APPLICATION OF ENZYME IMMOBILIZATION TO ACHIEVE RAPID LOW COST GLUCOSINOLATES ANALYSIS OF SEED AND MEAL OF CANOLA/RAPESEED

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INTRODUCTION

Accuracy, precision, speed, simplicity and low cost are desirable attributes of any analytical method, particularly when there is a need for large numbers of analyses. This is particularly true in breeding programs or when quality control is required in association with production, transportation and marketing. Over the past four decades many methods have been proposed or used to determine glucosinolate content (McGregor et al. 1983, Daun and McGregor 1990). In part this proliferation has been due to deficiencies in one or other of the above attributes. A simple, low cost method which can be completed in only a few minutes and which has sufficient sensitivity to identify seed or meal of low glucosinolate (<30 µmoles glucosinolate/g oil-extracted air-dried meal) canola rapeseed cultivars, or cultivars presently under development which are essentially glucosinolate-free, would facilitate production, transportation and marketing.

Recent studies on the immobilization of myrosinase on solid supports (Iori and Palmieri 1988, Wang et al. 1989a, 1989b) and on the coupling of immobilized myrosinase with immobilized glucose oxidase to measure glucose release from glucosinolate extracts (Koshy et al. 1989, Wang et al. 1989a) suggest that readily available commercial supports and bonding agents may be used to effect glucosinolate determination of seed or meal in only a few minutes time. Moreover, neither the myrosinase nor the glucose oxidase are consumed and may be reused as many as 500 times, substantially simplifying the analysis and reducing costs.

Studies were undertaken at the Agriculture Canada Research Station, Saskatoon to evaluate the use of immobilized myrosinase and glucose oxidase for glucosinolate analysis. A method was refined and evaluated employing a bioreactor, consisting of myrosinase covalently bonded to controlled-pore glass, in combination with a commercial glucose analyzer, consisting of a hydrogen peroxide sensitive electrode to which was attached two membranes containing entrapped glucose oxidase.

MATERIALS AND METHODS

Construction of the Bioreactor

The bioreactor was constructed in a 5 mL Eppendorf repeater pipette tip (Fig. 1). The portion of the plunger below the polyethylene seal was cut off. A disk of porous polyethylene (1.6 mm 35 µm pore size) was placed in the bottom of the barrel and a small (ca. 2 mm) hole drilled near the top of the barrel. CPG/Aminopropyl alkylamine glass, 125-177 µm particle size (Pierce Chemical Co.) was activated by placing 0.2 g in the

bioreactor and adding 2 mL 6.6 % glutaraldehyde in 0.05 M citrate-phosphate buffer pH 7. The bioreactor was sealed and shaken on a Vibrax shaker for 2 hours then washed with water to remove all unbound glutaraldehyde. Isolated myrosinase (Appelqvist and Josefsson 1967) was covalently bound to the activated glass by adding 2 mL of freshly dissolved myrosinase (7 mg/mL) to the bioreactor and shaking overnight at 4 °C. The glass containing the bound myrosinase was washed with 0.5 N NaCl, water, and 0.05 M citrate-phosphate buffer pH 7 and the bioreactor stored at 4 °C with sufficient buffer to cover the glass.

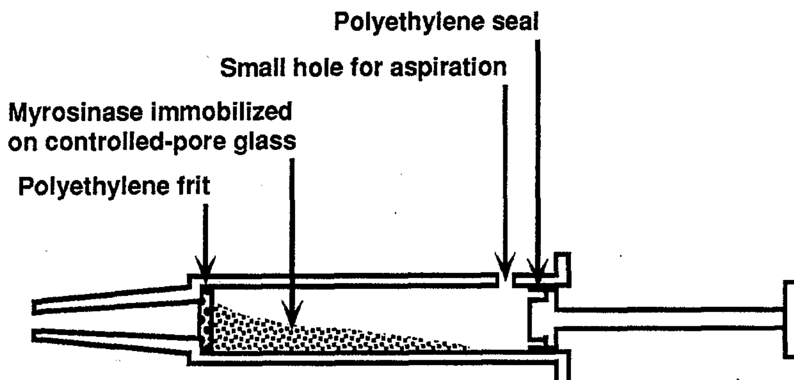

Fig. 1. Bioreactor constructed from a 5 mL Eppindorf repeater pipette tip.

Glucosinolate Analysis

Glucosinolate Extraction. Glucosinolate analyses were performed using oil-extracted air-dried meals prepared by the Swedish tube method (Troeng 1955). Endogenous myrosinase was inactivated and glucosinolates extracted by placing 250 mg of oil-extracted air-dried meal in a test tube, heating the meal to 90 °C in a boiling water bath, and adding 3 mL of hot (>80°C) water. The thoroughly wetted meal was heated for a further 3 minutes in the boiling water bath then centrifuged and the supernatant and two 1.5 mL washes made to 5 mL in a volumetric flask .

Myrosinase Hydrolysis. Prior to addition of the sample, the bioreactor was rinsed with water and excess water removed under suction on a water aspirator by drawing in air through the small hole at the top of the barrel. Extract, 0.5 mL, was drawn into the bioreactor and the contents then shaken on a Vibrax shaker for 5 minutes. The hydrolyzed sample was expelled into an 8 mL screw-capped vial, the vial capped, and heated for 1 minute in a water bath at 70°C.

Glucose Measurement. A Glucose analyzer (Yellow Springs Instrument Co. model 27) fitted with a glucose oxidase membrane (Yellow Springs Instrument No. 2365 glucose membrane) and flushed with 0.05 M citrate-phosphate buffer pH 7 was calibrated with a solution of 30 mg/dL glucose. A 25 µL aliquot of hydrolyzed sample was injected and the value displayed after 90 seconds for glucose content expressed in µmoles glucosinolate/g oil-extracted air-dried meal. To correct for

free glucose background in the sample a non-hydrolyzed aliquot of extract was also analyzed.

RESULTS AND DISCUSSION

In preliminary studies the bioreactor was prerinsed with water followed by an aliquot of glucosinolate extract to avoid subsequent dilution of the sample with rinse water held in the solid support, as previously observed (Wang et al. 1989a, 1989b). However, this lead to erroneously high results. It is thought that during the brief time the aliquot of prerinse extract was in the bioreactor a substantial amount of the glucosinolate became bound to the active sights of the myrosinase but was not hydrolyzed to release glucose. This would result in a substantial carryover of glucosinolate. To avoid this concentrating effect the bioreactor was rinse with water only and the excess water removed by aspiration before the sample was added.

Time course studies with purified allyl glucosinolate established that sufficient myrosinase activity was bound to 0.2 g of controlled-pore glass to effect complete hydrolysis in as little as 5 minutes (Fig 2). However, when a rapeseed extract was added, glucose release appeared to continue for an extended period of time. Subsequently it was determine that a similar rate of glucose release could be effected with the addition to the bioreactor of sucrose in an amount comparable to that found in the rapeseed extract. These results suggest that the crude myrosinase preparation contained invertase which was also bound to the controlled-pore glass. However, it was determined that the glucose released from the relatively low invertase activity did not unduly interfere with glucosinolate determination when the hydrolysis time was restricted to less than 5 minutes.

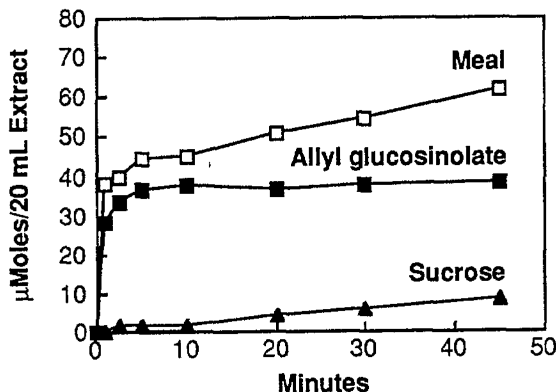

Fig. 2. Time course of glucose release in the bioreactor from a solution of sucrose, allyl glucosinolate and an extract of rapeseed meal.

Studies showed that care must be taken to allow for the beta form of glucose, released by myrosinase hydrolysis of glucosinolates, to equilibrate with the alpha form. At room temperature (ca. 25 °C) it was determined that equilibration took up to 2 hours. To allow for equilibration, standard

solutions of glucose were always prepared the day before being analyzed. However, it was found that glucosinolate analysis could be facilitated by heating the eluate from the bioreactor to 70 °C whereupon equilibration was effected in ca. 1 minute.

Using 10 oil-extracted air-dried meals of low glucosinolate strains and cultivars or mixtures thereof, the immobilized enzyme method of determining glucosinolate content was compared to gas chromatography of trimethylsilyl derivatives of glucosinolates and glucose, thymol and high performance liquid chromatography (Table 1). Pooled standard errors and overall means showed that the immobilized enzyme method had comparable accuracy and precision.

Table 1. Relative accuracy and precision of the enzyme immobilization method compared to four other methods of determining total glucosinolate content of canola/rapeseed

Cultivar or strain source	Immobilized enzymes	Thymol (1)	TMS glucose (2)	Canadian Grain Commission GLC-TMS (3)	HPLC (4)
	Micromoles / g oil-extracted air-dried meal				
Cultivars and strains					
BC86-18	7.9	4.2	5.5	4.7	5.1
Tobin (S)	16.1	14.7	15.8	14.1	14.0
Westar	29.4	25.2	28.1	28.2	24.2
Tobin (Cert.)	30.0	27.6	27.5	25.3	23.1
BL802	47.7	51.2	51.5	47.9	42.4
Meal standards					
226-1	18.3	14.1	14.3	16.0	14.5
226-2	37.9	34.5	31.4	34.5	31.9
Mixtures of BC86-18 and BL802					
BC:BL 3:1	17.9	14.9	16.4	14.7	14.2
BC:BL 1:1	29.6	28.2	26.5	26.1	23.5
BC:BL 1:3	40.3	39.6	39.9	36.7	33.0
S.E. (5)	0.57	0.49	0.33	0.34	0.67
Overall mean	27.5	25.4	25.7	24.8	22.6

(1) McGregor and Downey 1986.
(2) Olsson et al. 1980.
(3) Daun et al. 1989.
(4) McGregor 1985.
(5) Pooled standard error of the means of three determinations.

Immobilization of myrosinase in a bioreactor in combination with a hydrogen peroxide sensitive electrode onto which glucose oxidase is immobilized offers a number of advantages over other methods of glucosinolate analysis. Hydrolyzing the glucosinolates with exogenous myrosinase in a bioreactor facilitates correction for the free glucose content of the seed. This is particularly advantagous with low glucosinolate samples (<10 µmoles glucosinolate/g oil-extracted air-dried meal) as the background content of free glucose in rapeseed is usually 8 to

10 µmoles/g oil-extracted air-dried meal. Relying on exogenous myrosinase to hydrolyzed the glucosinolates, rather than the endogenous myrosinse in the seed, permits inactivation of other endogenous enzymes which can release as much as 8 µmoles/g oil-extracted air-dried meal of free glucose from non-glucosinolate sources and result in erroneously high estimations of glucosinolate content (Smith and Donald 1988). Employing a hydrogen peroxide sensitive electrode eliminates the need for, and problems associated with, the use of peroxidase, an enzyme often coupled with glucose oxidase to measure glucose colorimetrically. Seed extracts of rapeseed contain an inhibitor of peroxidase which can interfere with glucosinolate methods which utilize this enzyme. Substantial reduction in the cost of analysis can be realized by using immobilized enzymes. Since neither the myrosinase nor glucose oxidase are consumed the method is essentially a "reagentless" assay. Finally, since myrosinase hydrolysis in the bioreactor takes less than 5 minutes, and glucose measurement with the glucose analyzer only 90 seconds, enzyme immobilization has the potential to be a fast method of analysis. When combined with a rapid method of glucosinolate extraction it can be a simple, fast and sensitive method for determining the glucosinolate content of canola/rapeseed.

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