# Immobilization of Myrosinase on Membrane for Determining the Glucosinolate Content of Cruciferous Material†

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A novel detection system for the direct determination of the glucosinolate content in crude aqueous extracts from cruciferous material is described. In the proposed system the myrosinase from *Sinapis alba* (thioglucoside glucohydrolase, EC 3.2.3.1) is immobilized with high yield on a nylon 6.6 membrane. The optimized immobilization and experimental parameters are also described. The enzymatic assay, based on the initial-rate method, uses the pH-stat technique. The biosensor was evaluated for linearity using sinigrin, progoitrin, and increasing amounts of crude extracts and showed a linear correlation (near 1) up to 3.5 μmol of glucosinolate. The sinigrin recovery from a crude rapeseed extract ranged between 99 and 103%. Although the reproducibility appeared to be comparable to that of other time-consuming and more expensive techniques, it was affected by a certain difficulty of restoring the initial condition of the system after each measurement. A comparison of the data obtained by the biosensor with those of HPLC shows a good correlation ($y = -0.2158 + 0.9989x$, $r = 0.999$, $n = 12$). In addition, immobilized myrosinase, if stored wet at 4 °C, appears to be very stable, retaining nearly all of its activity for more than 15 months and after about 1000 assays.

## INTRODUCTION

Plant myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) is widespread in seeds and tissues of the family Cruciferae and catalyzes the hydrolysis of glucosinolates (GLs) which are also contained in cruciferous plants. This reaction produces goitrogenic and potentially hepatoxic compounds, e.g., isothiocyanates, thiocyanates, nitriles, and thiones. For this reason the European Community recommends the cultivation of double-zero rapeseed with a GL content below 20 μmol/g seed, instead of the older varieties containing high amounts of GLs, often more than 100 μmol/g (Wathelet and Wagstaffe, 1989).

To further the production and utilization of oilseed rape and its food and feed derivatives and to differentiate "low" from "double-low" rapeseeds varieties, the availability of a fast, simple, and cheap analytical method for total GL determination appears to be a very important goal. Current methods, which include temperature-programmed gas–liquid chromatography and HPLC of intact or desulfo GLs, are in general satisfactory with regard to precision and accuracy but not simplicity and cost. The literature is rather rich on the chemistry of the myrosinase–GL system, which is employed in the determination of GLs in natural products (Ettlinger and Lundeen, 1957; Kjaer, 1961; Björkman, 1976; McGregor et al., 1983). The most common rapid methods investigated to date for this purpose are (i) photometric or polarographic determination of released glucose after myrosinase-catalyzed hydrolysis (Heaney and Fenwick, 1981; Iori et al., 1983); (ii) spectrophotometric determination of released glucose by thymol after acidic hydrolysis (Brzezinski and Mendelewski, 1984); and (iii) photometric determination of the colored complexes formed between tetrachloropalladate(II) and GLs (Thies, 1982).

On the basis of the experience of many laboratories in the first half of the 1980s, the palladium test emerged as the most promising and suitable for determining the total GL content, especially in double-low rapeseed varieties and related products. However, Röbbelen (1987) reported that even the palladium method is not well suited for the quick, inexpensive determination of total GL content in large seed lots, i.e., for an initial discrimination between traditional "0" and "00" rapeseed varieties.

Following these indications, we studied the possibility of immobilizing myrosinase on a suitable membrane to create a biosensor able to measure the GL content of a crude aqueous rapeseed extract to provide a satisfactory alternative to traditional methods suitable for breeding programs, feed manufacture, and the storage and processing of plant material.

To the best of our knowledge, only one enzyme biosensor for determining GLs has been performed recently and described in the literature (Koshy et al., 1988). This electrochemical method is based on the determination of released glucose by the hydrolysis of GLs using free or immobilized myrosinase as biocatalyst and determining glucose amperometrically by immobilized glucose oxidase. In this paper we present a novel assay method for determining GLs in crude aqueous rapeseed and vegetable extracts, using a simple pH electrode coupled with a membrane supporting immobilized myrosinase. The enzymatic hydrolysis reaction produces one hydrogen ion for each GL molecule:

$$\text{glucosinolate} + H_2O + \text{Myr} \rightarrow \text{isothiocyanate} + \text{glucose} + \text{sulfate} + H^+$$

If the above is a pseudo-first-order reaction, the hydrolysis rate essentially depends on the GL concentration.

The aim of this study was to evaluate the characteristics of this assay compared to those of reference methods of analysis, such as HPLC of desulfo GLs.

## EXPERIMENTAL PROCEDURES

**Materials.** Sinigrin was obtained from Sigma Chemical Co. (St. Louis, MO). Progoitrin mixed with other minor rapeseed GLs was isolated according to the procedure reported by Bjerg and Sörensen (1987). Myrosinase, homogeneous in SDS–PAGE,

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was purified from white mustard seeds (*Sinapis alba*) according to the procedure reported by Palmieri et al. (1986). The myrosinase used for the immobilization trials had a specific activity of 22.4 units/mg. One myrosinase unit corresponds to the amount of enzyme that catalyzes the hydrolysis of 1 μmol of substrate (sinigrin) in the assay conditions reported in a previous paper (Palmieri et al., 1982). Three types of membrane were used for the immobilization trials: (i) a natural membrane of pig intestine, which was purchased from the local market, cleaned, and mechanically defatted; (ii) a FHUP Teflon filtering membrane obtained from Millipore (Bedford, MA); and (iii) a nylon 6.6 net (Eterlon) supplied by Gaudenzi (Padova, Italy). The other chemicals were of analytical grade and used without further purification. Rapeseed samples came from agronomic trials carried out in different Italian locations from 1987 to 1989. A sample of CRM190 was obtained from the Community Bureau of Reference (BCR) of the Commission of the European Community. Vegetables were purchased from the local market.

**Apparatus.** The apparatus consists of a Model pH85 pH meter connected to a Model GK2401C glass electrode (both from Radiometer, Copenhagen) plugged in a perforated plastic cylinder partially covered by the membrane containing the immobilized enzyme.

**Enzyme Immobilization.** The enzyme immobilization procedures for the three membranes were those reported by Nabi Rahni et al. (1987), Mascini and Botrè (1979), and Mascini et al. (1983). After the immobilization, the membranes were rinsed several times with 0.2 M phosphate buffer (pH 6.5) to remove free, unbound myrosinase. The membranes were stored at 4 °C in the same buffer.

**Determination of Immobilized Activity.** The amount of active enzyme on the membrane surface was determined spectrophotometrically with a discontinuous method using sinigrin as substrate (Iori et al., 1988). The assay was performed in a cell thermostated at 37 °C; the reaction mixture was stirred constantly and continuously. The membrane was immersed in 6 mL of sinigrin solution (0.2 mg/mL in 0.2 M phosphate buffer pH 6.5) for 10 min.

The absorbance values were determined with a Cary Model 219 recording spectrophotometer at 227 nm against phosphate buffer as reference in a 0.5-cm cell. The same procedure was followed to determine the absorbance of the unhydrolyzed substrate. The difference between these two values was proportional to the amount of active myrosinase bound to the membrane. The three different active membranes were compared per unit of surface according to this procedure.

**Electrode Measurements.** The analytical measurements were carried out in a cell thermostated at 37 °C (Thermostat Lauda Model K2), and the reaction mixture was stirred at a constant rate. The reaction mixture was 0.5% NaCl in boiled distilled water with 10–50 μL of sample GL solution or 100–500 μL of sample crude extract in a total volume of 6 mL. The reaction velocity was determined according to the pH-stat method (Palmieri et al., 1987) by measuring the volume of 0.001 N NaOH added per unit time to maintain a constant pH at the initial value of 6.5. The NaOH was added with a Model E457 Methrom Eraus microdispenser and the time measured with a stopwatch. The NaOH volume was recorded every 20 s, and the reaction velocity was calculated with an Olivetti M24 PC. The conversion of reaction velocity to GL content was done on the basis of a calibration curve determined using a suitable standard solution of a mixture of progoitrin and other minor GLs isolated from defatted rapeseed meal. This solution, titered by a polarographic method (Iori et al., 1983) and HPLC (Büchner, 1987), had a GL concentration of 17 μmol/mL.

**Electrode Reconditioning.** After each measurement, the electrode was thoroughly washed with distilled water, immersed for 10–20 s in 0.1 M phosphate buffer, pH 6.5, and washed again with a 0.5% NaCl solution.

**Sample Preparation.** One gram of rapeseed or 0.5 g of defatted rapeseed meal was added to 30 mL of boiling distilled water and maintained in a boiling water bath for 10 min to deactivate endogenous myrosinase. The sample was homogenized for ca. 2 min (Ultraturrax, Ika-Werk Model TP 18-10N) and then centrifuged at 38000*g* (Beckman Model J-21 centrifuge). A similar procedure was used for the crude extracts of fresh vegetables, in which case the ratio of GL extraction was 1:5 (w/v).

**Table I. Activity of Immobilized Myrosinase on Different Membranes**

| membrane | ΔABS, cm² | rel activity, % |
|---|---|---|
| pig intestine | 0.16 | 46 |
| Teflon | 0.23 | 66 |
| nylon 6.6 | 0.35 | 100 |

**Table II. pH Influence on Nylon Membrane Immobilization of Myrosinase**

| pH | ΔABS | rel activity, % |
|---|---|---|
| 5.5 | 0.54 | 80 |
| 6.5 | 0.67 | 100 |
| 7.5 | 0.49 | 73 |

## RESULTS AND DISCUSSION

The use of an enzyme electrode for determining the total GL content in crude extracts of cruciferous material without special and expensive instrumentation appealed to us as a method of analysis (Iori et al., 1988). The reaction scheme reported above shows that there are three ways to monitor this reaction enzymatically: (i) following the decreasing GL concentration by monitoring the absorbance at 227 nm; (ii) measuring the released glucose photometrically or polarographically; and, finally, (iii) titrating the released sulfate or hydrogen ions. If one uses an enzyme electrode, system iii appears to be simpler and less expensive than the others. In fact, it requires a membrane with only one immobilized enzyme (myrosinase), a common pH meter, a microdispenser, and a stopwatch.

**Optimization of the System.** The initial experiments were aimed at optimizing the myrosinase immobilization procedure, finding a suitable membrane for the electrode preparation, and determining the reaction parameters, viz. pH, ionic strength, and temperature. Table I shows the immobilized activity of the three chosen membranes. All are able to bind myrosinase, although the nylon membrane appears to be most active, presumably because a greater amount of enzyme can be bound. In addition, this membrane shows a better manageability and time stability, particularly compared to the Teflon membrane. To determine the optimal pH for immobilizing the maximum amount of enzyme, we used buffered solutions at different pHs with the same myrosinase concentration. The nylon membranes had exactly the same surface area. These trials were of great importance to maximize the number of immobilized units of myrosinase per surface unit. In fact, a greater amount of immobilized enzyme is necessary to determine the GL content using a kinetic method based on the determination of the velocity of a pseudo-first-order reaction. As one can easily observe in Table II, pH 6.5 appears to be optimum for immobilization. This pH also appears to be optimum for GL hydrolysis when myrosinase is used in immobilized form (Figure 1).

**Reaction Trend, Calibration Curves, and Linearity of the Time Response.** Figure 2 shows the reaction trend of GL hydrolysis with immobilized myrosinase, which produces one hydrogen ion per mole of substrate. Great pH variation was observed, especially during the first 5–6 min of the reaction. The reaction rate clearly decreases because during the reaction the pH shifts away from the optimum pH of the enzyme. Nevertheless, the reaction rate seems to be biphasic with a change of rate around 7–8 min, corresponding to ca. pH 5.0, which is the isoelectric point of the enzyme (Pessina et al., 1990). Using increasing

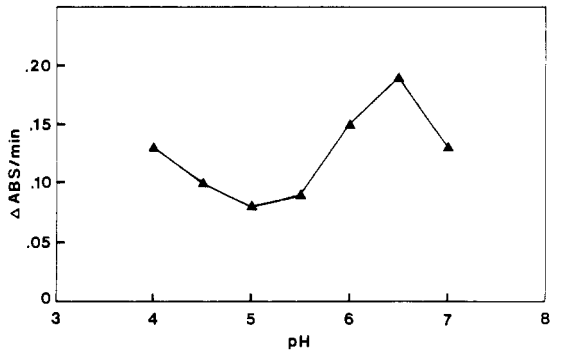


**Figure 1.** Effect of pH on myrosinase activity immobilized on nylon membrane.

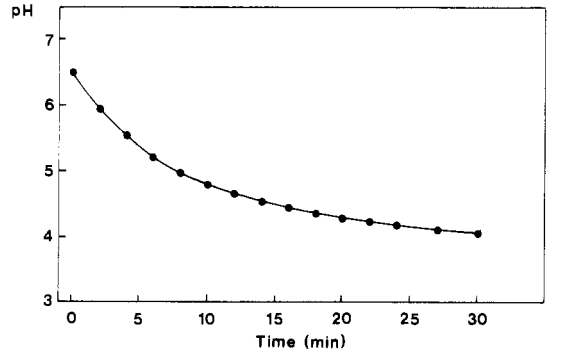


**Figure 2.** Reaction trend of sinigrin hydrolysis catalyzed by immobilized myrosinase.

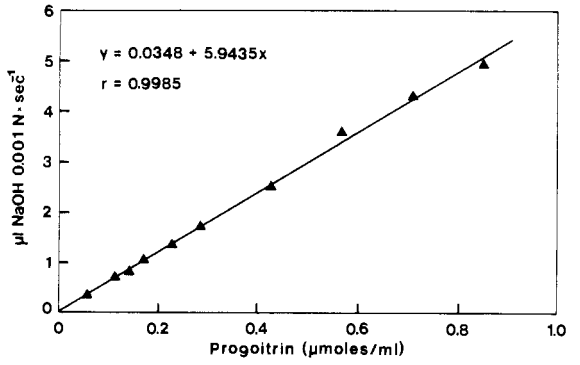


**Figure 3.** Calibration curve with progoitrin.

amounts of sinigrin as substrate, we obtained a calibration curve with a correlation coefficient of 0.9989, linear up to 3.5 μmol of sinigrin, which corresponds to 0.58 mM. This finding appears to be in disagreement with the properties of kinetic enzymatic methods of analysis, in which the concentration of the analyzed substrate should be a fraction of, or at most the same as, the enzyme $K_m$. The $K_m$ of free myrosinase is 0.16 mM (Palmieri et al., 1982). Since this value is lower than the limit value found for the linearity of our assay, it is reasonable to presume an increase of the Michaelis constant due to the immobilization process. In fact, as has been observed for many other enzymes (Guilbault, 1984), the apparent $K_m$ for myrosinase immobilized on the nylon membrane, determined using 12 different concentrations of sinigrin, notably increased at 5.54 ± 1.01 mM. On the basis of these considerations, a value of 0.5 mM as a limit of substrate concentration for GL analyses by the proposed system appears to be reasonable. Given the well-known fact that the GL of rapeseed is essentially progoitrin, we were also interested in verifying the linearity of the assay to create a calibration curve using progoitrin isolated from several rapeseed varieties (Figure 3). In this case, too, linearity was maintained in a wide range of GL

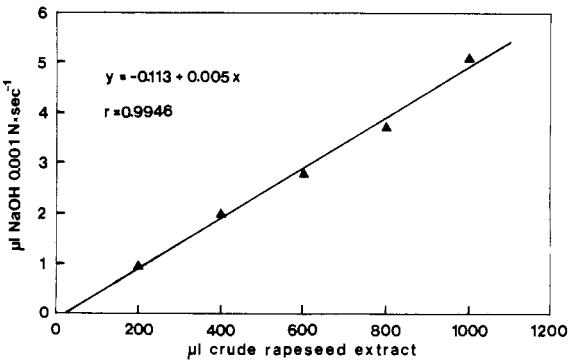


**Figure 4.** Linearity of the biosensor signal with crude rapeseed extract (cv. Jet Neuf).

**Table III. Recovery of Sinigrin Added to a Crude Extract of Rapeseed Meal (cv. Mikado)**

| sinigrin, μmol | | |
|---|---|---|
| added | recovered | recovery, % |
| 0.53 | 0.52 | 98 |
| 1.06 | 1.05 | 99 |
| 1.59 | 1.63 | 103 |
| 2.11 | 1.78 | 84 |

**Table IV. Reproducibility of Analytical Results Obtained with Immobilized Myrosinase[a]**

| sample | μmol/g | SD |
|---|---|---|
| rapeseed (CRM190) | 23 | 1.0 |
| rapeseed (cv. Mikado) | 51 | 1.5 |
| rapeseed (cv. Jet Neuf) | 89 | 3.6 |
| cabbage | 3.16 | 0.17 |
| progoitrin (50 μL, 17 μmol/mL) | 0.87 | 0.06 |

[a] Data are average of five determinations.

concentrations and the correlation coefficient was 0.9985. To determine if a matrix effect exists due to the presence of other compounds such as proteins, pigments, and phenolics, the linearity was also tested using increasing amounts of crude rapeseed extract (cv. Jet Neuf) (Figure 4). These three experiments should be sufficient to confirm the applicability of the system for GL analysis. One of the advantages of the proposed method is its high sensitivity, which allows the use of low substrate concentrations or small volumes of crude extract. This feature of the method avoids or reduces matrix effects when used for determining GLs in crude extracts as well as inhibition phenomena when high concentrations of reaction products are present in the reaction solution (Palmieri, 1988, unpublished results).

**Recovery and Reproducibility Studies.** Good linearity was also obtained when sinigrin was added to 1 mL of crude rapeseed meal extract (cv. Mikado, which contributed 1.9 μmol to the GL concentration in the assay mixture). Table III shows the recovery data obtained in this experiment. These values show a good recovery of sinigrin, especially for the first three data. The last result appears to be slightly but significantly lower than the previous ones, indicating that in this experiment the total GL amount (ca. 4 μmol) exceeds the maximum limit of the assay linearity (3.5 μmol). A study of reproducibility was performed using three rapeseed varieties with high, medium, and low GL contents, a sample of cabbage leaves, and 50 μL of a 17 μmol/mL progoitrin solution. The data presented in Table IV are the average of five determinations at different times. The standard deviation observed indicates a good reproducibility of the analytical data, which appear comparable to those of other time-consuming and more expensive techniques. Nevertheless,

**Table V. Comparison of Biosensor Analytical Results vs HPLC[a]**

| sample | glucosinolates, μmol/g | |
|---|---|---|
| | HPLC | biosensor |
| rapeseed (defatted meal) | | |
| cv. Santana | 7.5 | 4.5 |
| cv. Doral | 123.5 | 125.1 |
| cv. Tamara | 145.1 | 147.3 |
| rapeseed | | |
| cv. Sisforaggera | 19.1 | 20.7 |
| cv. Lingot | 46.6 | 48.5 |
| cv. Jet Neuf | 99.2 | 92.2 |
| vegetables | | |
| turnip | 2.28 | 2.45 |
| watercress | 2.25 | 2.25 |
| garden rocket | 3.08 | 2.70 |
| cabbage | 2.68 | 3.16 |
| cauliflower | 0.67 | 0.80 |
| radish | 0.88 | 0.75 |

[a] Data are average of three determinations.

there is a slight drawback due to the difficulty of consistently reproducing the same initial electrode conditions before the analyses, especially when crude rapeseed extracts are analyzed. It is not clear if this phenomenon is due to an uncontrolled interaction between particular compounds in the extract and the membrane, the glass electrode, or both. Studies in this direction are in progress in our laboratory.

**Stability Studies.** Calibration controls were carried out before every set of analyses with the same myrosinase electrode. After 15 months and about 1000 assays, we observed no loss of activity. We consider this observation an important aspect in the practical applicability of this system for routine GL determination.

**Comparison Studies.** To determine the reliability of this system for routine GL analyses of cruciferous material, several rapeseed cultivars (seeds and defatted meals) and other common cruciferous crops with different GL contents were analyzed in triplicate at least. Table V shows the data obtained with our technique compared to those obtained with HPLC. To verify whether the HPLC analyses were carried out in the right conditions, the CRM190 rapeseed reference sample obtained from the European Community Bureau of Reference was analyzed according to this technique. For this sample we found a GL content of 24.3 ± 0.9 vs 25.5 ± 0.9 μmol/g reported in the ECC certificate. In addition, most of the analytical results determined with the two methods compared are rather similar, although some samples show nonnegligible differences. However, the linear regression parameters ($y = -0.2158 + 0.9989x$; $r = 0.9989$; $n = 12$) indicate that the proposed method is sufficiently precise and reliable.

## CONCLUSIONS

The availability of large amounts of homogeneous (SDS–PAGE) myrosinase allowed us to obtain membranes with high specific activities per unit surface. This appears to be the most important aspect for obtaining an analytical system employing the kinetic method based on the determination of a pseudo-first-order reaction rate. Schwimmer (1981) reported that this method is more sensitive than the end-point method, without the problems of ensuring reaction completion and complications deriving from foreign activities. In addition, with the end-point method, phenomena of enzyme inhibition can occur when high final concentrations of the reaction products are formed.

The results presented here demonstrate that our system, which is based on the kinetic method and which uses myrosinase as the sole immobilized enzyme, while still open to improvement (especially regarding the reproducibility of the initial electrode conditions before analysis), promises to be particularly useful for the routine determination of GL content in crude extracts of cruciferous material.

Myrosinase shows extraordinary characteristics, given its simple immobilization on different types of membranes with negligible loss of activity and its stability in immobilized form when stored wet at 4 °C, where it retains nearly the same activity for more than 15 months.

The high specific activity of the myrosinase used, in addition to the good immobilization yield and the amplitude of the potentiometric signals obtained with normal glass pH electrodes, suggests it may be possible to miniaturize and automate the system. Automation and the simplicity of the instrumentation required would be important features for a hand-held instrument useful for rapid field and laboratory analyses.

In conclusion, the simplicity and speed of the determinations, the long lifetime of the membranes employed, the wide range of applicability, the absence of relevant matrix effects, the good linearity of the calibration curve and, finally, the good recovery and reproducibility demonstrate concrete advantages of the proposed method for determining the GL content in crude extracts of cruciferous materials.

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