkinson et al., 1984). Glucose can be measured using the test based on its enzymatic oxidation by glucose oxidase yielding in gluconic acid and H_2O_2. The latter then reacts with 4-aminoantipyrine and 4-hydroxybenzoic acid in the presence of peroxidase. The resultant coloured product N-(4-antipyryl)-p-benzoquinone imine is detected photometrically at 500 nm. In the presence of ascorbic acid this assay fails, as this compound scavenges H_2O_2 and consequently the formation of the coloured product is inhibited. To make matter worse, even when produced, it may be decoloured by further reactions with ascorbic acid. Therefore, the assay based on glucose determination is not very reliable for samples containing ascorbic acid, thus also for brassicas, which may contain up to 0.15% (w/w) of vitamin C (Davey et al., 2000; Vallejo et al., 2003). Another assay relying on glucose determination in which the formation of $NADH + H^+$ is monitored after glucose phosphorylation might not be suitable either. This is due to the fact that the absorbance at 340 nm may not only result from $NADH + H^+$ formed proportionally to the glucose present, but also reflect the decomposition products of dehydroascorbic acid, appearing after ascorbic acid oxidation. Various cases of indirect interferences occurring during determination of myrosinase activity by glucose release have been critically discussed by Kleinwächter and Selmar (2004). In addition, according to our experiments, myrosinase catalyses degradation of glycosidic bonds not only in GLS, but also in starch (data not shown). This suggest that glucose may arise also from the degradation of other sugars present in plants (3.3–7.1% of carbohydrates in brassicas (Leroux et al., 2002)). All in all, the determination of glucose seems not a very precise approach to myrosinase activity measurements.

In this research, the optimisation of spectrophotometric and pH-stat assays was carried out for a wide range of concentrations of two substrates, sinigrin and glucotropaeolin, with the use of commercial prep of myrosinase isolated from *Sinapis alba*. Also the influence of sample preparation method and measurement conditions on sensitivity and reproducibility of the assays were investigated. The optimised protocols were then verified for different types of plant samples prepared from edible parts of *Brassica* vegetables and seeds.

2. Methods

2.1. Materials

Myrosinase, sinigrin (SIN), allyl isothiocyanate (AITC) and benzyl isothiocyanate (BITC) used in the study were purchased from Sigma–Aldrich. NaCl and NaOH were from P.P.H Standard Sp. z o.o. Glucotropaeolin (GTL) was obtained from Plant Breeding and Acclimatisation Institute in Poznan (Poland). The UV–vis spectra of GLS and ITC standards were recorded for 0.1 mM water solutions with Cary 300 Bio spectrophotometer (Varian Inc.).

2.2. Plant material

Brassica vegetables and seeds used in the investigations of myrosinase activity were derived from a variety of sources. Brussels (*Brassica oleracea* L. var. *gemmifera*) and rutabaga (*Brassica napus* L. var. *napobrassica*) were harvested from organic plantation in Czapielsk (Northern Poland), turnip cabbage (*Brassica oleracea* var. *gongylodes* L.) was obtained from a farm in Osiek (Northern Poland), white cabbage (*Brassica oleracea* var. *capitata* f. *alba*) was grown by us in the vicinity of Gdansk University of Technology, Gdańsk (Poland). Savoy cabbage (*Brassica oleracea* L. var. *sabauda* L.), broccoli (*Brassica oleracea* L. var. *italica* Plenck) and daikon (*Raphanus sativus* var. *longipinnatus*) were purchased in a local shop. Seeds of *Brassica* plants were produced by PNOS (Ożarowice Mazowieckie, Poland) or Green-Land Service (Michałowice, Poland). Fresh vegetables were lyophilised, grounded and stored at 4 °C until investigation. The seeds were grounded right before use.

2.3. pH-stat method of determination of activity of purified myrosinase

The reaction mixture consisted of 15 mL 80 mM NaCl (pH 6.5), 0.15 mL of GLS solution (0.01–0.5 M SIN or GTL, final concentration 0.1–5 mM) and 0.15 mL of myrosinase prep solution (0.5–13 mg/mL, final concentration 0.005–0.13 mg/mL). Acidification of reaction mixture caused by GLS hydrolysis was counterbalanced by the addition of 1 mM NaOH to maintain pH at a constant level of 6.5. The reaction mixture was kept stirred at 37 °C if not indicated otherwise The GLS substrate hydrolysis was monitored for 1 h. Myrosinase activity was determined based on the measurement of NaOH solution consumption performed with T70 titrator with pH-stating option (Mettler Toledo).

The myrosinase activity was calculated using the formula:

$$\text{Activity} = 1000\frac{V_{\text{NaOH}}}{V_{\text{sample}} \cdot t \cdot C_{\text{MYR}}} \quad (1)$$

where V_{NaOH} denotes the volume of 1 mM NaOH used for titration (μL), V_{sample} the volume of investigated sample (μL), t the reaction time corresponding to the initial reaction rate characterised by linear consumption of the titrant (min), C_{MYR} the concentration of myrosinase prep (mg/mL). Myrosinase activity is given as μmol of hydrolysed GLS per min recalculated per 1 g of enzyme prep (U/g prep).

2.4. Determination of myrosinase activity in Brassica plants and seeds by pH-stat method

For the standard determination of myrosinase activity in different *Brassica* plants, 0.5 g of lyophilised plant material or grounded seeds was added to 15 mL of 80 mM NaCl and homogenised using Heidolph SilentCrusher M homogeniser (6500 rpm for 5 min with 12FG stainless steel generator) to ensure the complete liberation of the enzyme. During homogenisation, the samples were kept on ice to maintain low temperature. After hydrolysis of endogenous GLS and stabilisation of mixture pH at the level of 6.5, 0.15 mL of exogenous GLS was added (final concentration 2.5 or 5 mM if not stated otherwise).

To determine myrosinase activity in plant fresh matter, frozen cabbage leaves were used as a model sample. Chopped leaves were homogenised with 8 mL of 120 mM NaCl for 15 min (6500 rpm) to obtain uniform suspension. From leaves of the same cabbage, the juice was obtained using juice processor Omega 8004 (Omega Products Inc). In this case, the reaction mixture consisted of 4 mL of cabbage juice and 8 mL of 120 mM NaCl (final NaCl concentration 80 mM). It was not necessary to introduce the homogenisation step for this solution. Then, the measurements were carried out as described for lyophilised samples. The activity of myrosinase was calculated from Eq. (1) and recalculated per 1 g of dw or fw depending on plant material.

2.5. Spectrophotometric method of determination of purified myrosinase activity

The myrosinase activity was determined based on measurements of decomposition of the GLS by following the decrease in absorbance of reaction mixture at 230 nm in a cuvette with 1-cm path length (Brand) using spectrophotometer NanoDrop 2000/2000c (Thermo Scientific). The initial reaction mixture contained 80 mM NaCl (pH 6.5) and 0.05–0.3 mM GLS (SIN or GTL) preheated to 37 °C. After residual spontaneous decomposition of GLS and stabilisation of mixture temperature, 15 μL of dissolved myrosinase prep (0.5–13 mg/mL, final concentration 0.005–0.13 mg/mL) was added. Before measurements, myrosinase solution was kept at 4 °C to avoid enzyme inactivation. The total final volume of the reaction mixture was 1.5 mL. The reaction of GLS hydrolysis was carried out for 30 min at 37 °C.

The myrosinase activity was calculated using the formula:

$$\text{Activity} = \frac{1000V_A}{V_{\text{sample}}} \cdot \frac{2(A_0 - A_t)}{E \cdot t} \quad (2)$$

where V_A denotes the volume of reaction mixture (μL), V_{sample} the volume of investigated sample (μL), A_0 the initial absorbance, A_t the absorbance after time t, t the reaction time corresponding to the initial reaction rate characterised by a linear change in absorption (min), E the molar extinction coefficient, 7500 for SIN, 8870 for GTL (1/mol cm). Final myrosinase activity is given as μmol of hydrolysed GLS per min recalculated per 1 g of enzyme prep (U/g prep).

2.6. Determination of myrosinase activity in Brassica seeds by spectrophotometric method

To prepare water extracts, 50 mg of grounded seeds was mixed with 1 mL of water, homogenised (6500 rpm for 5 min, Heidolph SilentCrusher M) and centrifuged to remove debris (13,000 rpm for 5 min, Eppendorf 5415R). The obtained supernatants were filtered by passing through syringe filters (Chromafil xtra MV-45/25 0.45 μm for aqueous solutions). For the determination of activity of myrosinase, 15 μL of water extract of seeds was added to 1470 μL of 80 mM NaCl, followed by 15 μL of 20 mM SIN or GTL solutions (final GLS concentration 0.2 mM). The activity of myrosinase in plant material was determined as described before for enzyme prep and recalculated per 1 g of fw.

2.7. Statistical analysis

Results of determinations of myrosinase prep enzymatic activity obtained by spectrophotometric or pH-stat assays and the results of myrosinase activity determined by pH-stat assay in different forms of plant samples were examined by a two-tailed Student's *t*-test. All the statistical analyses were performed using the Prism 4.0 software package (GraphPad Software, Inc.). The level of statistical significance was set at $P \leq 0.05$.

3. Results and discussion

3.1. Optimisation of experimental conditions

The conditions of myrosinase activity determination were optimised to improve the sensitivity and reproducibility of present methods, so as to ensure the reliable results even in the case of plant samples containing low levels of this enzyme and to enable interlaboratory comparisons. As shown in Table 1 the published values of myrosinase activity differ vastly even for apparently similar plant samples reflecting the diversification of methodological details of current protocols.

First factor considered was a substrate with more favourable properties than currently used sinigrin (SIN). There are several reasons justifying common application of SIN for myrosinase activity assessment: it is commercially available at a relatively low cost and it occurs in many *Brassica* species, thus should be recognised as a natural substrate, regardless of the source of this enzyme.

As an alternative substrate in this research, glucotropaeolin (GTL) was tested, because of its spectrophotometric properties. Chemical structures and UV-Vis spectra of both GLS are given in Figs. 1 and 2A, respectively. As can be seen in Fig. 2A, GTL exhibits about 20% higher absorbance at 227–230 nm than SIN and consequently greater molar extinction coefficient (7500 M^{-1} cm^{-1} for SIN *vs.* 8870 M^{-1} cm^{-1} for GTL). Moreover, the change in absorbance between starting GLS and ITC formed as a result of degradation is also greater in the case of GTL–BITC pair. Therefore changes in absorbance of the latter during myrosinase catalysed hydrolysis can be measured more precisely. This results in the increase of the sensitivity of the assay; as can be seen in Figs. 4 and 5 the myrosinase activity determined with the use of GTL gives sightly higher values.

The next optimisation step concentrated on the influence of temperature on the kinetics of hydrolysis catalysed by myrosinase. Again, especially in the case of plant samples with low enzymatic activity, the velocity of the reaction can be important for the sensitivity of measurements of substrate consumption. Two temperature values were investigated: ambient temperature (20–25 °C) at which GLS hydrolysis naturally occurs in plants and elevated temperature (37 °C), that should speed up the reaction and

Table 1
Comparison of reported values of myrosinase activity in edible parts of *Brassica* plants used in this study determined with SIN as a substrate.

Method	Myrosinase activity		
	Spectrophotometric assay	Spectrophotometric coupled enzyme assay	pH-stat assay
Broccoli	0.32 U/mg protein (Singh et al., 2007) 3.3 U/g dw (Bellostas et al., 2008a) 9.87 U/mg protein (Travers-Martin et al., 2008)		56–91 U/g fw (Charron et al., 2005)
Brussels Savoy cabbage	0.34 U/mg protein (Singh et al., 2007) 0.04 U/mg protein (Singh et al., 2007) 24.2–33.2 U/g dw (Yen and Wei, 1993) 2.45 U/mg protein (Travers-Martin et al., 2008)	7.6 U/g dw (Shikita et al., 1999) 2.2 U/g dw (Shikita et al., 1999)	67–68 U/g fw (Charron et al., 2005)
White cabbage	0.11 U/mg protein (Singh et al., 2007) 6.08 U/mg protein (Travers-Martin et al., 2008)	5.2 U/g dw (Shikita et al., 1999)	21–50 U/g fw (Charron et al., 2005)
White mustard (seeds)	3.47 U/mg protein (Travers-Martin et al., 2008)	0.14 nmol/min/mg fw (Wilkinson et al., 1984)	69 U/mg protein (Bernardi et al., 2003) 101 U/g (Finiguerra et al., 2001) 4155 U/mg dw (Palmieri et al., 1987)

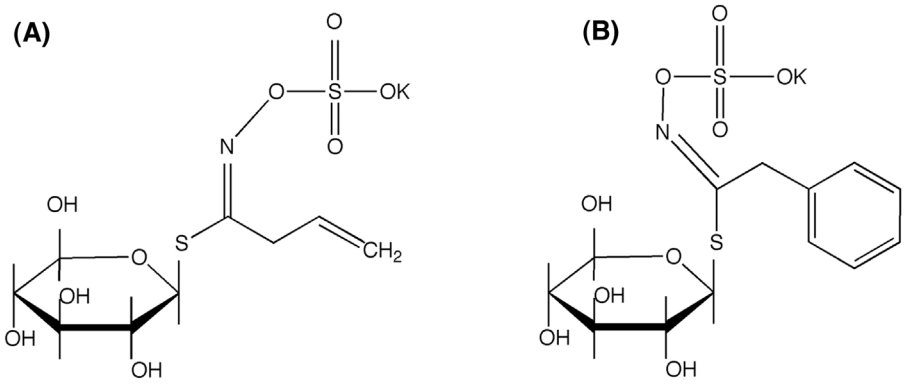

Fig. 1. Structures of sinigrin (A) and glucotropaeolin (B).

is known not to cause any damage to the enzyme (Finiguerra et al., 2001). As can be seen in Fig. 3 the kinetics of hydrolysis reaction was markedly accelerated at the higher temperature tested. The values of enzyme activity calculated based on the measurements at 37 °C were approximately 20–40% higher compared to those obtained for reactions conducted at ambient temperature. In procedures of myrosinase activity determination described in literature, different temperature values can be met: 23–25 °C (Anderton et al., 2003; Bellostas et al., 2008b; Charron et al., 2005; Wilkinson et al., 1984), 30 °C (Palmieri et al., 1987; Shikita et al., 1999; Singh et al., 2007; Travers-Martin et al., 2008) and 37 °C (Bernardi et al., 2003; Finiguerra et al., 2001). Our results suggest that this divergence in assay conditions might contribute to major variations among results reported by different researchers (compare Table 1) On the other hand, pH of the reaction mixture in the range of 6.5–7.5 had no influence on the enzymatic activity (data not shown). However, some indolyl GLS hydrolysis products, especially indole-3-carbinol, become unstable under even slightly acidic conditions and are converted to a variety of secondary products (Björkman and Lönnerdal, 1973). Therefore, pH 7–7.5 of reaction mixture would be preferable during experiments when myrosinase activity is measured along with indole concentration in *Brassica* plants.

During spectrophotometric follow-up of the kinetics of enzymatic GLS hydrolysis, we made another valuable practical observation. Usually absorbance measurements in spectrophotometric assay of myrosinase activity are carried out at 227 nm, the

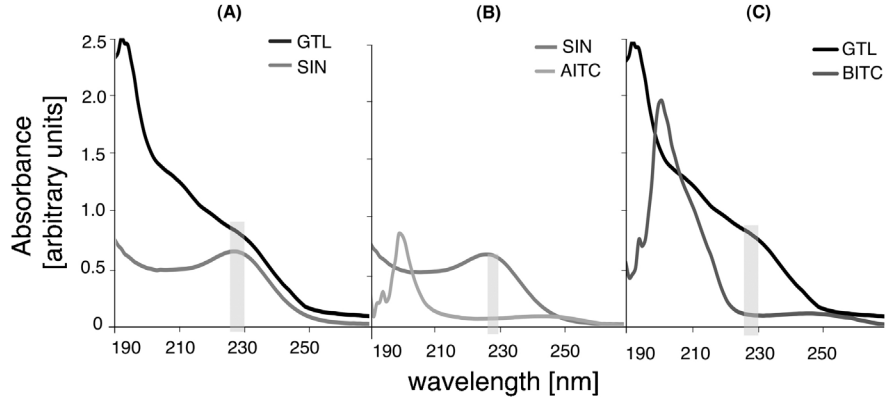

Fig. 2. UV–vis spectra of SIN and GTL (A) and comparison between spectra of GLS substrates and corresponding ITC: SIN and AITC (B) or GTL and BITC (C). The recommended range of wavelength for spectrophotometric method of myrosinase activity determination is marked as grey bar.

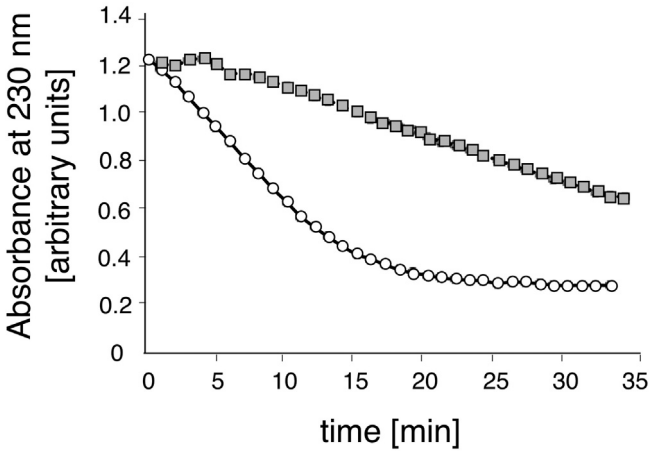

Fig. 3. Spectrophotometric follow-up of the kinetics of hydrolysis of GTL (0.2 mM) by purified myrosinase (0.13 mg/mL). The reaction was carried out at two temperatures: ambient (squares) or 37 °C (circles).

maximum of SIN absorption (Fig. 2). However, this wavelength seems not optimal for a spectrophotometric equipment and the measured values of absorbance tend to fluctuate. We noticed, that at 230 nm, these values were basically identical with those at 227 nm, but less dispersed (data not shown).

Taking all these findings into account, further experiments were carried out for both substrates (SIN and GTL) at 37 °C and pH 6.5. In the case of spectrophotometric assay, the reaction rate was followed at 230 nm. In calculations of myrosinase activity only the initial linear change either in absorbance or in NaOH consumption was considered.

3.2. Optimisation of reaction mixture composition

The optimised assay conditions, that is temperature of myrosinase catalysed reaction 37 °C, pH of reaction mixture 6.5 and wavelength 230 nm in the case of spectrophotometric method, were then used to select the most suitable concentration of SIN and GTL as substrates to determine enzyme activity. The concentration of reactants should be high enough to allow the enzyme to work with maximum velocity over reasonably long time enabling reliable measurements of changes in substrate concentration. In any case, even for samples with very high activity of myrosinase, the concentration of SIN or GTL should not be a factor limiting the reaction rate. However, overdosing may cause the situation in which the change in substrate amount during hydrolysis, reflected by the change in absorbance or NaOH consumption, is not visible enough to follow the kinetics and to precisely determine GLS consumption, especially in the case of samples with low enzymatic activity. To choose optimal amount of the two substrates tested, different ranges of SIN and GTL concentration in reaction mixture were compared using only one concentration of myrosinase prep - 0.065 mg/mL, corresponding to enzyme activity of 6.5 mU/mL of reaction mixture. This choice was based on literature data regarding protein concentration in different *Brassica* vegetables to range from 0.05 to 0.32 mg/mL of plant extract (Anderton et al., 2003). For the spectrophotometric method, additional restricting factors occur. Firstly, the measured values must be below the upper limit of linearity of GLS concentration–absorbance relationship. Secondly, the absorbance of substrate solution should not exceed the reliable operating range of the spectrophotometer. Consequently, the highest final GLS concentration in reaction mixture that could be applied during spectrophotometric measurements was 0.3 mM, which corresponded to absorbance of 1.9 and 1.6 for GTL and SIN, respectively. In pH-stat method, a wider range of GLS concentrations could be investigated (Fig. 4).

As can be seen in Fig. 4 the determined myrosinase activity, expected to be equal to 100 U/g prep according to the declaration of the producer, depends on substrate concentration. At the lowest concentrations of both substrates, apparently the active sites of myrosinase are not completely occupied and the enzymatic activity reaches about 21% and 37% of expected value in the case of pH-stat and spectrophotometric methods, respectively. The rate of reaction increases and the determined myrosinase activity approaches the proper level as substrate concentration increases. In the case of spectrophotometric method, the maximum of enzymatic activity is reached at the 0.2 mM GLS concentration and further increase of concentration of the substrates does not influence the determinations. This suggests that all enzyme molecules are bound with substrate molecules and the reaction proceeds with maximum velocity. Therefore, for subsequent measurements of myrosinase activity with spectrophotometric assay, the concentration of 0.2 mM was chosen.

In the case of pH-stat method, comparable (not different statistically) myrosinase activity was obtained with substrate concentration ranging from 1.0 to 5.0 mM for both substrates, that is for much higher contents than in spectrophotometric assay. However, myrosinase activity seemed to slightly decline when GLS concentrations were increased up to 3.0 or 5.0 mM. This inhibitory effect could be explained by the reaction of isothiocyanates, formed during GLS hydrolysis, with nucleophilic groups such as SH or NH_2 in myrosinase protein structure. Taking all these observations into account, further investigations carried out with the use of pH-stat method were suggested to employ 2.5 mM concentration of both GTL and SIN. This amount of GLS ensured that myrosinase could work with maximum velocity, yet there was no risk of overdosing, which disturbed measurements as could be seen for GLS concentration exceeding 3.0 mM (Fig. 4B).

Irrespective of the method used, higher myrosinase activity was determined with GTL as a substrate. It is a bit surprising, because this compound is not present in *S. alba*, from which the investigated enzyme was purified, thus it is not a natural substrate of this particular myrosinase. However, *S. alba* contains 4-hydroxybenzyl GLS (sinalbin), which is structurally similar to GTL (Bodnaryk, 1991; Kusznierewicz and Iori, 2013).

Next step of this study was to determine the sensitivity of both assays using chosen substrate concentrations (0.2 mM GLS for spectrophotometric and 2.5 mM GLS for pH-stat assay) and a wide range of myrosinase concentrations that can be anticipated in real plant samples (Fig. 5). Results obtained by the spectrophotometric method show that activity of the enzyme does not depend on its amount in the reaction mixture, which is in agreement with basic knowledge about enzymes. Only values calculated for the biggest concentration of myrosinase (0.13 mg/mL) are smaller than those for concentration 0.005–0.065 mg/mL. This may be explained by the limiting amount of substrate for such a high concentration of myrosinase, causing situation in which the enzymatic reaction cannot reach maximum velocity. Another reason of this effect might be too high absorbance of the reaction solutions, becoming on the border of reliable measurement range of the spectrophotometer as the addition of myrosinase also increases the absorbance of reaction mixture at 230 nm. Some authors use cuvettes with 0.5 cm path length while performing spectrophotometric assay, because then the measured absorbance of reaction mixture is lower and consequently the risk of exceeding spectrophotometer operating range is avoided (Anderton et al., 2003; Palmieri et al., 1987).

In contrast, the results from pH-stat method for low myrosinase concentrations (0.005–0.020 mg/mL) showed incorrect relation between the determined activity of the enzyme and its amount added to reaction mixtures. Only in the range of 0.025–0.13 mg/mL,

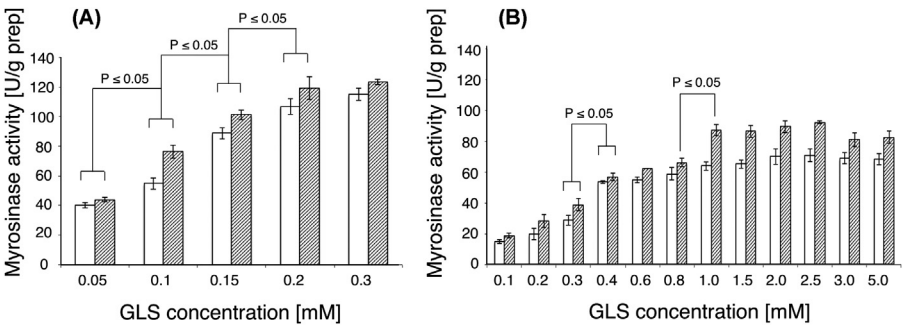

Fig. 4. Myrosinase activity determined by spectrophotometric (A) or pH-stat (B) assay for different ranges of GLS concentration: SIN (white bars) and GTL (dashed bars). The measurements were performed for commercial myrosinase prep (final concentration 0.065 mg/mL). The results are means of two independent determinations ± SD.

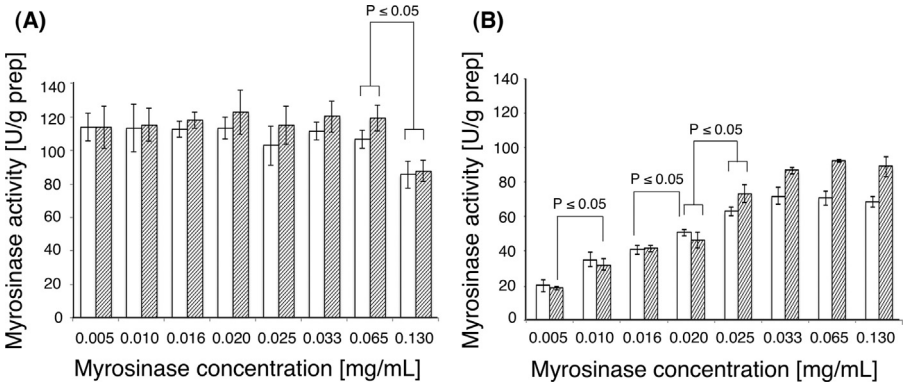

Fig. 5. Myrosinase activity determined by spectrophotometric (A) and pH-stat (B) assays for increasing enzyme prep concentrations and constant concentration of a substrate, respectively 0.2 mM and 2.5 mM (SIN – white bars, GTL – dashed bars). The results are means of two independent determinations ± SD.

the activity values are correct and independent of myrosinase prep concentration. This may suggest that pH-stat method is not sensitive enough and the assay response is not reflecting the true values when the myrosinase activity drops below 2 mU/mL of reaction mixture. However, this problem was solved by introducing appropriate mathematical correction in the form of a function $y = a + b \times e^{-x}$, where x denotes myrosinase activity calculated as U/mL of reaction mixture and y is corrected myrosinase activity expressed as U/g prep. Transforming the above nonlinear equation into a linear model, parameters a and b were calculated by the OLS estimation separately for each substrate based on experimental data presented in Fig. 5. In both cases, the coefficients of determination of linear regression are high, the R^2 value for SIN is 0.93 and for GTL is 0.97. Moreover, the statistical tests confirmed that proposed models are accurate (data not shown). The myrosinase activity values determined by pH-stat assay and corrected using model equations: $y = 72.599 - 56.645 \times e^{-x}$ for SIN and $y = 88.102 - 81.101 \times e^{-x}$ for GTL show proper and sufficiently stable values of activity expressed per g of prep irrespectively of the enzyme concentration in reaction mixture (Fig. 6). Here also the limiting influence of substrate concentration seems slightly visible in the case of the biggest enzyme amount.

Generally, myrosinase activity values determined by the spectrophotometric method are higher than those by the pH-stat assay. This observation refers to both commercial myrosinase prep, as well as real *Brassica* extracts (shown in Table 2). Considering activity values calculated for myrosinase prep determined using 0.2 mM GLS concentration, the activity obtained by spectrophotometric assay is almost 5 times higher than in pH-stat assay. We found only one paper comparing myrosinase activity determined simultaneously by both assays. The activity values described there, calculated for extracts from *Brassica* plants, were similar for both methods, however, in pH-stat assay much higher SIN concentration (5 mM) was used than in spectrophotometric assay (0.5 mM) (Palmieri et al., 1987). In our case, such a difference in substrate concentrations, *i.e.* 0.1 mM in spectrophotometric assay and 1.0 mM in pH-stat assay, also gave comparable values of myrosinase activity. Possible explanation of such a divergence might be different mechanisms underlying each method and disturbances during measurements. The core structure of GLS, *i.e.* C=N conjugated with S, is responsible for absorbance of these compounds at 225–230 nm (Durham and Poulton, 1990). Spectrophotometric method is based on the measurement of decreasing absorbance caused by degradation of this bond during GLS enzymatic hydrolysis. Any other

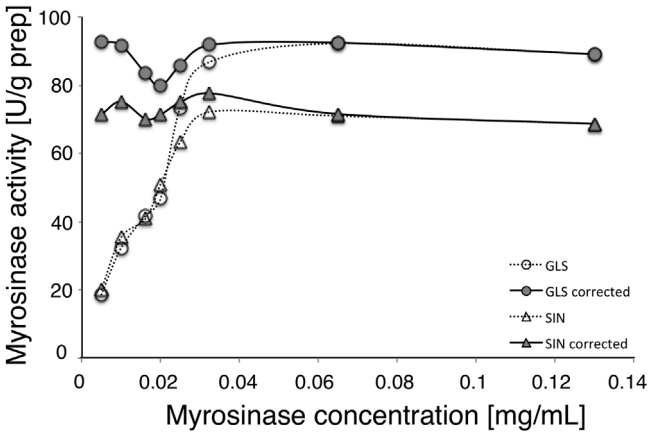

Fig. 6. Myrosinase activity determined by pH-stat assay for different myrosinase prep concentrations and 2.5 mM SIN or GTL as substrates, calculated with and without mathematical correction. The results are means of two independent determinations, SD was in the range of 5%. The used model equations are: $y = 72.599 - 56.645 \times e^{-x}$ for SIN and $y = 88.102 - 81.101 \times e^{-x}$ for GTL.

Table 2
Kinetic parameters of myrosinase determined by spectrophotometric or pH-stat assays.

Parameter	Method used			
	Spectrophotometric assay		pH-stat assay	
	SIN	GTL	SIN	GTL
K_m (mmol/dm^3)	0.229 ± 0.016	0.149 ± 0.023	0.187 ± 0.001	0.261 ± 0.017
V_{max} (μmol/min)	0.052 ± 0.002	0.056 ± 0.004	0.072 ± 0.002	0.093 ± 0.002

The results are means of two independent determinations ± SD.

reaction involving this bond may contribute to lowered absorbance of reaction mixture and be misinterpreted as resulting from myrosinase activity. On the other hand, in pH-stat method, myrosinase activity can be underestimated because of buffering effect of plant proteins other than myrosinase present in the sample. This effect is seen only during measurements of activity in plant samples, naturally containing a lot of additional proteins. This explains why higher concentration of substrate in myrosinase catalysed reaction must be used to overcome the buffering effect in pH-stat method.

The experiments described so far demonstrate that both compared methods of myrosinase activity determination, spectrophotometric and pH-stat assay, are reliable and highly reproducible when optimised reaction and measurement conditions described above are applied. However, to obtain enzymatic activity in both assays, different concentrations of GLS are needed. In addition, in pH-stat method, for low myrosinase concentrations, it is advisable to introduce the mathematical correction in order to obtain appropriate myrosinase activity values. Coefficients of correcting equations calculated in this paper can be applied, however, only if described conditions are used. Any change in reaction or measurement conditions requires recalculation of these coefficients.

3.3. Kinetic parameters of myrosinase

Based on the results presented in Fig. 4, the kinetic parameters of myrosinase prep and both substrates were calculated from Lineweaver–Burk plot (Fig. 7). The K_m and V_{max} values obtained are shown in Table 2.

As can be seen, V_{max} values are comparable in the case of both substrates and do not differ much between the applied methods of myrosinase activity determination. The greater variations, up to almost 2-fold, occur for K_m values which are influenced by both the kind of substrate and assay used. Such inconsistency is also reflected in literature, where reported K_m values for myrosinase from *S. alba* and SIN used as a substrate vary from 0.06 (Li and Kushad, 2005) to 0.2 mM (Bernardi et al., 2003). Our results fall in the range with those published. For enzymes from other *Brassica* plants, published K_m values determined with the same substrate fluctuate between 0.023 and 0.3 mM (Bellostas et al., 2008b; Pocock et al., 1987; Shapiro et al., 2001). From the methodological point of view, neither of two GLS used in this study as a substrate for

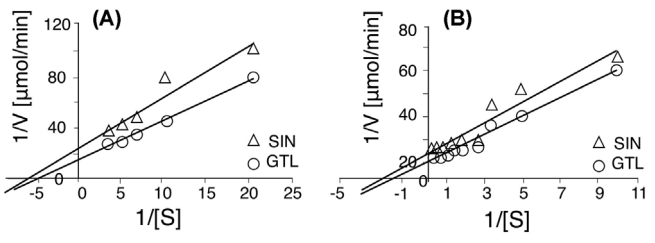

Fig. 7. Lineweaver–Burk plots for SIN or GTL and commercial myrosinase prep based on data derived from spectrophotometric (A) or pH-stat assay (B).

myrosinase activity measurements can be regarded as more recommended because of kinetic properties.

It is generally accepted that substrate concentration for enzymatic determinations should be about 10 times higher than K_m value. In our experiments, in the case of pH-stat method, the GLS concentration of 2.5 mM has been chosen as the most suitable, so it is in agreement with the above recommendations. However, in spectrophotometric assay, it is impossible to use such a high GLS concentration because of absorbance limitations. Therefore, theoretically when the myrosinase activity is high, 0.2 mM substrate concentration may be not saturating for the enzyme. Nevertheless, the calculated activity values seem to be correct, hence it can be presumed that the chosen GLS concentration should be sufficient for samples with low myrosinase activity, as can be expected in plant samples.

3.4. Myrosinase activity in Brassica plants

Using the reaction and measurement conditions described above, the myrosinase activity was determined in different types of samples prepared from *Brassica* plants by both assays with SIN and GTL as substrates. During preparation of these real samples an additional step, *i.e.* homogenisation was introduced. Lyophilised vegetable material or grounded seeds were mixed with 80 mM NaCl and homogenised (6500 rpm for 5 min) in order to efficiently release myrosinase from plant cells and foster its contact with GLS. Indeed, samples processed including homogenisation showed 20–30% higher enzyme activity in pH-stat assay compared to samples prepared without this step (data not shown). This indicates that for proper determination of activity, myrosinase should be thoroughly liberated from plant tissues. This observation points to yet another possible reason for the vast variation among literature values of myrosinase activity. Often in pH-stat method, lyophilised material is just mixed with the reaction solution (Finiguerra et al., 2001). Also in spectrophotometric assay, when water extracts are used (Charron et al., 2005; Travers-Martin et al., 2008; Wilkinson et al., 1984), there is a risk that not all myrosinase present in plant tissue was transferred to water phase. Some authors use crude enzyme preps isolated from *Brassica* plants, however, in this case steps taken during purification may cause losses of enzyme activity (Bernardi et al., 2003; Shikita et al., 1999). The simple homogenisation step proposed by us allow one to avoid these dangers.

Depending on purpose of application, myrosinase activity is determined in different forms of plant material. Therefore, we compared this enzyme activity in feasible preparations obtained from the same vegetable, that is in juice, fresh matter and lyophilised leaves of white cabbage. As mentioned above, the measurements of myrosinase activity in plant samples can be disturbed by the buffering effect of proteins present in plant material. To avoid this effect, we tested also higher concentrations of substrates (5 mM, 7.5 mM) than that chosen based on results obtained for purified myrosinase prep. In the case of all forms of plant samples, there is a significant difference between myrosinase activity calculated for 2.5 mM and 5 mM GLS. However, 5 mM GLS concentration seems to be sufficient to overcome the buffering effect as further increase of

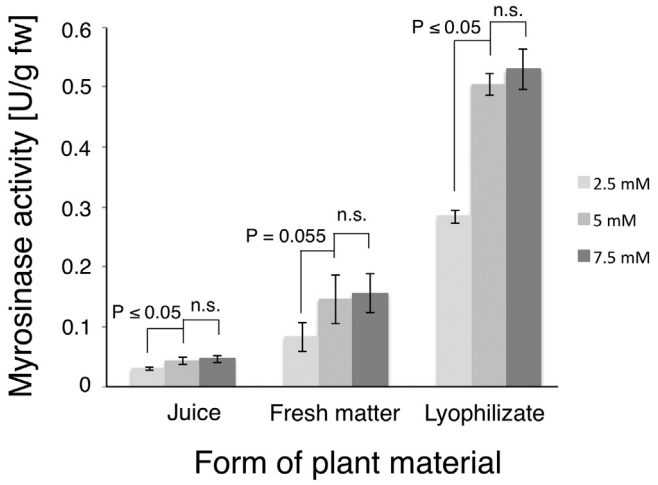

Fig. 8. The myrosinase activity determined by pH-stat assay for different forms of material obtained from white cabbage and using indicated concentrations of GTL as a substrate. The results are means of two independent determinations ± SD. Significantly different values determined by two-tailed Student's *t*-test were marked as $P \leq 0.05$, not significant as n.s.

substrate amount brings no statistically significant change in determined enzyme activity. The results indicate that in freeze-dried material, the myrosinase activity is the highest (Fig. 8). It is not surprising since during lyophilisation, protein structure is maintained and the loss of enzymatic activity is minimal. Moreover, sample of lyophilised leaves seems to be the most representative, as the material is taken from the whole investigated vegetable and mixed while grounding, whereas the myrosinase activity determined in fresh tissue depends on the chosen leaves and even their parts (flesh or veins). In addition, when fresh leaves are investigated, chopping and homogenising may cause some losses in activity due to mechanical and chemical (*e.g.* oxidative) damage of enzyme protein. The same effect occurs during preparation of juice. The lowest enzymatic activity determined in juice may be also explained by the fact that some of enzyme molecules remained in solid residue left after squeezing.

Finally, myrosinase activity was investigated by pH-stat assay in different *Brassica* vegetables using lyophilised plant material and 2.5 mM or 5 mM concentration of SIN or GTL as substrates (Fig. 9). Because of the lack of transparency of water extracts, it was impossible to carry out the corresponding spectrophotometric tests. However, it turned out possible to use seed extracts also in the case of spectrophotometric method. As seeds show greater myrosinase

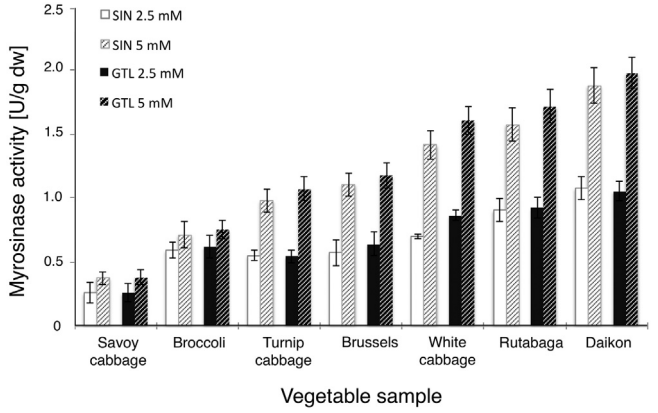

Fig. 9. Myrosinase activity in edible parts of *Brassica* vegetables determined by pH-stat assay with 2.5 or 5 mM SIN (white bars) or GTL (black bars). The results are means of two independent determinations ± SD.

activity, less extract is needed and therefore, the problem of transparency of reaction mixture was sufficiently diminished.

In pH-stat method both substrate concentrations were tested, while for spectrophotometric assay, the formerly chosen GLS concentration (0.2 mM) was applied. According to the results shown in Table 3 spectrophotometric assay gave higher values of myrosinase activity, but comparable to values obtained in pH-stat assay when 5 mM GLS concentration was used. Again in the case of each method, GTL as a substrate leads to determination of greater enzymatic activity. For rape samples, it could be partially explained by the fact that GTL naturally occurs in this plant, so myrosinase found in the samples derived from these species may recognise this substrate with better affinity. However, this compound is not present in the investigated vegetables, where similar correlation was observed. Irrespectively of the method used, the results clearly show that white mustard seeds contain higher activity of myrosinase than other seeds, these differences are especially clear for values of myrosinase activity obtained by spectrophotometric assay.

As mentioned before, the determined values of myrosinase activity in *Brassica* vegetables found in literature show major differences depending on the applied method, sample preparation protocol, substrate used and plant cultivar (Table 1). It is interesting that even among samples of the same vegetable, *e.g.* white cabbage, published activity values determined by two spectrophotometric tests and SIN as substrate range between 0.108 and 6.08 U/mg protein (Singh et al., 2007; Travers-Martin et al., 2008). There were also shown significant variations among plant cultivars: myrosinase activity in a few cultivars of *Crambe abyssinica* ranged between 71 and 102 U/g though measured by the same method and substrate (Finiguerra et al., 2001). The harvesting season influences myrosinase activity as well; activity values range from 21 U/g fw, determined in cabbage harvested in fall, to 50 U/g fw in the crop collected in spring (Larkin and Griffin, 2007). However, SD values in the both studies were as high as 28–29 U/g fw, so the used measurement technique might not be a very reliable one. Nonetheless, there is some pattern in cited data – savoy cabbage displayed the lowest myrosinase activity, cabbage, broccoli and brussels were in the middle and the highest values were obtained for white mustard. Similar relation can be observed in our results (Fig. 9), though mustard, not being a true vegetable, was not taken into consideration. The greatest enzyme activity was determined in daikon, which is said to contain only myrosinase and no additional protein factors affecting GLS hydrolysis. Myrosinase activity in the remaining *Brassica* plants investigated ranged between 0.37 and 1.71 U/g dw, with the lowest value determined in savoy cabbage. Definitely, higher activities were observed in seeds compared to corresponding vegetables.

To determine absolute myrosinase activity in brassicas, 5 mM GLS should be used. As can be seen, a very high GLS concentration is needed to determine reliably myrosinase activity by pH-stat assay, which makes these measurements quite expensive. However, the enzyme activities calculated for 2.5 mM and 5 mM substrate solutions show very good correlation (Fig. 10). Therefore, the former, lower substrate concentration can be used during measurements that are meant to compare the activity between plants or to follow the changes in activity during processing. Such solution is cheaper but still gives reliable results.

It can also be stated that enzyme activity tends to be higher when GTL is used as a substrate. A similar trend was shown by Travers-Martin et al.: the highest enzyme activity in *S. alba* extracts was observed with GTL used to monitor the rate of hydrolysis (0.2 nmol/min/mg fw), lower values were obtained for sinalbin and gluconasturtin (both 0.18 nmol/min/mg fw) and the lowest for SIN (0.14 nmol/min/mg fw) (Travers-Martin et al., 2008). In other studies involving *S. alba*, the application of GTL also resulted in

Table 3
Myrosinase activity in *Brassica* seeds determined by spectrophotometric or pH-stat assays with the use of SIN or GTL as substrates.

Seeds	Myrosinase activity (U/g fw)[a]					
	Spectrophotometric assay		pH-stat assay			
	0.2 mM GLS		2.5 mM GLS		5 mM GLS	
	SIN	GTL	SIN	GTL	SIN	GTL
White mustard	22.93 ± 0.70	25.60 ± 0.70	2.14 ± 0.11	2.98 ± 0.31	3.45 ± 0.37	3.88 ± 0.19
Rape	2.79 ± 0.25	3.52 ± 0.28	1.23 ± 0.34	1.66 ± 0.27	2.74 ± 0.15	2.92 ± 0.20
Broccoli	3.78 ± 0.21	4.21 ± 0.11	1.22 ± 0.14	1.47 ± 0.40	2.02 ± 0.09	2.22 ± 0.15
Savoy cabbage	2.43 ± 0.25	2.97 ± 0.11	0.96 ± 0.17	1.03 ± 0.08	2.00 ± 0.11	2.11 ± 0.41
Turnip cabbage	1.61 ± 0.15	2.26 ± 0.26	1.08 ± 0.12	1.29 ± 0.36	2.11 ± 0.27	2.25 ± 0.13
White cabbage	2.71 ± 0.58	3.16 ± 0.18	1.21 ± 0.07	1.38 ± 0.06	2.33 ± 0.35	2.74 ± 0.27

[a] The results are means of two independent determinations ± SD.

Table 4
Recommended parameters to be used for myrosinase activity determination in plant samples by spectrophotometric and pH-stat assays.

Parameter	Method of myrosinase activity determination	
	Spectrophotometric assay	pH-stat assay
Sample preparation	Homogenisation of plant material in reaction buffer, centrifugation, filtration of supernatant	Homogenisation of lyophilised plant material in reaction buffer
Reaction solution	80 mM NaCl, pH 6.5	80 mM NaCl, pH 6.5
Reaction temperature	37 °C	37 °C
Substrate concentration	0.2 mM	5 mM
Detection wavelenght	227–230 nm	Not applicable
Calculation of myrosinase activity	Based on the initial linear change of absorption at 230 nm	Based on the initial linear change of 1 mM NaOH consumption

higher myrosinase activity determinations (77 U/mg protein) in comparison to SIN (69 U/mg protein). However, myrosinase from *C. abyssinica* showed reversed relation, its activity measured with SIN reached the level of 7 U/mg protein, while with GTL 4 U/mg protein. The best substrate for *C. abyssinica* enzyme turned out to be epi-progoitrin (59 U/mg protein), because this GLS naturally occurs in this plant and might be additionally stabilised in the enzyme active site (Bernardi et al., 2003). These results are in agreement with other determined values, where epi-progoitrin also was demonstrated to be the best substrate for *C. abyssinica* myrosinase (600 U/g), much lower activity values were obtained for SIN (92 U/g) and GTL (47 U/g) as substrates (Finiguerra et al., 2001).

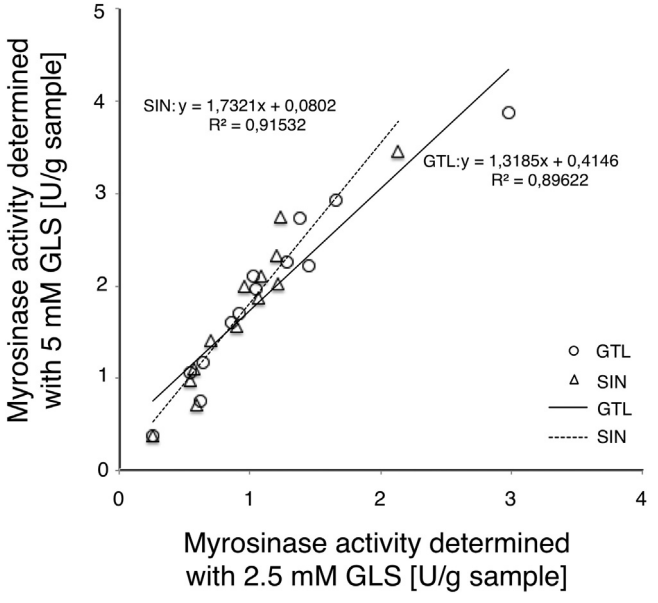

Fig. 10. Correlation between myrosinase activity determined in *Brassica* plants by pH-stat assay using 2.5 mM and 5 mM SIN or GTL. The phrase "U/g sample" means enzyme units per either gram of fresh weight in the case of seeds or g of dry weight in the case of edible parts of plants.

4. Conclusion

In summary, our research provides information concerning methodological details of the two most common methods of determination of myrosinase activity. As demonstrated by our results, to obtain correct activity values, it is important to ensure full liberation of the enzyme from plant tissue and to apply the appropriate concentration of a substrate and reaction temperature. It is also recommended not to be tempted to use random two time points, but to calculate the myrosinase activity based on the initial changes of either absorption at 230 nm or NaOH consumption corresponding to the linear part of reaction kinetics. The optimised reaction and measurement conditions, as well as sample preparation procedure are assembled in Table 4.

Although spectrophotometric assay seems to be more sensitive and consequently gives higher values of enzymatic activity, its main limiting factor is frequently insufficient transparency of investigated solutions. Thus, the preparation of plant samples require sadditional steps like centrifugation and filtering. Such a problem does not appear in pH-stat method, where samples do not have to be specifically processed before measurements to achieve appropriate clarity, thus this assay is more suitable for determination of myrosinase activity in real plant samples.

Considering literature data, there is no clear evidence which GLS can be regarded as the most universal substrate for monitoring the hydrolysis catalysed by myrosinases derived from different sources. Based on our results, it can be argued that with GTL slightly higher activity values, hence sensitivity, are observed for all investigated *Brassica* plants and seeds, using both assays. However, K_m or V_{max} values calculated for myrosinase prep point to SIN and GTL as comparably adequate for this enzymatic reaction.

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