Registry No. Adenopus breviflorus neutral proteinase, 99531-63-2.

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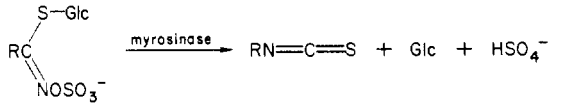
Myrosinase from *Sinapis alba* L.: A New Method of Purification for Glucosinolate Analyses

Sandro Palmieri,* Renato Iori, and Onofrio Leoni

Myrosinase from white mustard seeds (*Sinapis alba*) has been purified starting from aqueous crude extract in a single step by affinity chromatography on Con A-Sepharose. The specific activity, recovery, and binding capacity in four separate trials using glucose, mannose, methyl α-D-glucoside, and methyl α-D-mannoside for elution were also determined. The enzyme isolated by our approach showed a good degree of purification, appearing homogeneous on SDS-PAGE analyses. In the four trials of purification, the specific activity and recovery ranged from ca. 21 800 to 26 000 U/mg and 39.2 to 91.1%, respectively. The binding capacity of Con A-Sepharose for myrosinase was 6.6 mg/mL gel bed, which corresponds to 150 000 U/mL of chromatographic bed. In addition, the enzyme bound in a column to Con A-Sepharose remained active toward substrates. In this condition, myrosinase can therefore be useful for routine analyses of total glucosinolates.

INTRODUCTION

Meals of defatted rapeseed and other cruciferous seeds have a high protein content with good amino acid composition, which should make them suitable for animal feed. However, they normally contain large amounts (20–25 g/kg) of glucosinolates, which limits their use (Clandinin and Robblee, 1978; Thomke, 1981). Rapeseed also contains myrosinase (thioglucoside glucohydrolase 3.2.3.1) which along with glucosinolates, is widespread in Cruciferae. This enzyme catalyzes the hydrolysis of glucosinolates to form goitrogenic and potentially hepatotoxic isothiocyanates, glucose, and sulfate:



A selection aimed at lowering the total glucosinolate content while maintaining a good oil percentage (especially for rapeseed) appears inevitable today. Consequently a dependable, fast, and cheap analytical method is essential for screening in breeding programs.

In the last decade many useful analytical techniques for total and individual glucosinolate determination in cruciferous material have been proposed such as UV spectrophotometry (Wetter and Youngs, 1976), gas chromatography (Underhill and Kirkland, 1971; Thies, 1976), titrimetry (Croft, 1979), and ion-exchange chromatography (Heaney and Fenwick, 1981). However, many of these techniques, in addition to being long and rather tedious, require myrosinase for glucosinolates hydrolysis before analyses.

In previous paper (Iori et al., 1983) we described a method to determine glucosinolates and glucose content simultaneously using double-coupled enzymes such as myrosinase-glucose oxidase, with polarographic measurements of O_2 uptake. This method permits a significant reduction in the analysis time (ca. 4 min). It also affords the free glucose of the sample in the same analysis period of the total glucosinolate. In addition, it is suitable for analyzing samples with low glucosinolate content (<10 μmol/g). In our laboratory, we have carried out more than 1000 such glucosinolate and glucose analyses (Olivieri et al., 1982). The technique gives good results regarding quality of data, cost, and time saving. Nevertheless, the applicability of the described method is still greatly hindered by the availability of myrosinase, which requires a tedious, time-consuming purification procedure with a rather low activity recovery. In fact, myrosinase from white mustard seed (*Sinapis alba*), from rapeseed (*Brassica napus*), and from other cruciferous seeds has been purified until now by typical multicolumn systems as reported by Björkman and Janson (1972) and Ohtsuru and Hata (1979).

This paper describes a simple method, also suitable for large-scale preparations, for isolating myrosinase with high specific activity from crude extracts of white mustard seed by affinity chromatography.

MATERIALS AND METHODS

Materials. White mustard seeds (*S. alba*) were purchased from the local market. Con A-Sepharose was obtained from Pharmacia Fine Chemicals; the electrophoresis equipment and reagents were from Bio-Rad. The sinigrin used as the myrosinase substrate was obtained from K &

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Table I. Purification of Myrosinase

step	vol, mL	total protein, mg	total act., U	sp act., U mg^{-1}	purifcn, fold	yield, %
crude extract	80	243.9	164700	680	1	100
dialyzed–centrifuged extract	90	30.6	157500	5100	7	95.6
Con A-Sepharose						
glucose (A)	10	2.8	64600	23100	34	39.2
mannose (B)	10	4.4	114300	26000	38	69.4
methyl α-D-glucoside (C)	10	6.0	130900	21800	32	79.5
methyl α-D-mannoside (D)	10	6.6	150000	22700	33	91.1

K Laboratories (Plainview, NY). The other reagents were of analytical grade.

Preparation of Crude Extract. A 300-g sample of white mustard seeds was washed with distilled water and then homogenized with an Ultra-Turrax Model T.45 (IKA-Werk, Staufen, West Germany), with 2 L of distilled water. The nonsoluble material was removed by centrifugation at 17700*g* for 20 min; the supernatant was filtered through two sheets of filter paper and dialyzed thoroughly against distilled water. During dialysis the greater part of nonactive proteins precipitated and were easly removed by centrifugation. The light yellow, transparent crude extract (ca. 750 mL) was then dialyzed against 20 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl. All steps of extract preparation were carried out at 4 °C.

Chromatography. Four chromatographic trials were carried out at 4 °C simultaneously on 1 × 10 cm columns each loaded with 1 mL of Con A-Sepharose and then equilibrated with the same buffer solution used for last dialysis of crude extract. An aliquot of dialyzed–centrifuged extract sufficient to saturate each column was applied with a loading rate of 8 mL/h. The columns were then thoroughly washed with starting buffer until the absorbance reached at zero value at 280 nm. To establish the best elution system, myrosinase was then eluted from each column with a total volume of 10 mL of different eluent solutions, viz. 0.25 M glucose, mannose, methyl α-D-glucoside, and methyl α-D-mannoside, all in starting buffer. In all trials the flow was stopped for 30 min just after the collection of each fraction to improve the elution efficiency.

Enzyme Assay. Myrosinase activity was determined by measuring the decomposition of the substrate sinigrin by following the decrease in absorbance at 227 nm using quartz cells with a 5-mm path length on a Model 219 Cary recording spectrophotometer. One unit of myrosinase activity was taken as the amount of enzyme that catalyzed the hydrolysis of 1 nmol of substrate min^{-1} under the conditions described in previous paper (Palmieri et al., 1982).

Protein Measurement. Soluble-protein concentrations were determined by the Coomassie Brilliant Blue G-250 method using BSA as standard reference (Bio-Rad Laboratories, 1979).

Electrophoresis. SDS-PAGE was carried out with a Bio-Rad vertical cell system by the Laemmli (1970) procedure and with 7% polyacrylamide gels loading 25 μg of protein/lane. Protein was stained with Coomassie Brilliant Blue R-250. The protein markers used were the high molecular weight standard mixture containing myosin (200K), β-galactosidase (116K), phosphorylase (92K), BSA (66K), and ovalbumin (45K).

RESULTS AND DISCUSSION

Is well established that concanavalin A binds molecules that contain α-D-mannopyranosyl and α-D-glucopyranosyl residues with reactive hydroxyl groups in C-3, C-4, and C-5. Since white mustard myrosinase is a glycoprotein containing 18% carbohydrate as reported by Björkman and Janson (1972), it was reasonable to suppose that the enzyme binds to the gel when the carbohydrate moiety is in a suitable configuration. In fact, preliminary trials in batch showed an appreciable retention of activity. By the one-step Con A-Sepharose affinity column procedure as described in the Materials and Methods, the myrosinase was purified in high yields starting from the aqueous crude extract of white mustard seeds, resulting in a valuable specific activity. In fact, as reported in Table I, with a suitable eluent, e.g. 0.25 M methyl α-D-mannoside, it is possible to obtain a yield above 90%. In all cases we obtained high specific activities, ranging from 21800 to 26000 U mg^{-1}. Both specific activities and yields of enzyme were much higher than previously obtained with a multicolumn procedure involving DEAE-cellulose, CM-Sephadex C-50, and Sephadex G-200 gel filtration chromatography (Palmieri et al., 1982; Iori et al., 1983). In addition, considering that the maximum loading capacity of Con A-Sepharose reached 150000 U of myrosinase/mL of bed (corresponding to 6.6 mg) it is possible to obtain very large-scale, pure myrosinase preparations by this simple method using a greater gel quantity.

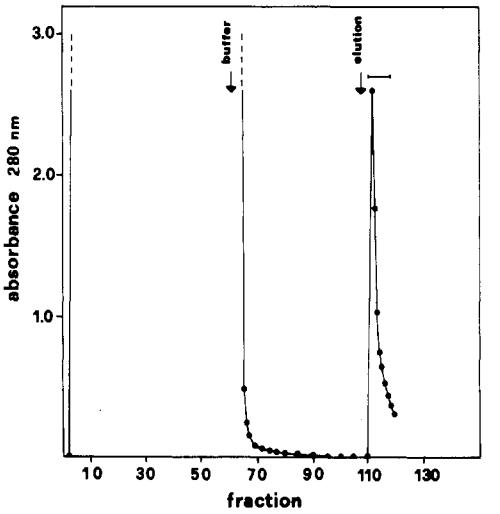

Figure 1. Dialyzed-centrifuged extract (90 mL) applied to 1 × 10 cm column containing 1 mL of Con A-Sepharose at a rate of 8 mL/h. The column was then washed with starting buffer. The fraction size was 1.5 mL. The elution with 0.25 M methyl α-D-mannoside in starting buffer was begun with fraction 108; the flow was stopped for 30 min after the collection of each 1.25 mL/fraction. The enzyme was pooled as indicated.

The chromatographic profiles obtained by the present method of myrosinase purification using solutions B–D (Table I) were typical of an affinity chromatography with an initial high, broad peak corresponding to nonadsorbed material and a second narrow peak containing the myrosinase activity (Figure 1). With glucose as eluent, only ca. 40% of myrosinase was recovered even at higher concentration. However, continuing the elution with α-D-mannoside the great part of the remaining activity was eluted in few milliliters.

Myrosinase isolated by this simple procedure from each trial migrated on SDS-polyacrylamide gel as a nearly homogeneous polypeptide as shown in Figure 2. The cal-

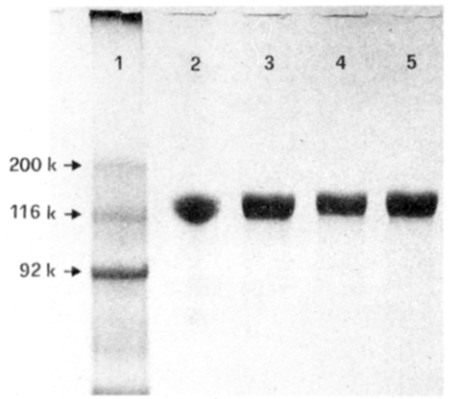

Figure 2. SDS-PAGE of myrosinase purified in a single step by Con A-Sepharose affinity chromatography. Samples were run in 7% gel and stained with Coomassie Brilliant Blue R-250. Key: molecular weight standards (lane 1); myrosinase eluted with glucose (lane 2), mannose (lane 3), methyl α-D-glucoside (lane 4), methyl α-D-mannoside (lane 5).

culated molecular weight was ca. 140 000.

The myrosinase retained by the Con A-Sepharose gel remained highly active toward its substrates. This observation can be usefully exploited for routine analyses of total glucosinolate content in crude extracts of cruciferous materials by determining the glucose concentration before and after the myrosinase-catalyzed hydrolysis of glucosinolates at room temperature and in a few minutes.

In conclusion, the procedure described here allows the preparation of hundreds of milligrams of pure myrosinase from aqueous crude extract of white mustard seeds in a few days. In view of the simple isolation procedure and the high yields, myrosinase isolated by this method can be used without further purification for glucosinolate analyses, particularly by the above-described polarographic technique (Iori et al., 1983). In addition, such high yields should allow a detailed structural characterization of the protein, improving our knowledge of chemical and biological properties of myrosinase.

ACKNOWLEDGMENT

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Registry No. Glucose, 50-99-7; mannose, 3458-28-4; methyl α-D-glucoside, 97-30-3; methyl α-D-mannoside, 617-04-9; myrosinase, 9025-38-1.

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Volatile Components of Salted and Pickled Prunes (*Prunus mume* Sieb. et Zucc.)

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Volatile components of salted and pickled prunes (*Prunus mume* Sieb. et Zucc.) were obtained by extraction and steam distillation of the prunes at pH 2.5 and 7.0. The volatile components were analyzed by capillary gas chromatography (GC) and identified by combined gas chromatography–mass spectrometry (GC–MS). There were 181 compounds detected by GC analyses, 92 compounds identified by GC–MS analyses, and some tentatively identified isomers of naphthalene and benzene derivatives. Aromatic compounds, monoterpenes, monoterpene alcohols, acids, and some aliphatic aldehydes and alcohols were the major components extracted at pH 2.5 of the salted and pickled prunes. The amount of most components extracted at pH 7.0 was less than those extracted at pH 2.5. Components that could be present in the salted and pickled prunes as glycosides were benzyl alcohol, 2-phenylethanol, monoterpene alcohols, linalool oxides and the tentatively identified isomers of (trimethylphenyl)but-3-en-2-one and a decahydronaphthol-like compound.

INTRODUCTION

The fruit of *Prunus mume* Sieb. et Zucc. is originally grown in the central and southern regions of China and is harvested in the late spring. The average weight of a fruit is about 9–14 g. The fruit is used to make wines, beverages, and pickles, but the usual metod to preserve this fruit is to make salted and pickled prunes.

Salted and pickled prunes (*P. mume* Sieb. et Zucc.) is a traditional food and has been consumed in China and Japan over 1000 years. The processing of fresh fruit of *P. mume* Sieb. et Zucc. under high salinity (ca. 20%) has been the traditional practice in order to obtain good flavor and inhibit the microbial growth (Lee et al., 1984). The "sour"

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