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

Anna Piekarska[a,*], Barbara Kusznierewicz[a], Magdalena Meller[b], Karol Dziedziul[b], Jacek Namieśnik[a], Agnieszka Bartoszek[a]

[a] Chemical Faculty, Gdansk University of Technology, ul. Narutowicza 11/12, Gdansk, Poland
[b] Faculty of Applied Physics and Mathematics, Gdansk University of Technology, ul. Narutowicza 11/12, Gdansk, Poland

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ABSTRACT

Myrosinase (EC 3.2.3.1) found in *Brassicaceae* plants, is the enzyme responsible for hydrolysis of glucosinolates. 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 methods 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 concentration 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.

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.

* Corresponding author. Tel.: +48 58 347 14 23; fax: +48 58 347 26 94.
E-mail addresses: anna.m.piekarska@o2.pl, p-anna1@wp.pl (A. Piekarska).

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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).