(Tanaka et al., 1984). While the mode of interaction seems to be somewhat flexible, there appears to be a minimum requirement to possess at least two of the three active sites: two electronegative and one steric bulkiness (or hydrophobicity) sites within a definite range of distance. Bridged bicyclic structures are suited for preparation of compounds which satisfy such structural requirements.

Registry No. 1, 53033-91-3; 2, 99618-55-0; 3, 99664-04-7; 4, 99618-56-1; 5, 99618-57-2; 6, 99618-58-3; 7, 99618-59-4; 8, 53033-90-2; 11, 99618-60-7; 12, 99664-05-8; 15, 99664-06-9; 16, 99618-61-8; 17, 99618-62-9; 18, 99618-63-0; 19, 99618-64-1; 20, 99664-07-0; 21, 99618-65-2; 23, 99618-66-3; 24, 70096-05-8; 25, 99618-67-4; 26, 99618-68-5; 27, 99618-69-6; 28, 99618-70-9; 29, 99618-71-0; 30, 99618-72-1; 31, 99618-73-2; 32, 99618-74-3; 33, 99618-75-4; 34, 99618-76-5; 35, 99618-77-6; 36, 99618-78-7; 37, 99618-79-8; camphor, 76-22-2; norcamphor, 497-38-1; norbornylene, 498-66-8; picrotoxinin, 17617-45-7; picrotin, 21416-53-5; α-hydropicrotoxinin, 17617-46-8; β-dihydropicrotoxinin, 62697-27-2.

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Purification of a Proteolytic Enzyme from *Adenopus breviflorus* Fruits

Raphael O. Adewoye[1]

A neutral protease with some similar physicochemical properties to papain was extracted and purified from the fruit of *Adenopus breviflorus*. The pH range of optimum activity in Tris-HCl buffer was between 7.0 and 9.0, and activity was enhanced by the addition of sodium cyanide, cysteine hydrochloride, 2-mercaptoethanol, poly(ethylene glycol)-6000, or polyclar AT, while some inhibition was noticed by the addition of common biocides. Degree of purification achieved was 100.4-fold while overall yield was 15.75%. Its molecular weight was estimated to be 69 500, and it was found to be highly thermostable and significantly stable toward acids and alkalies, organic solvents, freezing, and thawing. Its maximal UV absorption was at 275 nm.

The fruit of *Adenopus breviflorus* is a small green melon with irregular yellow markings and a bitter astringent taste. It belongs to the Curcurbitaceae family and its main distribution is in the tropical regions of Africa and Central America (Irvine, 1961). It is empirically used in Nigeria by rural tanners for the depilation of raw hides and skins, a process suspected to be enzymatic in nature. The objective of this paper is to report on systematic steps taken to extract and purify a proteolytic enzyme from the fruits in order to enhance its economic importance, particularly with respect to the replacement of toxic sodium sulfide in unhairing operations and the reduction of both the pollutional effects of tannery effluents and the number of unit operations required for modern leather manufacture, as earlier proposed by Adewoye and Bangaruswamy (1984).

MATERIALS AND METHODS

Materials. Fresh fruits of *A. breviflorus* were collected from forest clearings around Oyo City, Oyo State, Nigeria. Polyclar AT powder came from GAF Corp. NY, poly(ethylene glycol)-6000 from J. T. Baker Chemical Co., Phillipsburg, NJ, and the SDS-acrylamide kit from BIO-

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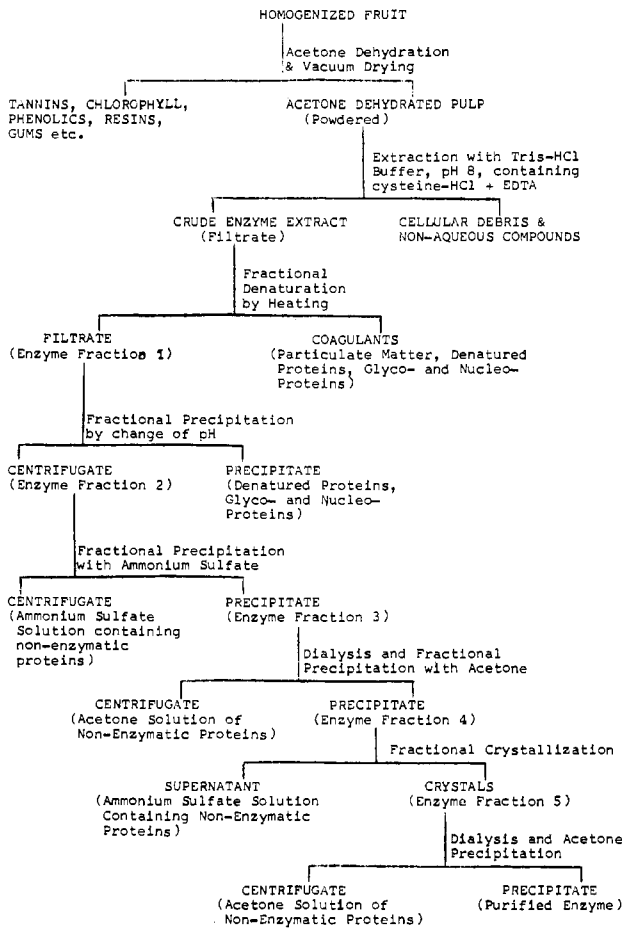

Figure 1. Schematic flow diagram illustrating the key steps in the purification of the enzyme from *Adenopus breviflorus* fruits.

RAD Laboratories, NY. Lysozyme and papain came from Sigma Chemical Co., St. Louis, MO, while bovine serum albumin was from Calbiochem, San Diego, CA. All other chemicals were of AnalaR grade and were obtained from local sources.

Fruit Preparation. The mesocarp and endocarp of the fruits where the enzyme was mainly confined were pulped and acetone dehydrated at about 2 °C.

Extraction and Purification Procedures. For extraction, 0.5 g of the acetone-dehydrated powder was leached for 24 h in 50 mL of Tris-HCl buffer solution (pH 8.0) at 37 °C and debris separated by centrifugation. Purification procedure was initiated through fractional denaturation by heating to 50 °C, since plant proteinases are known to be generally heat resistant (Greenberg, 1955). This was followed by fractional precipitation by change to pH 9.5 and the resultant gray precipitate discarded. Fractional precipitation with ammonium sulfate, primary standard, using Dixon's nomogram (Dixon, 1953) was the next major step, followed by fractional precipitation with 100% acetone (v/v), using the technique proposed by Kaufman (1971). Purification process was concluded by the application of fractional crystallization procedure as described by Jakoby (1971). The extraction and purification processes schematically followed for this study are as set out in Figure 1.

Enzyme Activity Assay. Colorimetric method, developed by Anson (1938), for estimating liberated tyrosine during proteolysis, as adopted by Greenberg (1955), was slightly modified and used, because it gave more reproducible results. Briefly, the reaction mixtures contained 5 mL of the enzyme extract in 0.2 M Tris-HCl buffer (pH 8.0) and 10 mL of 2.5% egg albumin solution. Digestion was carried out for 30 min at 45 °C after which 30 mL of trichloroacetic acid (5%) was added. The mixture was heated briefly over boiling water and cooled to room temperature, and the coagulated proteins were removed by filtration. Tyrosine and tryptophan liberated in the hydrolyzed protein were estimated by adding 5 mL of 0.5 M sodium hydroxide to 2.5 mL of the filtrate and 1.5 mL of twice-diluted Folin–Ciocalteau phenol reagent accompanied with swirling until blue color was fully developed. The color was measured on a UV spectrophotometer at 660-nm wavelength (results were expressed in terms of tyrosine only, as in the original method, for reproducibility purposes). A control in each case was run in an identical manner except that trichloroacetic acid solution was first added to the egg albumin solution before the addition of the enzyme extract.

Proteolytic activity was expressed in terms of units of enzyme activity. One unit was defined as the amount of the enzyme in 0.2 M Tris-HCl buffer (pH 8.0) that liberated 1 μg of tyrosine from 2.5% egg albumin solution in 30 min at 45 °C.

Protein Determination. Turbidimetric technique, involving sulfosalicylic acid, was the method employed for routine protein determination because it was quick and reliable as previously observed by Loomis (1974) and Layne (1957).

Assessment of Homogeneity. Homogeneity tests were based on SDS-polyacrylamide disk gel electrophoresis, according to the original descriptions by Shapiro et al. (1967), Ornstein (1964), and Davis (1964) and concentration–activity relationship.

Stability to Acid or Alkali. The effect of pH on the proteolytic activity of the enzyme was studied by taking 2-mL portions of the enzyme solution (1 mg/mL) in 5 mL of 0.15 M citrate–phosphate buffer having different pH values. They were then stored at 21 °C for 24 h, after which time their residual proteolytic activities were assessed.

Stability to Heat. Stability to heat was assessed by heating for 1 h aliquot portions of the enzyme solution at pH 8.0 in stoppered test tubes to different temperatures and cooling rapidly to room temperature; volumes were made up and residual proteolytic activities determined.

UV Absorption Measurement. Ultraviolet absorption spectrum was determined with the aid of a Hitachi Perkin-Elmer (139) spectrophotometer. For the purpose of comparison, the measurement was repeated on a solution of papain.

Molecular Weight Determination. The molecular weight of the enzyme was determined by SDS-polyacrylamide disk gel electrophoresis according to the procedure reported by Weber and Osborn (1975). The electrophoretic mobility of the protein on SDS gel was compared with those of marker proteins whose molecular weights were known.

Effect of Biocides. Commonly used biocides such as toluene, sodium chlorite, merthiolate, sodium silicofluoride, mercuric chloride, sodium pentachlorophenate, and hydrogen peroxide were separately added to aliquot portions of the enzyme solution to give different concentrations of 0.1%, 0.5%, 1.0%, and 2.0% and the residual proteolytic activities assessed.

RESULTS AND DISCUSSION

The enzyme exhibited a typical temperature–activity relationship, resulting in a bell-shaped curve (Figure 2) with the temperature of optimum activity in the range of 40–45 °C. Similarly, the pH range of maximum activity was between 7.0 and 9.0 (Figure 3). Addition of 2-

Table I. Effect of Different Periods of Extraction and Chemical Additives on *Adenopus breviflorus* Enzyme (in enzyme units/mL)

extractn med	periods of leaching, h						
	1	6	12	18	24	36	48
Tris-HCl buffer alone	226.8	393.0	601.3	735.2	745.2	725.1	678.5
buffer plus 0.005 M Cys-HCl	20.4	24.3	447.4	743.3	753.3	719.0	639.9
buffer plus 0.033 M NaCN	34.3	479.8	563.1	741.3	757.5	702.8	704.7
buffer plus 0.1 M 2-mercaptoethanol	18.1	571.2	761.4	866.7	879.0	947.7	939.6

Table II. Fractional Crystallization of *Adenopus breviflorus* Enzyme

ammonium salt soln, %	total proteolytic act., enzyme units	total protein, mg	sp act., enzyme units/mg of protein
50	2733.8	0.24	11391
45	2096.0	0.18	11644
40	5012.0	0.09	55689
35	7685.0	0.14	54893
30	34566.8	0.68	50834
35 and 40 (combined)[a]	15339.5	0.20	76698
30[a]	36450.0	0.48	75938
crude extract	11074.5	9.81	1129

[a] Fractions dialyzed and reprecipitated with acetone.

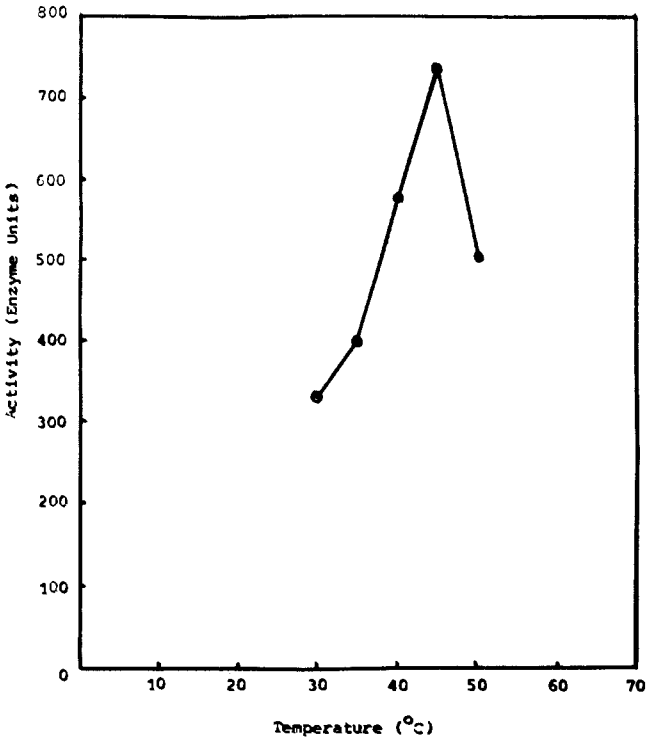

Figure 2. Temperature-dependent activity profile of the *Adenopus breviflorus* enzyme.

mercaptoethanol significantly enhanced proteolytic activity of the enzyme in about 12 h of extraction, much more than sodium cyanide or cysteine-HCl did in 24 h (Table I). Its suspected carcinogenic effect, however, led to its discontinued application during subsequent investigations.

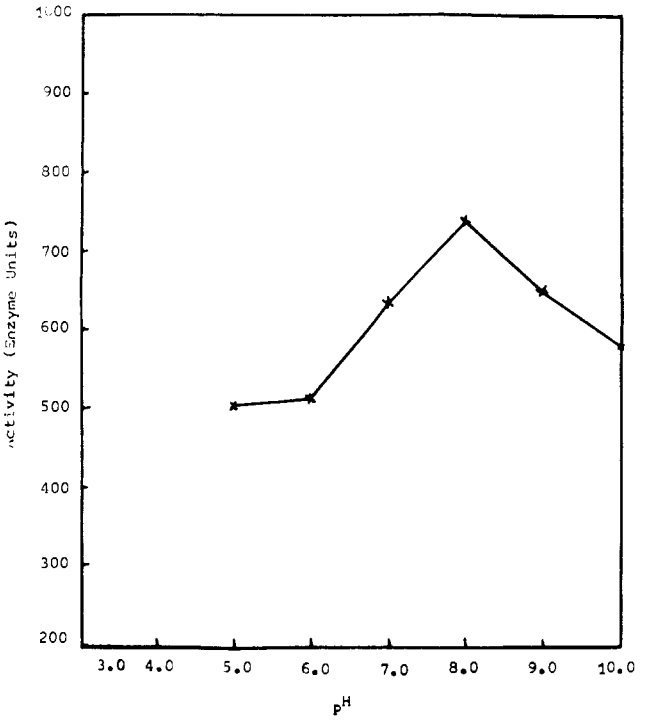

Figure 3. pH-dependent activity profile of the *Adenopus breviflorus* enzyme.

Tables II and III contain typical protein and activity data for the various stages of purification of the enzyme. The bulk of the enzyme protein crystallized and came down at ammonium sulfate saturation level of 30% as shown in Table II. In view of the high specific activity of 35% and 40% salt fractions and their low protein contents, they were first combined with each other and later with the 30% salt fraction for the same reasons. As a result of fractional precipitation with saturated ammonium sulfate solutions (stage III, Table III), the specific activity was increased more than 26-fold over the initial crude enzyme extract, while precipitation with acetone process (stage IV) increased the activity to approximately 45-fold. Finally, activity was increased to more than 100-fold in the final stage of purification by fractional crystallization (stage V). The enzyme's yield of proteolytic activity in the final purification stage was, however, about 16% of the original activity. It should be noted that purification stages III and V of Table III are not the same fractions, hence the difference in concentration.

Fractional crystallization procedure was used only as a step to remove minor impurities in the final stage of pu-

Table III. Purification of *Adenopus breviflorus* Enzyme

stage/procedure	total vol, mL	total protein, mg	total proteolytic act., enzyme units	sp act., units/mg	yield, %	purifcn, fold
I. crude extract	1000	654	738300	1129	100	1.00
II. fractnl denaturn by heating (to 50 °C)	974	498	694111	1395	94.02	1.24
III. fractnl precipn with ammonium sulfate (40–45%)	50	4.35	128737	29493	17.44	26.13
IV. fractnl precipn with 100% acetone (v/v)	50	2.45	123756	50646	16.76	44.86
V. fractnl crystalizn (from 30–40% ammonium sulfate)	45	1.04	116291	113344	15.75	100.40

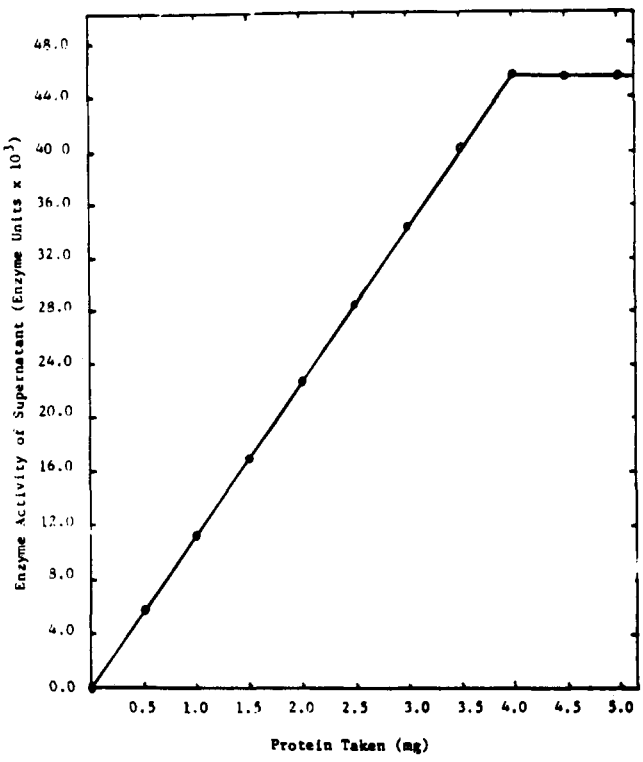

Figure 4. Homogeneity assessment of *Adenopus breviflorus* enzyme based on solubility/activity tests.

rification and not necessarily to prepare a product meant for structural studies. Formation of enzyme crystals set in some of the saturated ammonium sulfate solutions containing the enzyme protein within minutes of transfer to other centrifuge tubes at room temperature. In most cases, however, crystallization was only signalled by the appearance of turbidity, but the crystals' formation was not complete until 1 h or more later. The significant increase in specific activity was possibly aided by substantial removal of lipid materials at the solvent fractionation stage. Recrystallization, however, did not substantially improve activity. Further increase in activity was only observed after dialysis of the dissolved crystals and reprecipitation with acetone as noted in Table II.

SDS-polyacrylamide disk gel electrophoresis showed a single band of the protein while solubility and activity tests revealed that the amount of enzyme protein in solution, and its proteolytic activity, was directly proportional to the amount added to the solvent up to the saturation point, at which point no more enzyme got dissolved and therefore there was no noticeable increase in activity. Thus, only one inflection point was obtained on each of the curves plotted as shown in Figure 4. These tests indicated that the purified enzyme was reasonably pure.

Critical inactivation temperature was found to be around 85 °C at pH 8. The high thermostability exhibited by the enzyme seems to confirm that plant proteases are generally stable to heat. This finding is particularly noteworthy in respect of this enzyme in view of its relatively large molecular size, since comparatively smaller molecules are more associated with strikingly high heat-stability according to Greenberg (1955). The enzyme's stability to acid and alkali can be described as broad since most of its proteolytic activity was retained between the pH 4.5 and 11.5. The ultraviolet light absorption showed a maximum absorption at a wavelength of about 275 nm and a minimum at about 255 nm. Figure 5 shows an electrophoretic mobility–molecular weight curve for some selected enzyme proteins consisting of lysozyme, papain, trypsin, pepsin, and bovine

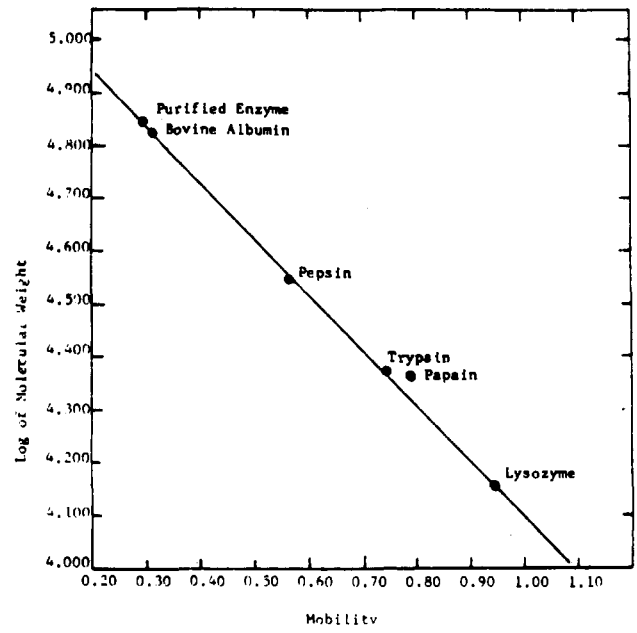

Figure 5. Molecular weight estimation of *Adenopus breviflorus* enzyme based on electrophoretic mobilities of selected marker proteins.

serum albumin from which the molecular weight of the purified enzyme was estimated to be 69 500. This is a reasonably high molecular weight when compared to most other enzymes, particularly those of plant origin. Sodium pentachlorophenate, a biocide easily regarded as conventional preservative for raw hides and skins, exhibited one of the most drastic effects on the enzyme's activity, even at low concentrations. This may suggest the need for a selection of more tolerable biocides as hide preservatives whenever this enzyme is to be used for unhairing. Sodium silicofluoride, which had least inhibition on the activity of the enzyme, would be a probable choice.

The enzyme appears to have exhibited a number of similarities in physicochemical properties with papain, despite the fact that it is derived from the juice of vine fruit while papain is from the latex of a tree fruit. Such similarities include the exhibition of optimum activity in the pH range of 7.0–9.0 and maximum UV absorption at a wavelength of between 275 and 278 nm. In addition, both enzymes have their activity enhanced by the addition of such chemicals as cysteine hydrochloride, 2-mercaptoethanol, or sodium cyanide, especially after the addition of an appropriate amounts of EDTA (Kimmel and Smith, 1957). Both of them also seem to have their activity inhibited by the addition of mercuric chloride or hydrogen peroxide. Some dissimilarities are, however, also noticeable. For instance, Tris-HCl buffer (pH 8.0), on its own, does not efficiently extract papain whereas it extracted about 90% of the enzyme under investigation. The molecular weight of 69 500 is about 3 times that of papain, widely accepted as 23 000 (Arnon, 1970; Drenth et al., 1968). The observed similarities in chemical behavior of the two enzymes, despite apparent dissimilarities in some of their physical characteristics, may therefore be due to probable similar constitution of their active centers.

Conclusion. Some reasonable degree of purification and characterization has been achieved for a proteolytic enzyme from the fruits of *A. breviflorus*, which has considerable potential as a depilatory and bating agent for leather processing with the accompanied prospect for curtailing pollution from tanneries by substituting toxic sodium sulfide and possibly saving on chemicals, processing time, and inventories.

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

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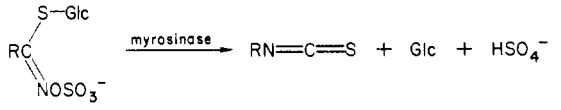
Myrosinase from *Sinapis alba* L.: A New Method of Purification for Glucosinolate Analyses

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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