Method for Myrosinase Activity Assessment in *Brassicaceae* Products

Alain Quinsac[1], Joëlle Garrioux[2], Morgane Citeau[3], Khawla Zayani[3], Patrick Carré[3]

1 TERRES INOVIA - 11 rue Monge - 33600 PESSAC
2 TERRES INOVIA - 270 avenue de la Pomme de pin - 45166 ARDON
3 OLEAD - 11 rue Monge - 33600 PESSAC
a.quinsac@terresinovia.fr

Objectives

Determination of the myrosinase activity in products from rapeseed and other Brassicaceae, for a better prediction of the glucosinolates (GSL) breakdown routes. The method shall be robust and applicable in a large range of enzyme activities to be useful for the process monitoring and the feed evaluation.

Principle :

The proposed method uses ion pairing HPLC (Helboe *et al.*, 1980) to directly measure sinigrin (substrate of myrosinase) during hydrolysis. Several determinations of the remaining amount of sinigrin are carried out on the same chromatogram with multiple injections of the enzyme-substrat mixture (Bellostas *et al.*, 2006) and give the kinetics of the reaction (V_{max}) and the activity level of the enzyme

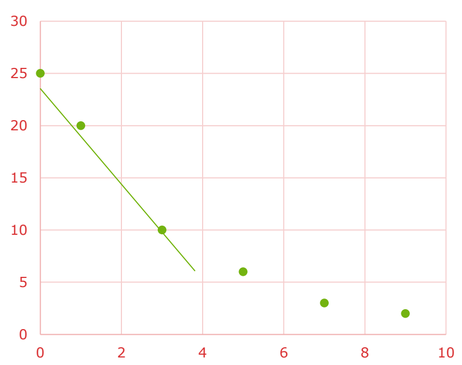

Fig. 1 : Kinetics of sinigrin hydrolysis

Kinetics of sinigrin hydrolysis (Fig. 1) is given by 3 measurements (initial value + 2 analyses) of the sinigrin amount during the reaction.
The measurement of the V_{max} of the hydrolysis at 25°C (slope of the curve when the substrate is still in excess) allows to calculate the activity according to the definition:

1 activity unit hydrolyses 1 µmol sinigrin per min at 25 °C, pH 7.0

Background

The degradation rate of GSL in the seeds of mustard or rape during the crushing process may be very different according to the initial activity of the myrosinase and the applied conditions of temperature, moisture, etc. (Fig. 2).

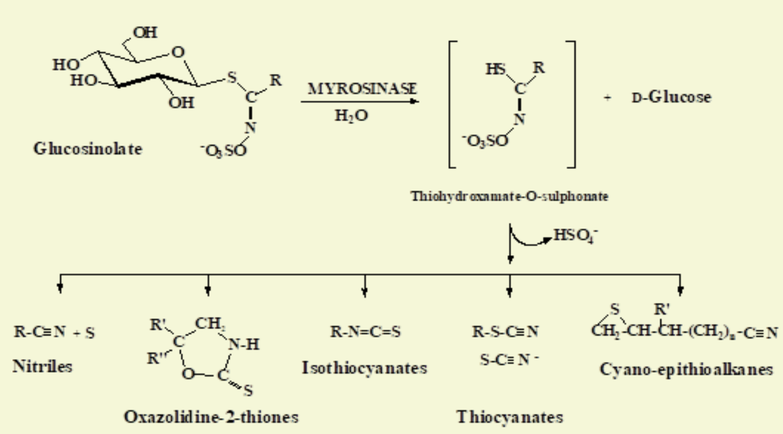

Fig. 2 : General scheme of Glucosinolates hydrolysis by myrosinase

Monitoring the activity of myrosinase is then necessary to predict the behaviour of glucosinolates and the release of degradation compounds in cakes or meals during the process and finally, the quality of these products.

Usual methods are based on the determination of the glucose released by the hydrolysis of myrosinase. Application to crude extracts remains difficult because the enzymatic reactive systems and spectrophotometry used can often be disrupted by the matrix, resulting in low sensitivity and specificity.

Materiel & Methods

HPLC equipment with isocratic elution and UV detection at 235 and 330 nm. Column : Nucleosil C18, 5 µm, 250 mm length, 4 mm ID. Eluent : Acetonitrile 45%, tetraheptylammonium bromide 5mM in phosphate buffer, 10 mM, pH 7,0, flowrate 1 ml/min. Injection (20 µL loop). Data acquisition system allowing several injections on the same chromatogram.

Substrate : Sinigrin solution at 0.3 mM in water.

Myrosinase extraction : 1,0 g of the finely ground solid sample was extracted by gentle agitation with 20 ml of phosphate buffer (10 mM, pH 7,0) during 30 min at 4°C. The extract was then centrifuged or filtered to discard the solid phase and the volume was adjusted to 20 mL with buffer pH 7,0.

HPLC separation of GSL and other compounds : An aliquot (20 µL) of the Myrosinase extract was injected in HPLC after filtration at 0,45 µm. As the sample was not purified, peaks from not yet degraded GSL and other compounds (flavonoids) could be observed. Identification of these compounds was carried out by UV detection at 235 nm and 330 nm (absorption at 235 nm for GSL ; at 235 and 330 nm for flavonoids). Injection of a solution of pure sinigrin allowed to check that this compound (used as substrate of myrosinase) was well separated and quantified during the measurement of the activity.

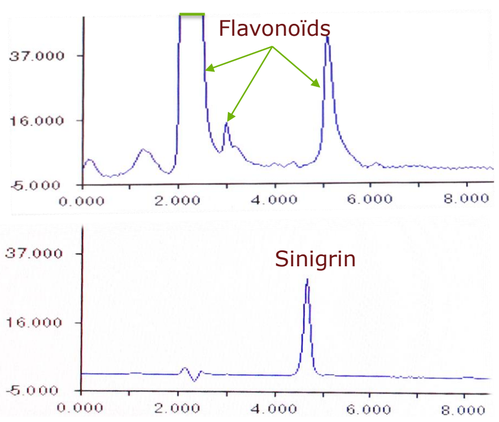

Fig. 3 ***Chromatogram (UV det. 235 nM) of extract of rapeseed cake without intact GSL (top) and sinigrin solution (down)***

HPLC analysis of myrosinase extract (Fig.3 top) only showed peaks from flavonoïdes GSL were degraded during the extraction in cold conditions.

HPLC analysis of the substrate (Sinigrin) (Fig.3 down) showed that its separation from the flavonoïds could be achieved if it was mixed with the myrosinase extract.

As the chromatographic system is able to well separate the peaks of Sinigrin and flovonoids, **several injections (every 5 min for instance) of the same solution allow to monitor on the same chromatogram, the content of the substrate during its degradation** (Fig.4)

Sinigrin hydrolysis : Basic operating mode consisted in mixing 1 mL of the sinigrin solution (0.3 mM) with 1 mL of the myrosinase extract. Dilution of the myrosinase extract or extended time of hydrolysis were applied to measure very high or low myrosinase activities, respectively.

As the reaction conditions were set to be in substrate excess (Fig; 1), only few injections (3 at least) are needed to determine Vmax with the kinetics curve.

Application

Activity measurement

In this example, activity was measured on a rapeseed cake. A preliminary test was necessary to check the presence of endogenous sinigrin in the sample, and the approximate level of the myrosinase activity. No sinigrin was found and because added sinigrin was very rapidly degraded, the myrosinase extract was then 10 fold diluted. The time interval between injections (5 min) was chosen to avoid overlapping of peaks from successive injections.

The hydrolysis was initiated by mixing 1 mL sinigrin at 0.3 mM and 1 mL of the diluted myrosinase extract. Immediately, 6 injections (20 µL each) of the mixture were carried out in HPLC every 5 min.

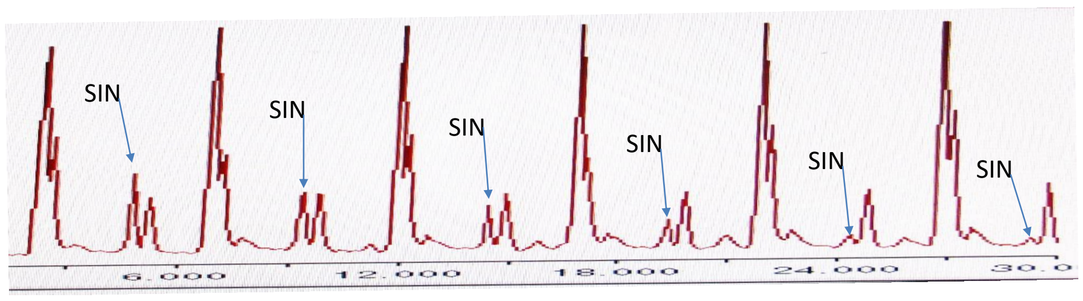

Fig. 4 ***Chromatogram with 6 successive injections of the same solution at 5 min interval***

The chromatogram (Fig. 4) directly showed the decrease of the amount of sinigrin and the quantification was made on the basis of the peak heights. The retention times of sinigrin peaks were used to calculate the reaction time and a correction was achieved to take in account that when the myrosinase-sinigrin solution was injected, the substrate was very quickly separated from the enzyme. In this example, the reaction time for each injection was reduced by the value of the retention time of sinigrin for the 1st injection (4.64 min).

The slope of the decreasing of the sinigrin concentration in the reacting volume (2mL) was -0.0051 mM/min. The activity of myrosinase was therefore 0.0102 u.a. (0.0102 µmol/min). As the enzyme solution used was extracted from 0.005 g of cake (1 g in 20 mL, with 10 fold dilution), the specific activity of the myrosinase in the cake was 2.04 u.a/g.

Conclusion

This method based on the chromatographic separation of the substrate (sinigrin) before its determination allows a very specific monitoring of the reaction kinetics. Determination of very low or high level of Myrosinase activity is possible by adapting the conditions. The principle of the method is in favour of applications on various crude extracts and the use of other glucosinolates as substrates.

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Helboe P., Olsen, O. and Sørensen, H., 1980. Separation of glucosinolates by high-performance liquid chromatography. *J. Chromatogr.*, 197, 199-205.
Bellostas N., Sørensen J.-C., Sørensen H., 2006, Micellar electrokinetic capillary chromatography—Synchronous monitoring of substrate and products in the myrosinase catalysed hydrolysis of glucosinolates, *J. Chromatogr.*, 1130, 246-252.