The isolation and purification of Brassica juncea myrosinase and a study of its glycoprotein nature
by Kenneth Nordahl Thompson

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Chemistry
Montana State University
© Copyright by Kenneth Nordahl Thompson (1966)

Abstract:
Myrosinase of high purity was isolated from seeds of yellow Oriental mustard,. Brassica juncea, by the following sequence of purification steps: (1) water extraction of the milled oil-free seeds, (2) ammdnium sulfate precipitation (40-80% saturated fraction recovered), (3) ethanol precipitation (50% fraction recovered), (4) step-wise elution from carboxymethylcellu-lose, and(5) gel filtration through Sephadex G-200. The enzyme-preparation, appeared to contain thioglucosidase and sulfatase activity when incubated with sinigrin solution at 3 7°C; however, separation of the two enzymes was not accomplished. A 250-fold purification of thioglucosidase and a 115-fold purification of sulfatase were obtained.

Myrosinase isolated in this work was estimated to have a molecular weight of 320,000 ± 15,000 by a gel filtration technique-and contained 19.2% carbohydrate. Analyses by paper and gas-liquid chromatography showed the carbohydrate to include residues of L-arabinose, L-fucose, D-xylose, D-mannose, D-galactose, D-glucose,-D-glucosamine, and N-acetyl-neuram-inic acid. Amino acid analysis revealed a high glutamic and aspartic acid content and a low percentage of sulfur-containing amino acids.

Digestion of myrosinase with a Streptomyces griseus protease and subsequent fractionation by a series of gel filtrations served to partially characterize the carbohydrate present. Major amounts of the carbohydrate appeared to be present as an associated heteropolysaccharide with an approximate molecular weight of 55,000. This polysaccharide contained L-arabinose, D-xylose, D-glucose, and D-galactose. Carbohydrate moities with molecular weights of approximately 1000 were indicated as present.. These contained D-xylose, L-fucose, D-mannose,. D-glucose, D-glucosamine , and N-acetyl-neuraminic acid; however, specific assignment of sugars to a particular moiety, or moieties to a particular enzyme, was not accomplished. The high carbohydrate content (85%) and the presence of serine, threonine, aspartic and glutamic acids in this oligosaccharide mixture suggested covalently-bound carbohydrate in myrosinase similar to that in known glycoproteins.

Treatment of myrosinase with 8 M urea solution followed by gel filtration appeared to free a large polysaccharide from most protein present. Studies with urea supported the results obtained from the protease digest.

THE ISOLATION AND PURIFICATION OF *BRASSICA JUNCEA* MYROSINASE AND A STUDY OF ITS GLYCOPROTEIN NATURE

by

KENNETH N. THOMPSON

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Chemistry

Approved:

Head, Major Department

Chairman, Examining Committee

Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

March, 1966

ACKNOWLEDGEMENT

I wish to express my sincere gratitude to Dr. K. J. Goering for his guidance and consideration during my years of graduate study.

I wish to acknowledge the financial assistance provided by the National Science Foundation research grant GB647 (a continuation of G7497), and by the Montana State University Chemistry Department and Chemistry Station.

I wish to thank the M. S. U. Chemistry Department staff and graduate students for their advice and assistance.

I am indebted to Dr. John Robbins of the University of Oregon for obtaining the sedimentation patterns contained in this thesis.

And last, but most of all, I wish to thank my parents for the help and encouragement which they have generously given.

TABLE OF CONTENTS

	Page
LIST OF TABLES	v
LIST OF FIGURES	vi
ABSTRACT	viii
INTRODUCTION	1
MATERIALS AND METHODS	5
Assay for Myrosinase Activity	5
Protein Assays	6
Carbohydrate Assay	6
Preparation of Ion-Exchange Celluloses	7
Preparation of Gel Filtration Media	7
Electrophoresis Procedures	8
Paper Chromatography Techniques	10
Amino Acid Analysis	10
Sedimentation Studies	11
Gas-Liquid Chromatography of Carbohydrates	11
EXPERIMENTAL	14
Extraction and Purification of Myrosinase	14
Chemical Composition of Myrosinase	20
Molecular Weight of Myrosinase	32
Pronase Digestion of Myrosinase	35
Dissociation of Myrosinase	49
DISCUSSION	53
Extraction and Purification of Myrosinase	53
Chemical Composition of Myrosinase	56
Molecular Weight of Myrosinase	58
Pronase and Dissociation Studies on Myrosinase	59
SUMMARY	64
LITERATURE CITED	65

LIST OF TABLES

TABLE Page

I. Purification of Brassica juncea Myrosinase 21

II. Chemical Composition of Myrosinase 22

III. Amino Acid Composition of Myrosinase 24

IV. Composition of the Carbohydrate Associated with Myrosinase 28

V. Carbohydrate Composition of the Pronase-Myrosinase Digest Fractions 44

VI. Amino Acid Composition of the Pronase-Myrosinase Digest Fractions 48

LIST OF FIGURES

FIGURE — Page

1. Fractionation of crude myrosinase by step-wise elution from CM-cellulose 16
2. Sedimentation patterns of myrosinase preparations 17
3. Gel filtration of CMC-2 on Sephadex G-200 18
4. Elution of purified myrosinase from DEAE-cellulose with 0.05 M sodium citrate buffer, pH 6.1 19
5. Disk electrophoresis of purified myrosinase on polyacrylamide gel at pH 8.3 20
6. Gas chromatogram of the trimethylsilyl derivatives from the 24-hour myrosinase hydrolyzate 25
7. Gas chromatogram of the trimethylsilyl derivatives from the 48-hour myrosinase hydrolyzate 26
8. Gas chromatogram of the trimethylsilyl derivatives from the 72-hour myrosinase hydrolyzate 27
9. R_{BD} vs. molecular weight for Bio Gel P-300 (41x2.5 cm) equilibrated with 0.05 M sodium citrate buffer, pH 6.1 34
10. Gel filtration of the pronase-myrosinase digest on Sephadex G-25 37
11. Gel filtration of the pronase blank on Sephadex G-25 38
12. Gel filtration of PM-25-1 on Sephadex G-75 39
13. Gel filtration of P-25-1 on Sephadex G-75 40
14. Gel filtration of P-25-2 on Sephadex G-75 40
15. Gel filtration of PM-25-2 on Sephadex G-75 41

16. Gel filtration of PM-75-2 (A) and PM-75-3 (B) on Sephadex G-15 42

17. Scheme for fractionation by gel filtration of the carbohydrate fractions in the pronase-myrosinase digest or in the pronase blank 43

18. Gas chromatogram of the trimethylsilyl derivatives from the 24-hour PM-75-1 hydrolyzate 45

19. Gas chromatogram of the trimethylsilyl derivatives from the 24-hour PM-15-1 hydrolyzate 46

20. Gas chromatogram of the trimethylsilyl derivatives from the 24-hour PM-15-2 hydrolyzate 47

21. Gel filtration of an 8 M urea solution of myrosinase on Sephadex G-200 (58 x 1.6 cm) 50

22. Gel filtration of an 8 M urea solution of myrosinase on Sephadex G-200 (47 x 1.6 cm) 52

ABSTRACT

Myrosinase of high purity was isolated from seeds of yellow Oriental mustard, *Brassica juncea*, by the following sequence of purification steps: (1) water extraction of the milled oil-free seeds, (2) ammonium sulfate precipitation (40-80% saturated fraction recovered), (3) ethanol precipitation (50% fraction recovered), (4) step-wise elution from carboxymethylcellulose, and (5) gel filtration through Sephadex G-200. The enzyme preparation appeared to contain thioglucosidase and sulfatase activity when incubated with sinigrin solution at 37°C; however, separation of the two enzymes was not accomplished. A 250-fold purification of thioglucosidase and a 115-fold purification of sulfatase were obtained.

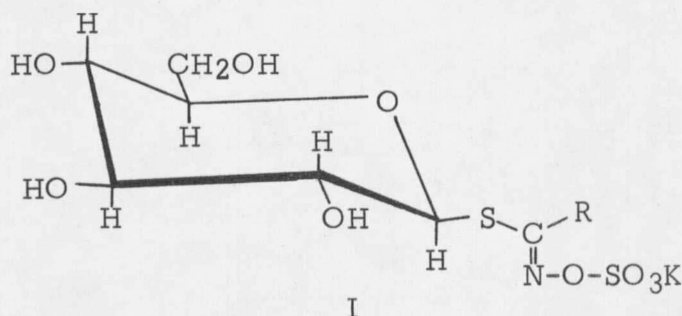
Myrosinase isolated in this work was estimated to have a molecular weight of 320,000 ± 15,000 by a gel filtration technique and contained 19.2% carbohydrate. Analyses by paper and gas-liquid chromatography showed the carbohydrate to include residues of L-arabinose, L-fucose, D-xylose, D-mannose, D-galactose, D-glucose, D-glucosamine, and N-acetyl-neuraminic acid. Amino acid analysis revealed a high glutamic and aspartic acid content and a low percentage of sulfur-containing amino acids.

Digestion of myrosinase with a *Streptomyces griseus* protease and subsequent fractionation by a series of gel filtrations served to partially characterize the carbohydrate present. Major amounts of the carbohydrate appeared to be present as an associated heteropolysaccharide with an approximate molecular weight of 55,000. This polysaccharide contained L-arabinose, D-xylose, D-glucose, and D-galactose. Carbohydrate moities with molecular weights of approximately 1000 were indicated as present. These contained D-xylose, L-fucose, D-mannose, D-glucose, D-glucosamine, and N-acetyl-neuraminic acid; however, specific assignment of sugars to a particular moiety, or moieties to a particular enzyme, was not accomplished. The high carbohydrate content (85%) and the presence of serine, threonine, aspartic and glutamic acids in this oligosaccharide mixture suggested covalently-bound carbohydrate in myrosinase similar to that in known glycoproteins.

Treatment of myrosinase with 8 M urea solution followed by gel filtration appeared to free a large polysaccharide from most protein present. Studies with urea supported the results obtained from the protease digest.

INTRODUCTION

Myrosinase is the enzyme system present in several members of _Cruciferae_, _Tropaeolaceae_, _Capparidaceae_ and _Resedaceae_ which is responsible for the breakdown of mustard oil glucosides (I) into glucose, inorganic sulfate and organic isothiocyanate (Gmelin and Virtanen, 1959).



I

Allyl isothiocyanate can be isolated from mustard seed, but observations over a century ago on black mustard revealed that this volatile mustard oil did not occur free in the intact seed (Boutron-Charland and Robiquet, 1831; Faure, 1831). These workers showed that isothiocyanate was liberated only when crushed seeds were suspended in water. In addition, they observed that if the crushed seeds were pretreated with protein-denaturing reagents, mustard oil liberation was prevented in the subsequent water suspension.

A glucoside isolated from black mustard seed provided evidence that glucose and inorganic sulfate were associated with the isothiocyanate in the seed (Bussy, 1840). Bussy discovered that a mixture of this glucoside

and a water-soluble protein extract from mustard seed caused the appearance of glucose, inorganic sulfate and allyl isothiocyanate. The glucoside was named as the salt of myronic acid and this later led to naming its hydrolyzing enzyme, myrosin. Today this glucoside is known as sinigrin (I, where $R = CH_2{=}CH{-}CH_2{-}$. As a result, the enzyme system has been variously referred to as myrosin, myrosinase or sinigrinase.

Initial studies on myrosinase suggested the presence of two enzymes, a thioglucosidase and a sulfatase (von Euler and Erickson, 1926). Sandberg and Holly (1932) confirmed these results and, shortly thereafter, actual separation of thioglucosidase activity from sulfatase activity was reported (Neuberg and Schoenebeck, 1933).

After two decades of apparent acceptance, the two-enzyme character of myrosinase was questioned (Ettlinger and Lundeen, 1956; 1957). One enzyme, a thioglucosidase, was postulated to act followed by a stabilizing non-enzymatic rearrangement that liberated sulfate and formed allyl isothiocyanate. Experimental data appeared later to support this premise (Nagashima and Uchiyama, 1959a-d).

New evidence for a two-enzyme system was obtained when methods different from those of Neuberg and Schoenebeck allowed separation of the two apparent enzyme activities in yellow Oriental mustard seed, Brassica juncea (Gaines and Goering, 1960; Gaines, 1960). When incubated individually with sinigrin, these enzymes released only one of the characteristic products, glucose or inorganic sulfate; however, if they were mixed together prior to incubation complete hydrolysis of sinigrin was achieved.

Each enzyme was shown to possess hydrolytic activity toward several synthetic substrates (Gaines, 1960; Gaines and Goering, 1960; 1962).

The disagreement between the one-enzyme and the two-enzyme theories is still not settled.

The thioglucosidase preparation of Gaines and Goering (1962) behaved generally as a β-glucosidase and in common with some other β-glucosidases (Helferich, 1943) was shown to contain carbohydrate (Gaines, 1960). Many glycosidases are associated with polysaccharides which seem to stabilize these systems. If for some reason this carbohydrate is eliminated during purification frequently the enzyme system becomes unstable. In most cases it is not known how these polysaccharides are attached to the protein moiety and it is conceivable that many of these systems are mixtures of polysaccharides and protein rather than specific molecular entities (Fisher and Stein, 1960).

Huotari (1962) obtained evidence for covalently-bonded carbohydrate in the thioglucosidase from yellow Oriental mustard seed. This carbohydrate appeared to be present as an oligosaccharide which was free of sialic acid and accounted for one percent of the thioglucosidase preparation. In the same work, galactose, arabinose, hexosamine and uronic acid were reported to be present in the carbohydrate moiety.

Proteins containing tightly-bound carbohydrate are numerous and have been known for many years. They are constituents of blood plasma, mucous, cell wall material, connective tissue and include some hormones, blood group substances, antibodies, as well as enzymes. Since these compounds are part of many vital processes in living things, their structure and

metabolism are of interest. Enzymes containing carbohydrate are of added interest since their carbohydrate moieties may serve in the enzymes' active sites. Helferich *et al.* (1938) suggested that the carbohydrate grouping of β-glucosidases may act as a "seeding" site necessary for enzyme activity. However, preliminary studies involving a mild periodate treatment of carbohydrate-containing enzymes seemed to indicate that the carbohydrate was not essential for enzyme activity (Pazur *et al.*, 1963).

Prior to 1950, studies on proteins containing carbohydrate were primarily descriptive and have been adequately reviewed (Levene, 1926; Meyer, 1945; Stacey, 1946; Bettelheim-Jevons, 1958). These authors point out the confused nomenclature which exists in this field. Schmid (1964) has used the term "glycoproteins" to describe ". . . proteins, excluding nucleoproteins, that carry covalently-bound carbohydrate". Numerous glycoproteins have been discovered, but only three of these are known to have enzyme activity. They are Taka-amylase A (Tsugita and Akabori, 1959), acid deoxyribonuclease (Bernardi *et al.*, 1965) and glucose oxidase (Pazur *et al.*, 1965). The significant amount of carbohydrate that is apparently linked covalently in the enzyme myrosinase suggests that this system might be classified as a glycoprotein.

The purpose of this research was to further investigate the two-enzyme character of myrosinase and the nature of the carbohydrate-protein complex which is present.

MATERIALS AND METHODS

Assay for Myrosinase Activity

Enzymatic hydrolysis of sinigrin by myrosinase liberates glucose, inorganic sulfate and allyl isothiocyanate. Enzyme assays were made on incubation mixtures prepared by warming 0.2 ml of myrosinase solution with 0.5 ml of sinigrin solution (20 mg per ml) at 37°C. Solutions were in 0.05 M sodium citrate buffer, pH 6.1. Incubation times were varied to prevent total hydrolysis of substrate. The reaction was stopped with dinitrosalicylic acid solution when assaying thioglucosidase activity and with trichloroacetic acid solution when assaying sulfatase activity.

Sinigrin was obtained from Mann Research Laboratories, California Corporation for Biochemical Research, and Aldrich Chemical Company, Inc. Each sinigrin preparation was checked for purity by paper chromatography, along with a blank reaction containing assay reagents.

Glucose liberated by the water enzyme extract was determined by quantitative paper chromatography (Whistler and BeMiller, 1962) using n-butanol-pyridine-water (6:4:3, v/v) solvent and the phenol-sulfuric acid colorimetric procedure. The dinitrosalicylic acid method (Summer, 1925) was used to assay glucose at the other stages of enzyme purification. Sulfate was analyzed turbidimetrically as barium sulfate (Dodgson, 1961). Allyl isothiocyanate was not determined, although its odor was evident during every incubation in which glucose and sulfate were liberated.

Specific activity of myrosinase was calculated as previously done by Gaines and Goering (1962), wherein a unit of enzyme activity was defined

as that amount of enzyme which catalyzed the hydrolysis of 5 mg of substrate per hour at 37°C.

Protein Assays

Quantitative analysis for protein (Lowry *et al.*, 1951) was accomplished using phenol reagent obtained from the Hartman-Leddon Company, Philadelphia, with egg albumin (California Corporation for Biochemical Research, B Grade, 5X recrystallized) as the standard. In dilute solution (<0.10 mg protein/ml), Kalckar's (1947) approximation provided a convenient and adequate protein determination.

Protein eluted from various chromatographic columns was followed by by optical density measurements at 280 mμ in either a Beckman Model DB or Model DK-2 spectrophotometer.

Carbohydrate Assay

The phenol-sulfuric acid method of Dubois *et al*. (1956) was used for quantitative analysis of carbohydrate in myrosinase. This method allowed the determination of carbohydrate without interference from protein (Keen and Opie, 1957). A composite sugar mixture containing galactose, mannose, arabinose, xylose, glucose, fucose and glucosamine was used to prepare a standardization curve for this technique. The reaction mixture was prepared by mixing 1 ml of solution containing carbohydrate (10-100 μg) with 1 ml of 5% redistilled phenol and then rapidly adding 5 ml of 95.0-98.0% sulfuric acid. After 10 minutes the solution was cooled for 30 minutes in running tap water. The optical density of the amber-colored solution was read at

490 mμ on a Beckman Model DB spectrophotometer. Carbohydrate eluted during column chromatography was followed by the same method.

Preparation of Ion-Exchange Celluloses

Diethylaminoethylcellulose (DEAE-cellulose) obtained from Eastman Organic Chemicals and carboxymethylcellulose (CM-cellulose) obtained from Sigma Chemical Company were both prepared for use in the recommended manner (Peterson and Sober, 1962) prior to equilibration in the desired buffer.

An ion-exchange cellulose column was packed by filling the glass tube to one-third its volume with starting buffer and then adding the slurry of equilibrated ion-exchange material. The column stopcock was opened and the column allowed to pack with occasional tapping to remove occluded air bubbles. Every column was equilibrated by allowing buffer to flow through it overnight at the rate used during chromatography. CM-cellulose was regenerated (Peterson and Sober, 1962) once; i.e., used two times, then discarded because its ion-exchange properties seemed to change with extended use. All elutions were run at room temperature.

Preparation of Gel Filtration Media

Two types of gel filtration material were used in this work: dextrans of various cross linkage (Sephadex) purchased from Pharmacia Fine Chemicals, Inc. and polyacrylamide (Bio-Gel P-300, 50-150 mesh) purchased from Bio-Rad Laboratories.

These materials were suspended in the eluting solvent to be used and were allowed to swell at least 3 hours in the case of gels with small pore

size (Sephadex G-15 and G-25) or at least 18 hours in the case of the gels with larger pore size. Sephadex preparations had to be "washed" free of fine particles to prevent plugging of their packed columns. "Washing" was accomplished in three repeated steps: (1) Suspension of Sephadex in the elution solvent, (2) a 30-minute settling of heavier particles, and (3) decantation of the supernatant solution which contained fine particles. Five or six repetitions of these steps were usually sufficient.

A column was packed by filling the glass tube to one-third its volume with eluting solvent and then adding a slurry of the gel to be used. After the gel had settled to a depth of 1-2 cm, the eluting solvent was allowed to flow. The formed column was equilibrated before use by passing solvent through it for at least 12 hours. All elutions were performed at room temperture.

Electrophoresis Procedures

A. Ionic nature of crude myrosinase

The ionic nature of the components in crude myrosinase was checked in a Gelman electrophoresis apparatus. This unit was equipped with Seprphore III (microporous cellulose acetate) strips in sodium barbitol-acetate buffer, pH 8.2, and run at 100 volts and 4-5 milliamps per strip for two hours at room temperature. Approximately 25 μg of protein in 0.05 M sodium citrate buffer, pH 6.3, were applied per strip. Protein was detected on the undeveloped cellulose acetate strip with Amido Schwarz stain (100 mg Amido Schwarz per 100 ml 10% acetic acid).

B. Qualitative analysis for hexosamine

Paper electrophoresis has been used to separate and identify hexosamines (Ohkuma and Shinohara, 1963). This procedure was used with slight modification in the present investigation. A Spinco Model R paper electrophoresis apparatus was equipped with eight Beckman #320046 paper strips and 0.025 M sodium tetraborate electrolyte, pH 9.2. After allowing this system to equilibrate for an hour, the sample was applied as a stripe with a Spinco sample striper (Part No. 300-805), immediately covered with a stripe of 1% (v/v) acetic anhydride in acetone solution, and allowed to stand for 10 minutes before subjecting it to 200 volts for six hours at room temperature. The paper strip was dried for five minutes at 95°C, cooled and the N-acetyl-hexosamine bands located by staining with p-dimethylaminobenzaldehyde reagent (Partridge, 1948). This reagent caused darkening of the entire paper strip after 10-15 minutes, thereby masking the bands which were initially evident. In this system N-acetyl-D-glucosamine migrated about 1 cm from the origin toward the negative electrode. N-acetyl-D-galactosamine migrated about 1.5 cm toward the positive electrode. Ohkuma and Shinohara (1963) have reported that N-acetyl-D-mannosamine migrated to the positive electrode more rapidly than N-acetyl-D-galactosamine.

C. Homogeneity of purified myrosinase

Disk electrophoresis on polyacrylamide gels was used to check homogeneity of the purified myrosinase. Preparation and use of 2-amino-2-(hydroxymethyl)-1,3 propanediol (TRIS)-glycine buffer, polyacrylamide gels and Amido Schwarz staining solution for protein have been previously described (Davis, 1964). Approximately 1.4 mg of purified myrosinase were

equilibrated overnight with TRIS-glycine buffer, pH 8.3, before being subjected to 160 volts at 28 milliamps per 8 tubes for 55 minutes at room temperature.

Paper Chromatography Techniques

Four solvent systems were used for descending paper chromatography during this investigation. Normal butanol-pyridine-water (6:4:3, v/v) was used for most routine sugar analyses. Ethyl acetate-pyridine-water (8:2:1, v/v) and n-butanol-acetic acid-water (5:1:2, v/v) served to confirm the identity of sugars present. Uronic acid chromatograms were run in isopropyl alcohol-pyridine-acetic acid-water (8:8:1:4, v/v) solvent. Whatman #1 filter paper was used throughout this work and the elutions were usually run for 24 hours.

Reducing sugars were revealed on paper chromatograms by the CD-1 reagent of Gordon _et al_. (1956). The air-dried chromatograms were dipped in CD-1 reagent, dried at room temperature in the hood and the spots developed by heating with a Master blower heater. Unknown sugars were classified by color of their CD-1 spots. A sugar was considered identified if, when a known sugar was spotted on the paper with the unknown mixture, intensification of the same spot occurred in three of the solvent systems mentioned previously.

Amino Acid Analysis

Amino acids were determined by the method of Moore and Stein (1954), with an Auto Analyzer (Technicon Chromatography Corporation). Samples were hydrolyzed in sealed evacuated tubes with constant boiling

hydrochloric acid at 110°C. Hydrolyzed samples were freeze-dried, taken up in distilled water and freeze-dried a second time. The aqueous solution (0.6 ml) of the HCl-free hydrolyzate was applied to the Dowex 50x12 column (60°C) and eluted from the column with a pH gradient provided by 0.05 M sodium citrate buffer from pH 2.88 to pH 3.80 and by 0.05 M sodium citrate-0.6M sodium chloride buffer from pH 3.80 to pH 5.00.

Sedimentation Studies

The homogeneity of the myrosinase preparation was studied by recording its sedimentation at 59,780 rpm in a Spinco Model E analytical ultracentrifuge equipped with a single sector cell maintained at 3-5°C. Solutions were prepared by dissolving the enzyme in 0.05 M sodium phosphate buffer, pH 5.7-5.9, at 5°C. The sample was place in the centrifuge following a one-hour dialysis against the same phosphate buffer. Pictures of the developing sedimentation pattern were taken at various time intervals after the rotor had reached 59,780 rpm.

Gas-Liquid Chromatography of Carbohydrates

Gas-liquid chromatography of their O-trimethylsilyl derivatives has provided separation and identification of a number of carbohydrates (Hedgley and Overend, 1960; Sweeley et al., 1963; Bentley et al., 1963; Sweeley and Walker, 1964). This technique has been recently applied to investigations on glycoproteins (Bolton et al., 1965). A similar approach was taken in the present investigation when analyzing the monosaccharide units of purified myrosinase.

A sample (usually containing 200-400 μg of carbohydrate) was hydrolyzed by refluxing with 5 ml of 3.1% (w/w) methanolic hydrogen chloride prepared by bubbling anhydrous HCl gas into anhydrous methanol at $0^{o}C$. Unless otherwise specified, refluxing lasted for 24 hours. After evaporation under a stream of nitrogen gas, the residue was dried for six hours in a vacuum desiccator equipped with anhydrous $CaCl_2$ and NaOH pellets. The resultant methyl glycosides were treated with 1 ml of methanol-acetic anhydride (3:1, v/v) solution containing a few milligrams of silver acetate. This treatment lasted 24 hours at room temperature and was performed in order to acetylate any material that might have been de-N-acetylated during hydrolysis, (White, 1940). Following filtration through sintered glass, the filtrate was evaporated in a stream of nitrogen gas and dried in a vacuum desiccator for 24 hours. Trimethylsilyl ethers of the glycoside mixture were prepared by a 10-minute treatment with 0.10 ml of a pyridine-trimethylchlorosilane-hexamethyldisilazane (5:1:1, v/v) mixture. (Reagent grade pyridine was redistilled from BaO and stored over KOH pellets prior to use. Trimethylchlorosilane and hexamethyldisilazane were used as received from K and K Laboratories, Inc.). The trimethylsilylation mixture contained a gray to brown precipitate which was probably ammonium chloride and reduced silver ion. Kagan and Mabry (1965) have reported that this precipitate does not interfere with quantitative results obtained.

All analyses were performed in a F and M Biomedical Model 400 gas chromatograph equipped with temperature programming, a flame ionization detector and a U-shaped glass column (105 x 0.4 cm) packed with 3.8% SE-30

silicone on Gas Chrome Q (100-120 mesh). Helium served as carrier gas and was maintained at a flow rate of 70-72 ml per minute. Aliquots (0.8-1.1 μl) of the trimethylsilylation mixture were injected into the column at 100°C and, after a 2-minute delay, the column temperature was programmed (3°C per minute) to 200°C. Qualitative analyses were initiated at 150°C and, after a 10-minute delay, programmed (7.5°C per minute) to 200°C. Quantitative determinations required greater separation of peaks provided by the lower initial temperature.

The peaks obtained were identified by comparing their retention times with those of known substances prepared in the manner described above. Relative amounts of the sugars present in various unknown preparations were determined directly from integrator readings on a Sargent Model SR recorder.

EXPERIMENTAL

Extraction and Purification of Myrosinase

Thirty-one kilograms of yellow Oriental mustard seed, *Brassica juncea*, were ground into a coarse meal and defatted with Skelly B solvent in a Soxhlet extractor. After a ten-hour air drying, the 10 kg of defatted meal were milled to a powder, suspended in 91 liters of distilled water (44°C) and mechanically stirred for seven hours. A major portion of the debris in the resulting slurry was removed by centrifugation. The remaining debris was allowed to settle for 12 hours at 5°C. A clear amber-colored supernatant solution resulted and was collected by decantation. This supernatant solution plus one water wash constituted the enzyme-containing water extract. The water extract (44 liters) was cooled to 5°C and treated with 10.7 kg $(NH_4)_2SO_4$ to give 40% saturation. Precipitated protein was removed in a Westphalia centrifuge and discarded. Ammonium sulfate (11.6 kg) was added to the 40% saturated solution to give 80% saturation. The enzyme-containing precipitate was collected by centrifugation, dissolved in 22 liters of distilled water and dialyzed for 24 hours against cold running tap water. An equal volume of absolute ethanol (5°C) was slowly added to the protein solution (15°C). After mixing for 30 minutes, the precipitate was recovered by centrifugation, dissolved in distilled water (5 liters) and dried under forced air at room temperature in aluminum trays. After 36 hours, 87 grams of clear, amber-colored, flaky crude myrosinase were obtained.

Fractionation of crude myrosinase on DEAE-cellulose was used in an attempt to separate thioglucosidase and sulfatase activity as previously

reported (Gaines and Goering, 1960). Protein was recovered as a single fraction when eluted from the column with 0.05 M sodium citrate buffer, pH 6.3. Electrophoresis on cellulose acetate strips (See "Materials and Methods") showed this protein in pH 6.3 solution to behave as a cation. This information suggested the use of ion-exchange techniques on CM-cellulose.

Four-gram portions of crude myrosinase were dissolved in approximately 75 ml of 0.05 M sodium citrate buffer, pH 4.3, and placed on CM-cellulose columns (30 x 3.4 cm) equilibrated with the same buffer. Protein was eluted from the columns (2 ml/min) in three steps: (1) 0.05 M sodium citrate buffer, pH 4.3, (2) 0.05 M sodium citrate buffer, pH 5.8, and (3) 0.05 M sodium citrate-1 M sodium chloride buffer, pH 6.8. Figure 1 illustrates the typical elution pattern obtained. Major amounts of enzyme appeared in the protein eluted at pH 5.8 and this was dialyzed, concentrated by blowing air over the dialysis bag containing the enzyme, and freeze-dried. Approximately 2 g of enzyme-containing protein (CMC-2) resulted from fractionation of 30 g of crude myrosinase. Sedimentation in an ultracentrifuge revealed at least three components in CMC-2 (Figure 2A). Gel filtration through Sephadex G-200 (60 x 3.9 cm) equilibrated with 0.05 M sodium citrate buffer, pH 6.1, separated CMC-2 into at least three fractions (Figure 3). Ten-milliliter aliquots were collected at a rate of 30 ml per hour. Enzyme activity appeared entirely with the protein eluted in the first peak. An ivory-tan, flaky material (305 mg) was obtained after dialyzing, concentrating and freeze-drying this portion. Homogeneity of the enzyme was again checked in an ultracentrifuge (Figure 2B). Ion-exchange chromatography was

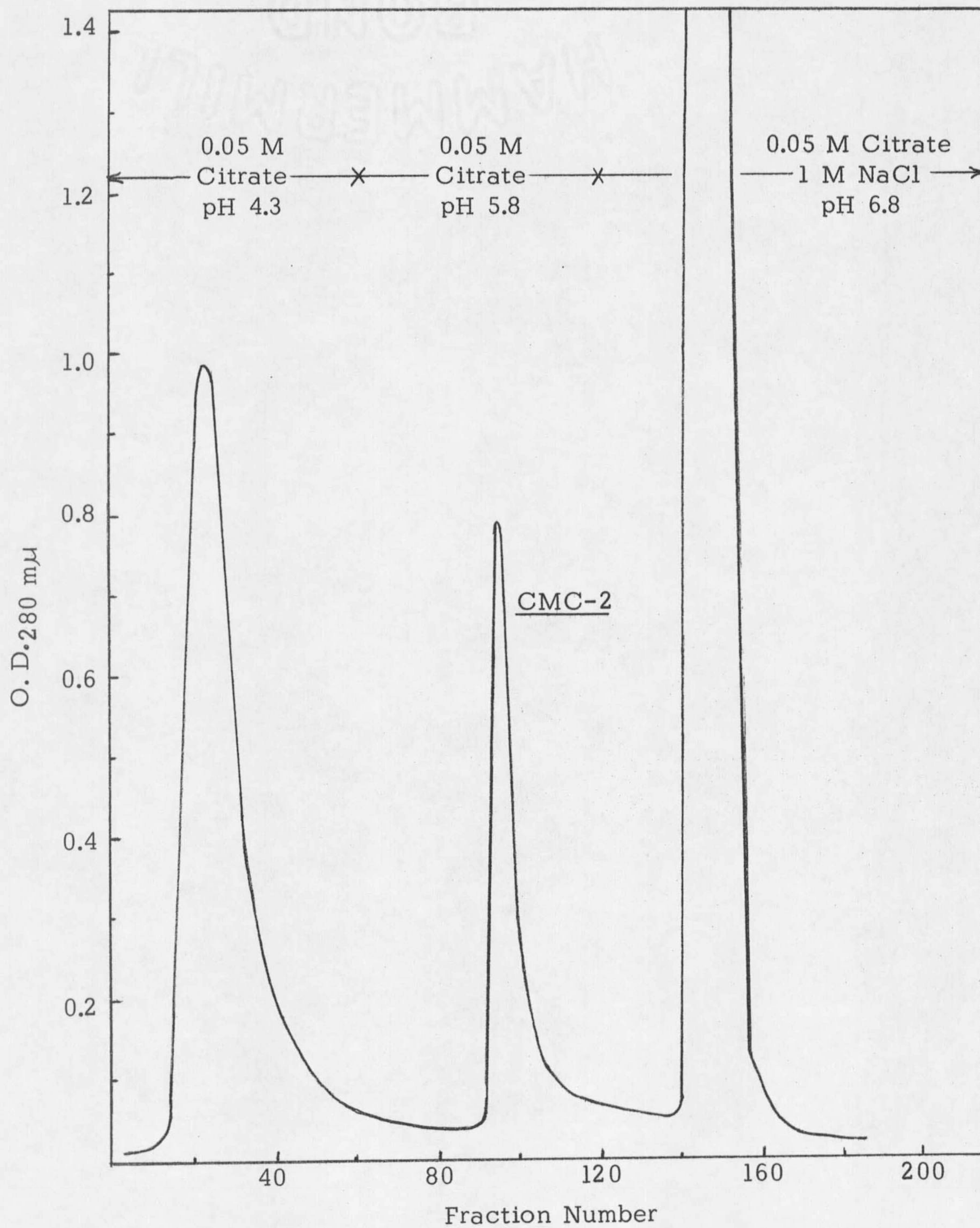


Figure 1. Fractionation of crude myrosinase by step-wise elution from CM-cellulose.

attempted on DEAE-cellulose using sodium citrate buffer, pH 6.1, as the eluting agent; but again, this failed to separate thioglucosidase from sulfatase activity (Figure 4).

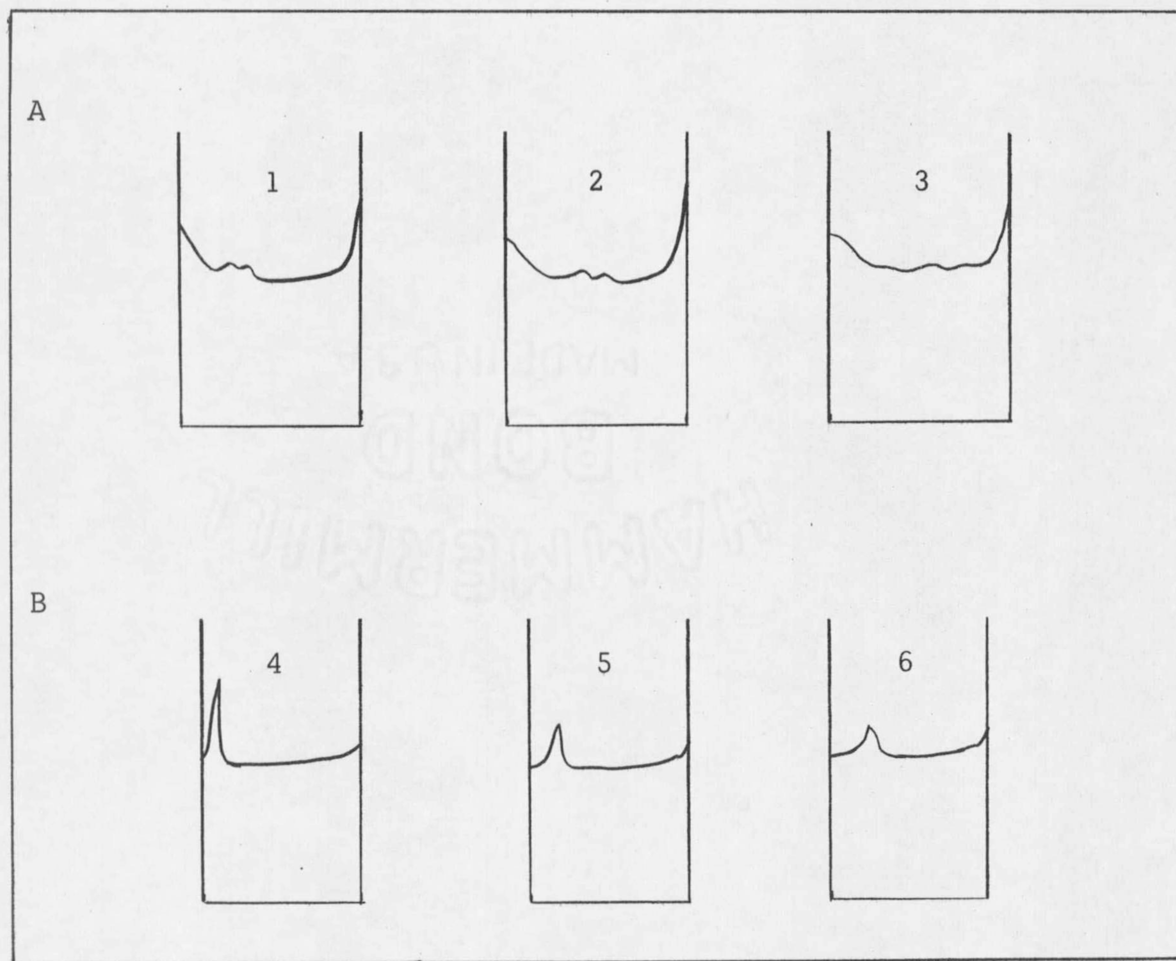


Figure 2. Sedimentation patterns of myrosinase preparations. Centrifuge speed was 59,780 rpm. Time of photographs after attaining speed given in parentheses: 1 (15 min), 2 (23 min), 3 (31 min), 4 (3 min), 5 (11 min), 6 (19 min). A. Experiment conducted at $5^{o}C$ in 0.1 M phosphate buffer, pH 5.7, with CMC-2 concentration of 9 mg/ml. B. Experiment conducted at $3^{o}C$ in 0.05 M phosphate buffer, pH 5.9, with purified myrosinase concentration of 6 mg/ml.

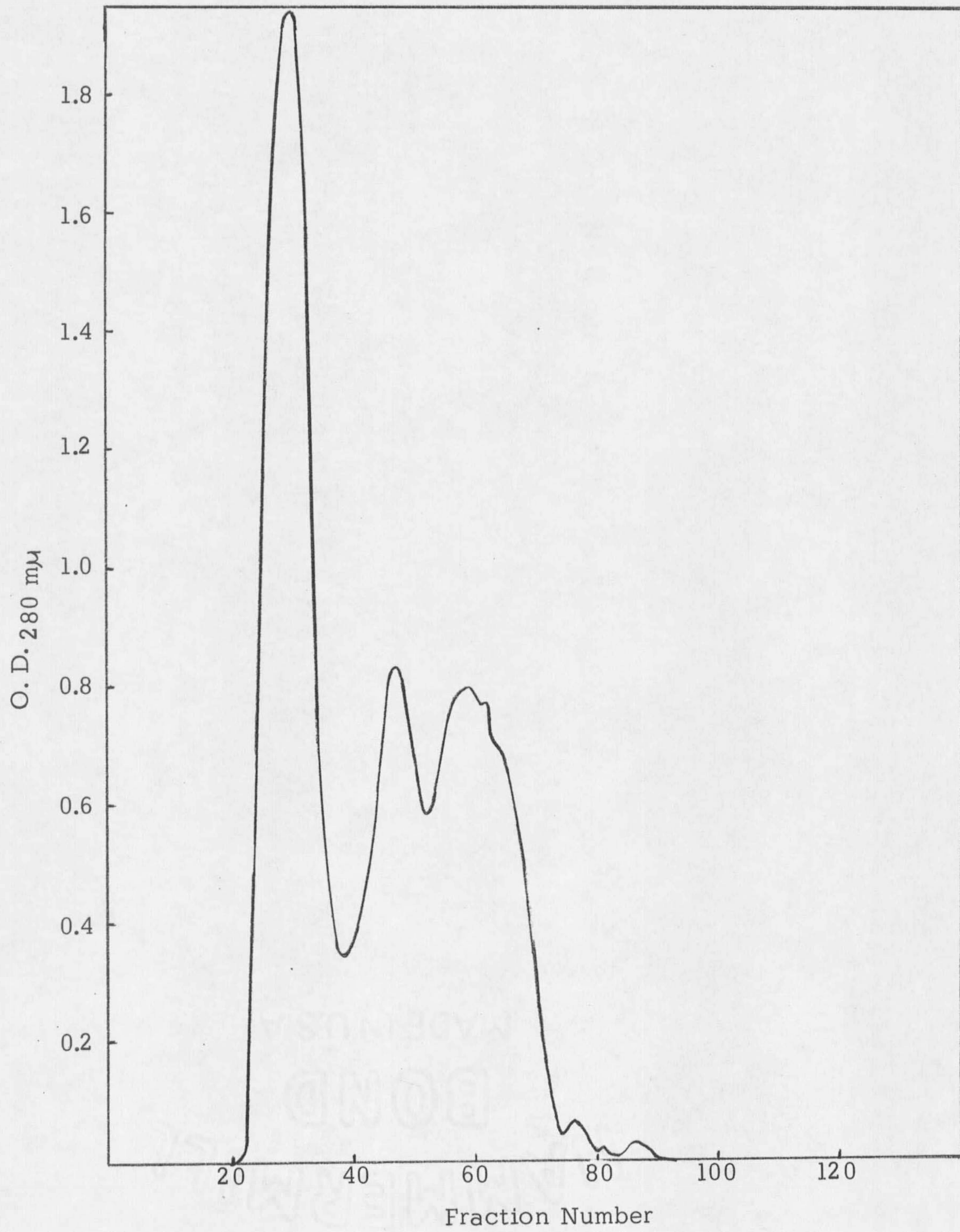


Figure 3. Gel filtration of CMC-2 on Sephadex G-200.

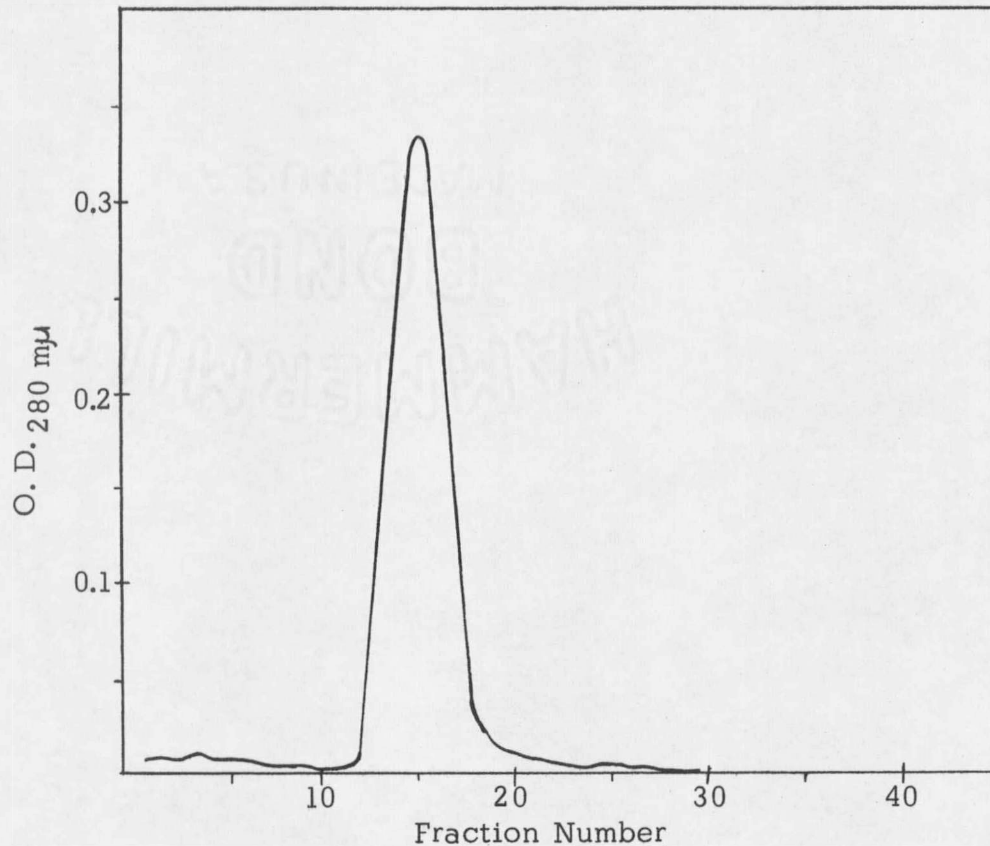


Figure 4. Elution of purified myrosinase from DEAE-cellulose with 0.05 M sodium citrate buffer, pH 6.1.

Disk electrophoresis performed on polyacrylamide gel in 0.10 M TRIS-glycine buffer, pH 8.3, revealed heterogeneity in the enzyme preparation (Figure 5). Each of the five bands appeared to exhibit myrosinase activity. Enzyme activity was ascertained by cutting the bands from a gel using a stained gel as reference. Each band was suspended in 1.0 ml 0.05 M sodium citrate buffer, pH 6.1, which contained 10 mg of sinigrin and incubated at 37°C for 36 hours. Enzyme activity was detected by smelling the incubation vial.

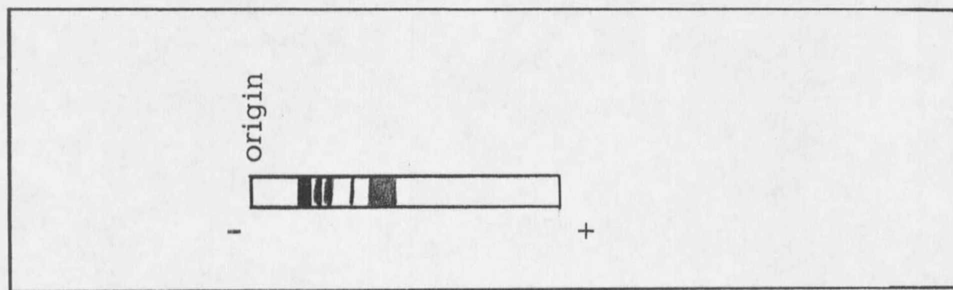


Figure 5. Disk electrophoresis of purified myrosinase on polyacrylamide gel at pH 8.3.

No further purification of the enzyme was attempted. Table I summarizes the purification of myrosinase from a second batch of mustard seed (36 kg) treated in the above manner. The enzyme preparation from gel filtration on Sephadex G-200 was referred to as "purified myrosinase" during this work.

Chemical Composition of Myrosinase

A. Moisture content

Duplicate samples of purified myrosinase were analyzed for moisture content by an indirect weight-loss method. The samples were weighed in a previously weighed vial and placed in an oven at 104°C for 12 hours. Following a one-hour cooling period in a desiccator the samples were weighed and dried an additional six hours. No additional water was lost during the second drying time. Duplicate samples dried for 17 hours at 100°C in a vacuum oven gave comparable results. Moisture content of the enzyme preparation is given in Table II.

Table I. Purification of Brassica juncea Myrosinase

Step	Volume (liters)	Units[a]/ml T[b]	Units[a]/ml S[c]	Total Units T	Total Units S	Protein[d] (mg/ml)	Specific Activity (units/mg protein) T	Specific Activity (units/mg protein) S	Specific Activity Ratio T/S	Yield (%) T	Yield (%) S	Purification T	Purification S
1. Water Extract	149	3.87×10^{-1}	6.12×10^{-1}	57,700	91,200	30.60	1.28×10^{-2}	2.00×10^{-2}	0.64	100	100	1	1
2. 40-80% $(NH_4)_2SO_4$ Precipitation	32.0	1.65	2.42	52,900	77,400	26.40	6.26×10^{-2}	9.18×10^{-2}	0.68	91.6	85.0	4.9	4.6
3. 50% Ethanol Precipitation	4.40	5.42	7.15	23,800	31,400	15.00	3.61×10^{-1}	4.76×10^{-1}	0.76	41.2	34.5	28.0	24.0
4. CM-Cellulose	2.84[e]	2.77	1.95	7,900	5,550	1.56	1.78	1.25	1.42	13.7	6.1	139	62
5. Gel Filtration Sephadex G-200	0.92[e]	5.00	3.62	4,600	3,330	1.57	3.20	2.30	1.40	8.0	3.6	250	115

[a] Unit defined as that amount of enzyme required to hydrolyze 5 mg sinigrin per hour at 37°C. [b] Thioglucosidase activity. Glucose determined by quantitative paper chromatography in Step 1. Dinitrosalicylic acid reagent was used to determine glucose in all other steps. [c] Sulfatase activity. Sulfate determined turbidimetrically as $BaSO_4$. [d] Protein determined by method of Lowry et al. (1951). [e] Value extrapolated from portions of the 50% ethanol precipitate which were further purified.

Table II. Chemical Composition of Myrosinase

	Percent	Number of Methods Used
Protein	55.8	2
Carbohydrate	13.3	2
Water	7.4	2
Citrate (Possible)[a]	8.2	1
Sodium ion (Possible)[b]	6.4	1
Total[c]	91.1	

[a] Estimation based on amounts of arginine and lysine in myrosinase.
[b] Estimation based on amounts of aspartic, glutamic, sialic and citric acid present. [c] Analyses for lipid, P, inositol were all negative.

B. Protein-amino acid content

Total nitrogen (8.92%) in purified myrosinase was determined by the micro-Kjeldahl method at Galbraith Laboratories, Inc., Knoxville, Tennessee. The usual conversion factor (6.25) was used in calculating a percent protein from the total nitrogen content of the enzyme preparation. This result is included in Table II.

Amino acids were analyzed by the method described in "Materials and Methods". Percent protein (55.5%) based on amino acids recovered during this analysis substantiated the results obtained by the micro-Kjeldahl method. Three myrosinase samples were hydrolyzed for 24 hours and one sample for 72 hours prior to analysis. The 72-hour hydrolysis revealed partial destruction of some amino acids. Correlation of results from the

72-hour and 24-hour hydrolyzates allowed extrapolation to zero time of hydrolysis when determining amounts of these labile amino acids. Tryptophan was completely destroyed during hydrolysis and, therefore, was determined independently by the spectrophotometric method of Goodwin and Morton (1946). The amino acid composition of myrosinase as determined in this investigation is given in Table III.

C. Carbohydrate content

Analyses of carbohydrate in the presence of protein have usually been accompanied by many problems arising from chemical interaction between amino acids and carbohydrate during hydrolysis (Gottschalk, 1963). In addition, separation of the resultant monosaccharides from these side products and amino acids has been time-consuming and prone to experimental error. In the present investigation a method (see "Materials and Methods") was used which overcame some of these problems. Hydrolysis of myrosinase with methanolic HCl gave the free monosaccharides as their methyl glycosides and partially degraded protein. Subsequent formation of their O-trimethylsilyl ethers and analysis by gas-liquid chromatography allowed separation, identification and determination of the quantitative ratios of sugars present in the enzyme preparation.

Samples of myrosinase were hydrolyzed for periods of 24, 48 and 72 hours before preparing them for gas-liquid chromatography. Typical chromatograms obtained from these preparations are shown in Figures 6, 7 and 8. Table IV gives values, as determined by gas-liquid chromatography, for the relative percentages of each sugar unit present in the total carbohydrate.

Table III. Amino Acid Composition of Myrosinase[a]

Amino Acid	Amino Acid (g/100g Glycoprotein) 24 hr.	72 hr.	0 hr.[b]	Amino Acyl Residue (g/100g Glycoprotein)	Amino Acid (mol/10,000g Glycoprotein)	Nitrogen (% of Total N)[c]
Aspartic acid	10.16	10.17	10.16	8.79	7.6	8.25
Threonine	3.49	3.94	3.94	3.34	3.3	3.55
Serine	5.09	4.58	5.35	4.44	5.1	5.47
Glutamic acid	15.77	16.99	16.99	14.91	11.5	12.56
Proline	6.68	5.76	7.14	5.41	6.2	6.71
Glycine	5.15	4.98	5.24	3.99	7.0	7.56
Alanine	4.06	3.64	4.26	3.40	4.8	5.17
Valine	4.44	5.32	5.32	4.51	4.5	4.93
Half-cystine	1.00	--	1.00	0.86	0.8	0.92
Methionine	1.73	--	1.73	1.53	1.2	1.23
Isoleucine	3.08	3.96	3.96	3.42	3.0	3.24
Leucine	5.91	6.90	6.90	5.95	5.3	5.70
Tyrosine	3.71	2.85	4.13	3.72	2.3	2.45
Phenylalanine	4.20	4.38	4.38	3.90	2.6	2.85
Ammonia	1.79[d]	1.48[d]	1.94[d]	--	--	--
Lysine	3.64	4.71	4.71	4.13	3.2	6.90
Histidine	2.04	2.08	2.08	1.83	1.3	4.32
Arginine	5.25	5.15	5.35	4.80	3.1	13.26
Tryptophan[e]	--	--	1.83	1.67	0.9	1.93
Totals	85.40	85.41	94.47	80.60	--	97.00

[a] Corrected to include only protein-carbohydrate content of preparation. [b] Results expressed as extrapolations to zero time hydrolysis or at maximal recovery. [c] 12.98% N in preparation. [d] This value omitted from total. [e] Determined by the method of Goodwin and Morton (1946).

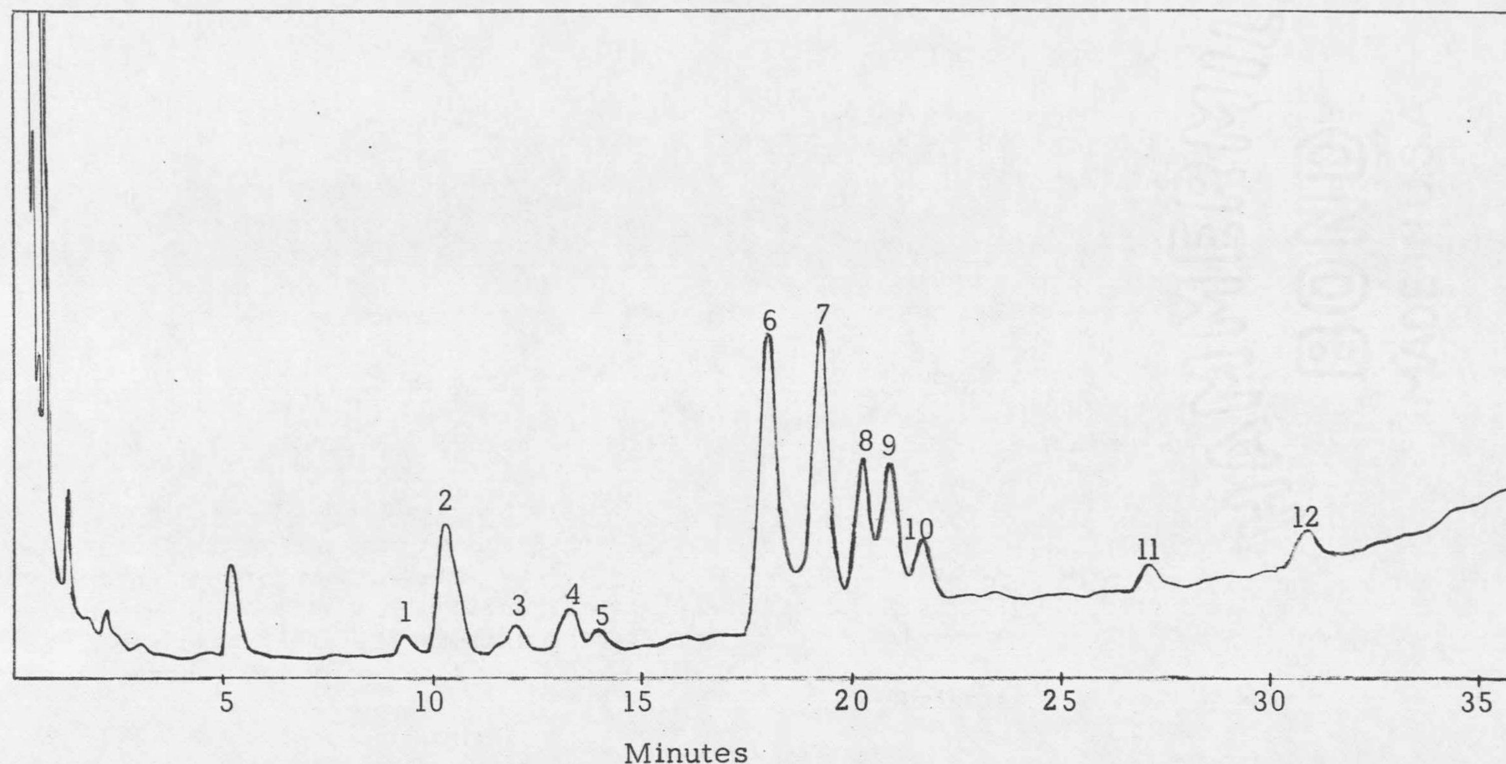


Figure 6. Gas chromatogram of the trimethylsilyl derivatives from the 24-hour myrosinase hydrolyzate. (1) ? Methyl citrate (2) Methyl β-L-arabinoside (3) Methyl β-L-fucoside (4) Methyl α-D-xyloside (5) Methyl β-D-xyloside (6) Methyl α-D-mannoside (7) Methyl α-D-galactoside (8) Methyl β-D-galactoside (9) Methyl α-D-glucoside (10) Methyl β-D-glucoside (11) Methyl 2-acetamido-2-deoxy-D-glucoside (12) N-acetyl-neuraminic acid.

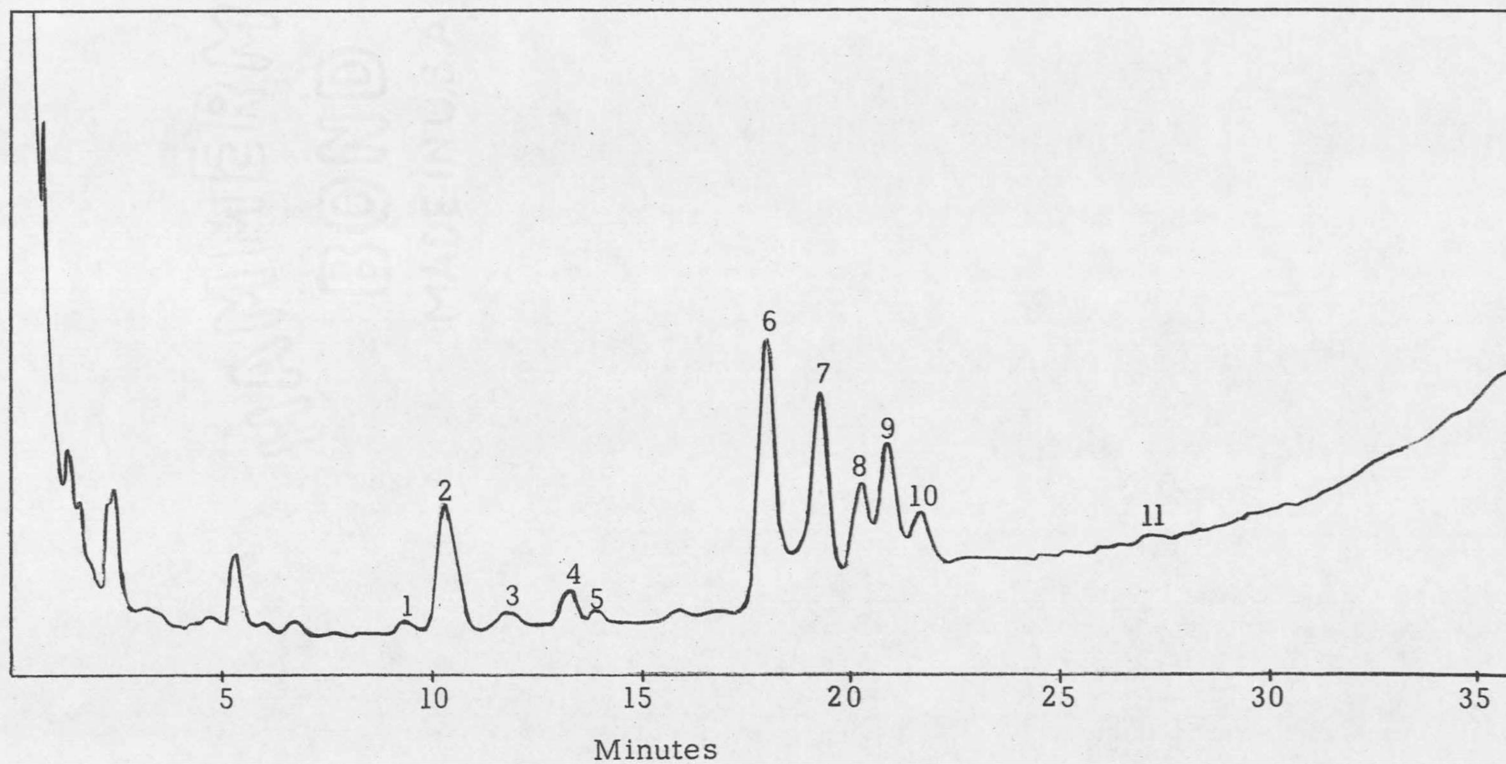


Figure 7. Gas chromatogram of the trimethylsilyl derivatives from the 48-hour myrosinase hydrozate. See Figure 6 for identification of peaks.

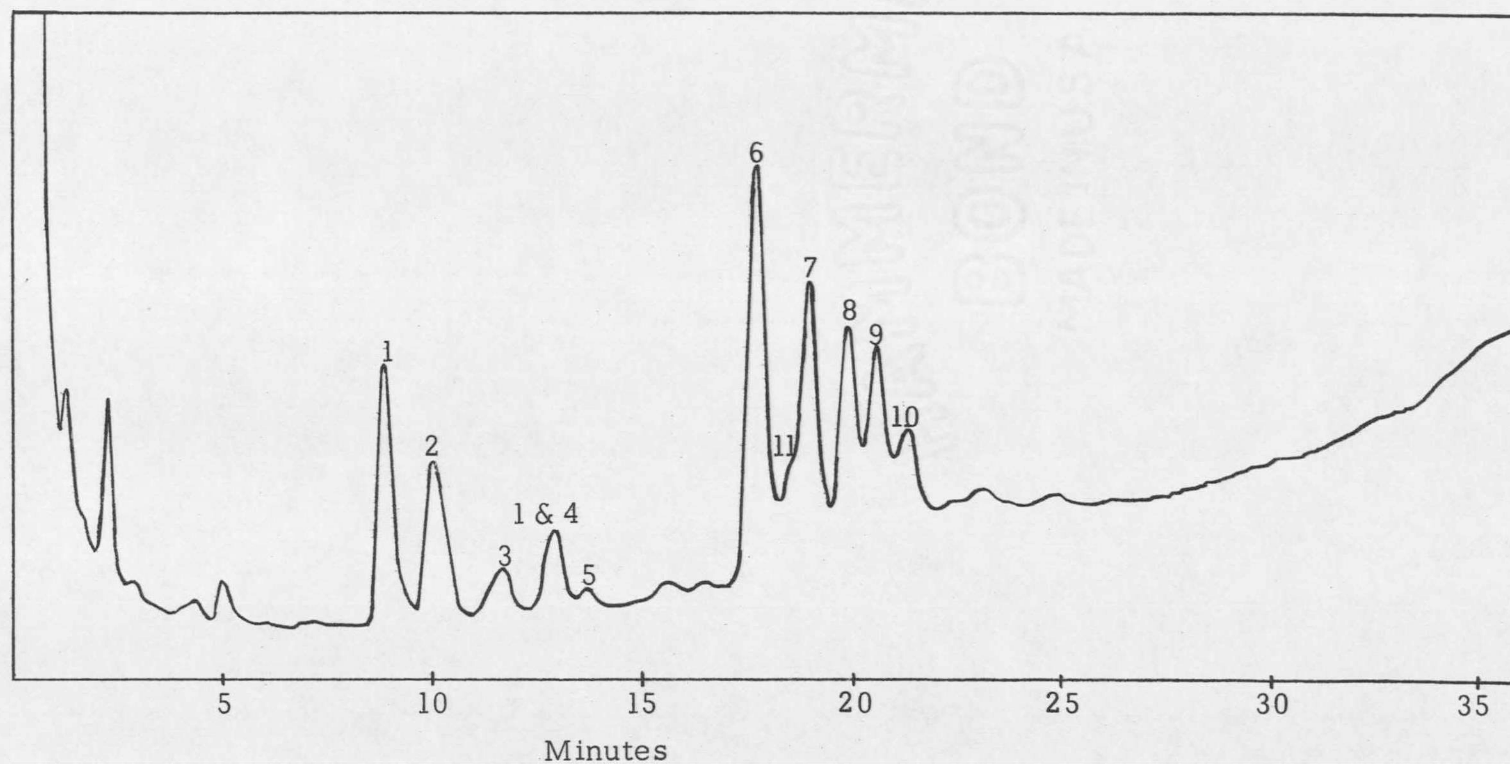


Figure 8. Gas chromatogram of the trimethylsilyl derivatives from the 72-hour myrosinase hydrozate. See Figure 6 for identification of peaks.

Table IV. Composition of the Carbohydrate Associated with Myrosinase.

Sugar	Hydrolyzed Sugar [a] (g/100g Carbohydrate) 24 hr.	48 hr.	72 hr.	Average Sugar Content (g/100g Carbohydrate)	Alternate Qualitative Analysis [b]
L-arabinose	15.2	17.6	16.2	16.3	+
L-fucose	2.6	2.4	2.2	2.4	+
D-xylose	5.9	4.8	(10.3)[c]	5.5	(+)
D-mannose	(26.8)[c]	31.9	33.0	32.5	+
D-galactose	(31.5)[c]	28.2	25.9	27.0	+
D-glucose	10.9	13.9	11.7	12.2	+
D-glucosamine [d]	2.7	1.1	0.9	1.6	+
Sialic acid [e]	4.9	--	--	4.9	-
Totals	100.5	99.9	100.2	102.4	--

[a] Obtained by gas-liquid chromatography of residues from myrosinase as described in text. [b] Obtained by paper chromatography of CMC-2 hydrolyzate as described in text; + = identified, (+) = tentative identity, - = not detected. [c] Values in parentheses not used in calculating average sugar content. [d] Analyzed as the N-acetyl derivative. [e] N-acetyl-neuraminic acid.

CM-cellulose and Sephadex G-200 might have been degraded in some manner while being used in the purification of myrosinase and, therefore, might have contaminated the enzyme preparation with carbohydrate. In order to check this possibility, a blank was prepared by subjecting a sample which did not contain protein to the procedures which were used to purify crude myrosinase. Analysis of this blank by gas-liquid chromatography showed only minor amounts of D-glucose to be present.

A standardization curve for the phenol-sulfuric acid method for total carbohydrate analysis was prepared by using a composite standard composed

of the sugars (minus sialic acid) in their weight ratios after 24-hour hydrolysis as given in Table IV. Percent carbohydrate in myrosinase was determined in triplicate and is recorded in Table II. Estimates (approximately 11.5%) from gas chromatograms substantiated these results.

Data from gas-liquid chromatography supported earlier qualitative evidence gained by acid hydrolysis of CMC-2, separation by means of ion-exchange resins (Simkin et al., 1964) and subsequent analysis of the carbohydrate residues by paper chromatography and paper electrophoresis. One hundred milligrams of CMC-2 were dissolved in 15 ml 0.2 N HCl containing 0.95g Dowex 50W x 8 (H+) ion-exchange resin and refluxed for 24 hours. The mixture was quantitatively filtered through a sintered glass funnel unto a Dowex 50W x 12 (H+) column (18 x 1.6 cm) which dripped unto a Dowex 2 x 8 ($HCOO^-$) column (24 x 1.6 cm). After five 5-ml washes the funnel was removed and the two-column system eluted with distilled water. The first 30 ml were discarded and the next 120 ml collected. This neutral sugar fraction was evaporated in a rotary evaporator, transferred in 6-7 ml of aqueous solution to a small vial and dried under a stream of nitrogen gas. The two ion-exchange columns were disconnected and mounted for individual elution. Hexosamine and some amino acids were eluted from the Dowex 50W x 12 with 2 N HCl. The first 20 ml of eluant were discarded and the next 120 ml were retained and concentrated in the same manner as the neutral fraction. This was the hexosamine fraction. The Dowex 2 x 8 column was eluted with 1.2N formic acid to remove any uronic or sialic acids that may have been retained from the original hydrolyzate. After discarding the first 20 ml of eluant, the next 100 ml constituted the uronic acid fraction

and were concentrated in the usual manner. Methods which have been previously described were used to analyze each of these three fractions. Neutral sugar and uronic acid fractions were analyzed by paper chromatography and the hexosamine fraction was subjected to paper electrophoresis. All migrating spots were identified and the results are included in Table IV.

D. Miscellaneous analyses

Protein, carbohydrate and moisture accounted for about 76% of the total weight of freeze-dried purified myrosinase. To account for the remaining 24% some additional analyses were attempted.

Lipid material frequently accompanies protein during protein isolation. Since the mustard seed had a high oil content (approximately 30%), such material could likely be present in the enzyme preparation. A sample of purified myrosinase (1.87 mg) was hydrolyzed with methanolic HCl in the manner used during preparation for gas-liquid chromatography of carbohydrates. After hydrolysis the dried residue was dissolved in 0.05 ml chloroform-methanol solvent (2:1, v/v). A 3-μl sample and a 1-μl sample of this solution were spotted on a thin-layer chromatography plate coated with Absorbosil-3 containing 0.02% Rhodamine 6-G. Samples of stearic acid and various standard fatty acid esters were spotted on the same plate. The plate was developed for 30 minutes in a hexane-diethyl ether (7:3, v/v) solvent. Detection of migrating species was made under ultraviolet light and also by exposing the plates to iodine vapor. The solvent caused extensive migration of the fatty acid esters and a slight migration of the stearic acid. No migrating components emerged from the myrosinase hydrolyzate. To confirm these results a modification of the lipid extraction procedure of Folch

et al. (1957) was then attempted. A suspension of myrosinase (6.75 mg) in 4.5 ml chloroform-methanol solvent (2:1, v/v) was agitated at intervals for 2 hours at $5^{o}C$. After centrifugation at slow speed, the supernatant solution was decanted into a weighed vial and dried in a stream of nitrogen gas. A fresh portion of solvent was added to the precipitate and the procedure repeated 3 times. Each of the 3 supernatant solutions was combined with the residue of previous supernatant solutions. After drying 8 hours in a vacuum desiccator, no residue was detected by weighing. Therefore, lipid was ruled out as a major component in the myrosinase preparation.

Inositol is also a common component of plant seeds and is usually present as its hexaphosphate ester (Pigman, 1957). Gas-liquid chromatography ruled out the presence of inositol, and phosphorus was shown to be absent by a modified method of Fiske and Subbarow (1925).

Citrate was present in the myrosinase preparation (Figure 8); however, estimations from the gas chromatogram suggested that it would make up only about 1.5% of the total preparation. There was a possibility that the citrate peak obtained represented a more volatile citrate derivative and that additional citrate may have been retained by the column. Prior to freeze-drying the myrosinase solution was dialyzed against distilled water to remove citrate ions from the pH 6.1 solution. At this pH lysine ($pKa_3 = 10.5$) and arginine ($pKa_3 = 12.5$) would possess positively charged groups ($-NH_3^+$) which could serve to hold ions such as citrate. With this in mind, a calculation based on lysine and arginine content in the myrosinase preparation, was performed to see how much citrate could possibly have been bound by these groups in a 1:1 molar association of $-NH_3^+$ and citrate. This

calculation suggested that 8.2% of the enzyme preparation could have been citrate.

The high aspartic and glutamic acid content of myrosinase offered many free carboxylate groups capable of binding metal ions. In addition, sialic and citric acid residues which were present would bind cations. Sodium ion was the most prevalent metal ion encountered by the enzyme during purification. Calculations were made to see what percent of the myrosinase preparation could be due to the presence of sodium. These calculations assumed that sodium was bound by each free carboxylate group of aspartic, glutamic and sialic acids known to be present and of citric acid which was probably present. Table II includes these results.

Molecular Weight of Myrosinase

Separation of molecules by gel filtration is believed to result from differences in size of the various molecules and their restricted diffusion into the gel pores. Molecules appear in the effluent in order of decreasing size. Although molecular size is not necessarily a good approximation for molecular weight, molecular size and weight are closely related for a series of similar macromolecules; e.g., a series of proteins or polysaccharides (Granath and Flodin, 1961). As a result gel filtration has been accepted as a comparative method for estimating molecular weight (Andrews, 1964; Pristoupil, 1965; Iwatsubo and Curdel, 1963; Whitaker, 1963; Wieland et al.,1963). In this method a working curve was prepared by plotting relative migration rates of standard compounds through a gel filtration medium versus their

molecular weights. Unknown molecular weights were then estimated on the basis of their relative migration rates.

A 41 x 2.5 cm Bio-Gel P-300 column was prepared using 0.05 M sodium citrate buffer, pH 6.1, as the eluting agent. Three-milliliter solutions of the following materials in citrate buffer served as standards: Blue Dextran 2000 (Pharmacia Fine Chemicals, Inc.), beta-amylase, beef liver catalase, glucose oxidase, yeast hexokinase and muscle phosphorylase (all from Nutritional Biochemicals Corporation). These solutions contained approximately 10 mg of solute, except in the case of phosphorylase which proved quite insoluble in citrate buffer. These standard solutions were placed on the Bio-Gel column and eluted in 2-ml aliquots at a rate of 8 ml/hr. Five milligrams of myrosinase dissolved in 3 ml of citrate buffer were treated in a similar manner.

Blue Dextran 2000 (approximate molecular weight of 2×10^6) was not retarded by Bio-Gel P-300 and was a convenient reference standard. The rate of protein migration through the column was determined relative to Blue Dextran 2000 and expressed as R_{BD} where:

$$R_{BD} = \frac{\text{Elution volume of Blue Dextran 2000}}{\text{Elution volume of protein}}$$

The elution volume was defined as that volume of eluting agent required to reach the midpoint of the eluted peak.

Figure 9 shows the R_{BD} versus molecular weight calibration curve used in this work. A second column of Bio-Gel P-300 (38 x 1.6 cm) using 0.5-ml samples in the same buffer as before and eluted in 5-ml aliquots gave essentially the same calibration curve.

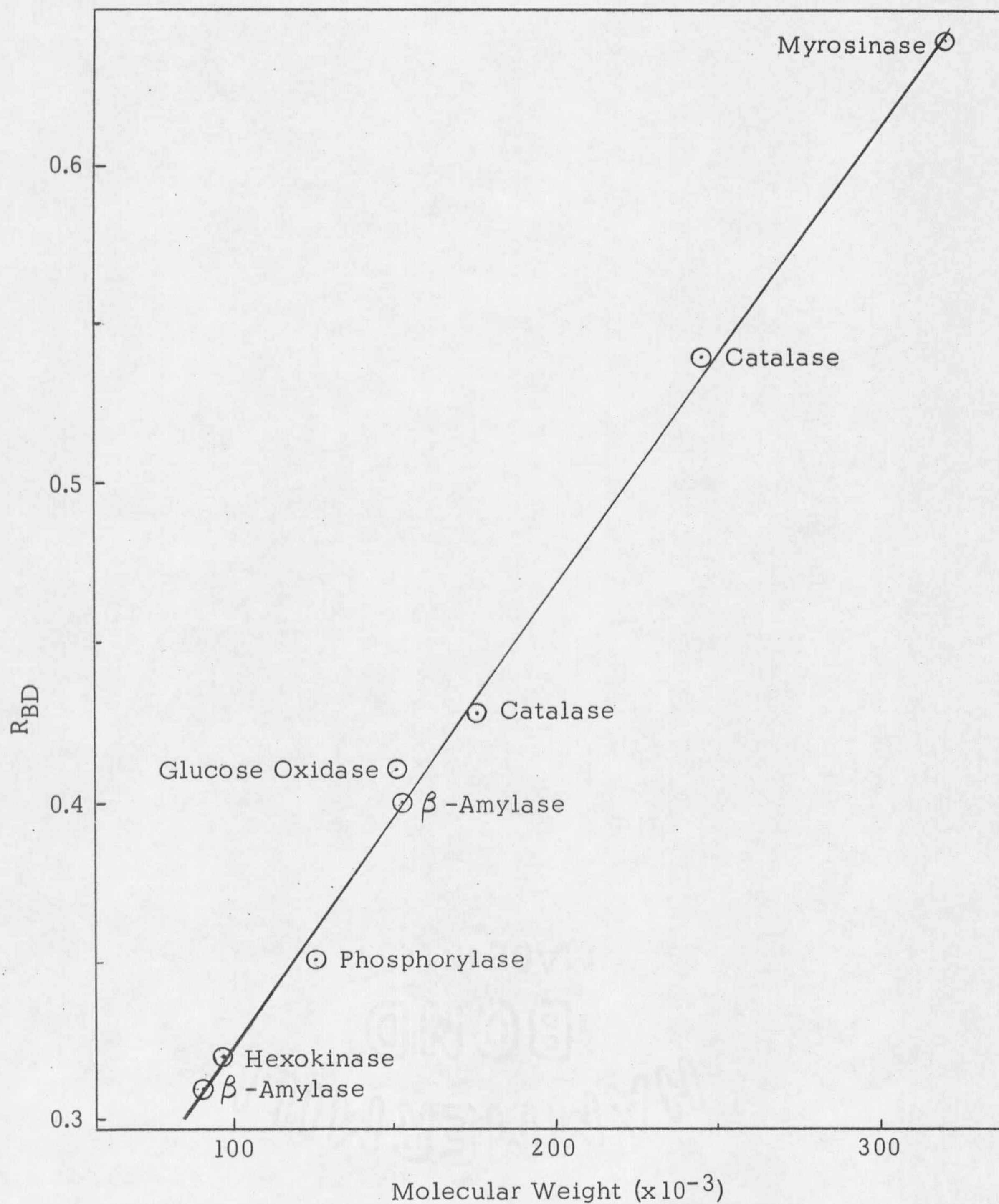


Figure 9. R_{BD} vs. molecular weight for Bio-Gel P-300 (41 x 2.5 cm) equilibrated with 0.05 M citrate buffer, pH 6.1.

A molecular weight of 320,000 ± 15,000 was estimated for myrosinase by this method.

Pronase Digestion of Myrosinase

Isolation of a fragment from myrosinase that contained carbohydrate linked to a short peptide chain would further substantiate the covalent linkage of carbohydrate to protein. Speculation as to what amino acid was involved in the linkage would also be possible. In addition, the number of different carbohydrate moieties present and their approximate size could be determined. A low-specificity protease preparation from *Streptomyces griseus* (pronase) has found use in degradation of glycoproteins to glycopeptides with the carbohydrate moieties apparently intact (Tsugita and Akabori, 1959; Nomoto *et al*., 1960; Eylar, 1962; Kamiyama and Schmid, 1962; Fletcher *et al*., 1963; Jamieson, 1965; Montgomery *et al*., 1965; Pazur *et al*., 1965).

Purified myrosinase (135 mg) was suspended in 3 ml of 0.05 M sodium borate buffer, pH 8.0, 0.015 M in $CaCl_2$ and warmed to 40°C. One-tenth milliliter of 2% pronase (Protease type VI, Sigma Chemical Company) in the same buffer was warmed to 40°C, added to the myrosinase suspension and incubated at 40°C for 24 hours. Another 0.1 ml of pronase solution was added and the incubation resumed for 137 hours. The incubation mixture was cleared by centrifugation and 1 ml of sodium borate buffer was used to wash the residue. This solution was combined with the original supernatant solution.

The combined supernatant solutions from the pronase digestions (4 ml) were applied to a Sephadex G-25 column (44 x 1.4 cm) equilibrated with 0.1 N acetic acid and eluted with the same solvent in 4-ml aliquots at a rate of 8 ml per hour. In like manner, a blank solution containing 0.2 ml of the pronase solution diluted to 4 ml with sodium borate buffer was applied to the same Sephadex G-25 column and eluted with 0.1 N acetic acid. Figures 10 and 11 show these elution patterns. The carbohydrate-containing peaks from the pronase-myrosinase digestion were designated PM-25-1 and PM-25-2 and were recovered by freeze-drying fractions 6-9 and 10-15, respectively. In the pronase blank elution, the same fractions were designated P-25-1 and P-25-2, respectively. (This separation and subsequent separations which are described below are schematized in Figure 17.)

Further fractionation of the carbohydrate present in PM-25-1, PM-25-2, P-25-1 and P-25-2 was accomplished by suspending each in 0.1 N acetic acid, centrifuging to remove minor amounts of insolubles and subjecting the supernatant solutions to gel filtration on Sephadex G-75 (60 x 1.6 cm). PM-25-1 and P-25-1 were applied as 2-ml samples (including a 1-ml 0.1 N acetic acid residue wash in each case) and collected in 3-ml aliquots. PM-25-2 and P-25-2 were applied as 3-ml samples (including 0.1 N acetic acid wash portions) and collected in 3-ml aliquots. The column flow rate in every case was 11 ml per hour. Figures 12-15 show these results. It was apparent that only partial separation of the carbohydrate fractions was achieved by the previous gel filtration through Sephadex G-25. Carbohydrate-containing peaks from both PM-25-1 and PM-25-2 were

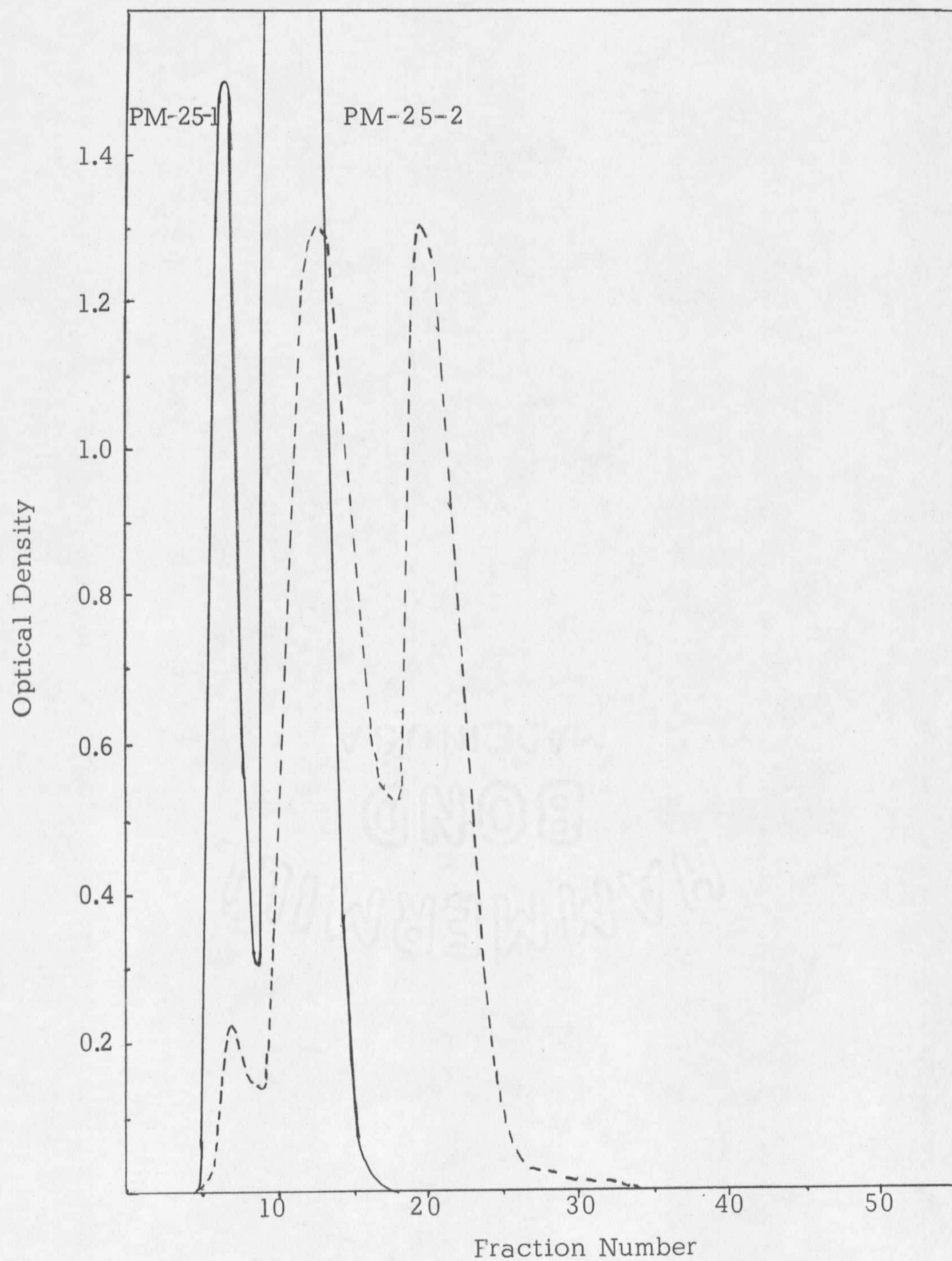


Figure 10. Gel filtration of the pronase-myrosinase digest on Sephadex G-25. —— Total carbohydrate; --- O. D. 280 mμ.

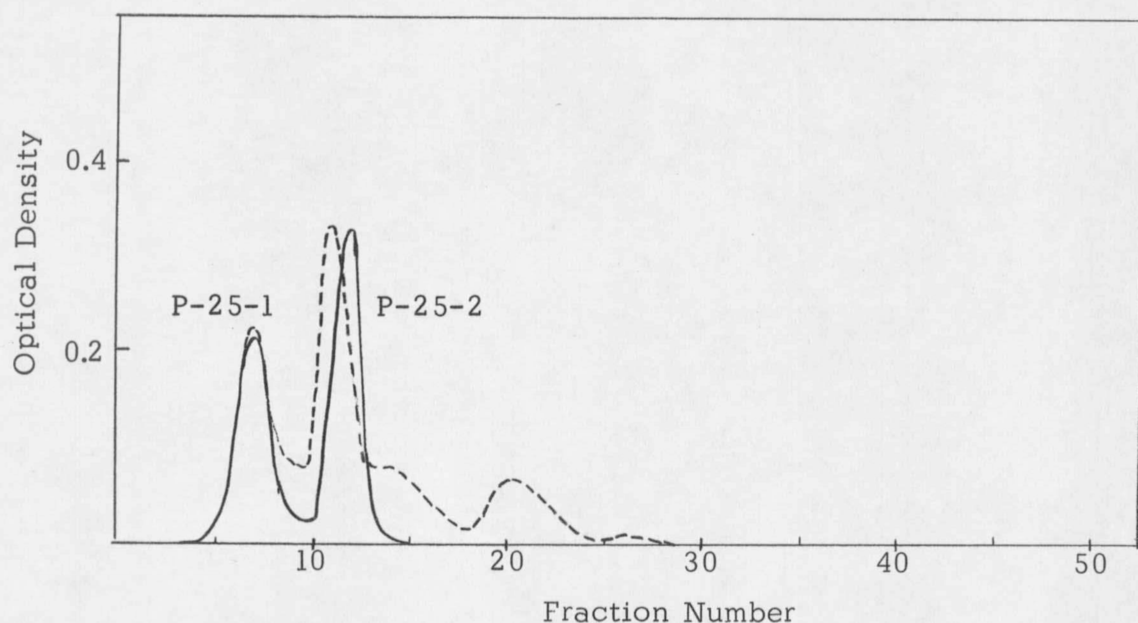


Figure 11. Gel filtration of the pronase blank on Sephadex G-25.
—— Total carbohydrate; ---- O. D.$_{280\ m\mu}$.

designated PM-75-1, PM-75-2 and PM-75-3. They were recovered by freeze-drying fractions 11-13, 32-36 and 38-42, respectively.

PM-75-1 was taken without further attempts at purification and analyzed for amino acid and monosaccharide content. A portion of PM-75-1 which was dissolved in 0.1 N acetic acid was eluted from Sephadex G-100 (40 x 2.5 cm) as a single, slightly-retarded peak. Portions of PM-75-2 and PM-75-3 were dissolved separately in 1 ml 0.1 N acetic acid and purified by gel filtration on Sephadex G-15 (48 x 1.6 cm). The effluent was collected in 2-ml aliquots at a rate of 8 ml per hour. These results are shown in Figure 16. The carbohydrate was recovered by freeze-drying and designated as PM-15-1 (fractions 17-23 from PM-75-2) and as PM-15-2 (fractions 30-34 from PM-75-3).