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
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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 thioglicosidase and sulfatase activity when incubated with sinigrin solution at 3°C; however, separation of the two enzymes was not accomplished. A 250-fold purification of thioglicosidase 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.
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
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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 moieties 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).

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 Robert, 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-denaturating 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 $R = \text{CH}_2\text{CH-CH}_2\text{-}$. 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 thiogalactosidase 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 carbohydate (Gaines, 1960). Many glycosidases are associated with polysaccharides which seem to stabilize these systems. If for some reason this carbohydate 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 carbohydate in the thiogalactosidase from yellow Oriental mustard seed. This carbohydate appeared to be present as an oligosaccharide which was free of sialic acid and accounted for one percent of the thiogalactosidase preparation. In the same work, galactose, arabinose, hexosamine and uronic acid were reported to be present in the carbohydate moiety.

Proteins containing tightly-bound carbohydate 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 optical density measurements at 280 m全媒体 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 m\(\mu\) 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 temperature.

**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 Sepraphore 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 50 × 12 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 placed 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°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 CaCl₂ 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 (NH₄)₂SO₄ 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°C in 0.1 M phosphate buffer, pH 5.7, with CMC-2 concentration of 9 mg/ml. B. Experiment conducted at 3°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.
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>
<thead>
<tr>
<th>Step</th>
<th>Volume (liters)</th>
<th>Units$^a$/ml</th>
<th>Total Units</th>
<th>Protein$^d$ (mg/ml)</th>
<th>Specific Activity (units/mg protein)</th>
<th>Specific Activity Ratio T/S</th>
<th>Yield (%)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Water Extract</td>
<td>149</td>
<td>$3.87 \times 10^{-1}$</td>
<td>57,700</td>
<td>30.60</td>
<td>$1.28 \times 10^{-2}$</td>
<td>0.64</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. 40–80% (NH$_4$)$_2$SO$_4$ Precipitation</td>
<td>32.0</td>
<td>2.42</td>
<td>52,900</td>
<td>26.40</td>
<td>$6.26 \times 10^{-2}$</td>
<td>0.68</td>
<td>91.6</td>
<td>49.0</td>
</tr>
<tr>
<td>3. 50% Ethanol Precipitation</td>
<td>4.40</td>
<td>7.15</td>
<td>23,800</td>
<td>15.00</td>
<td>$3.61 \times 10^{-1}$</td>
<td>0.76</td>
<td>41.2</td>
<td>28.0</td>
</tr>
<tr>
<td>4. CM-Cellulose</td>
<td>2.84$^e$</td>
<td>1.95</td>
<td>7,900</td>
<td>1.56</td>
<td>1.78</td>
<td>1.42</td>
<td>13.7</td>
<td>62.0</td>
</tr>
<tr>
<td>5. Gel Filtration Sephadex G-200</td>
<td>0.92$^e$</td>
<td>3.62</td>
<td>4,600</td>
<td>1.57</td>
<td>3.20</td>
<td>1.40</td>
<td>8.0</td>
<td>115.0</td>
</tr>
</tbody>
</table>

$^a$ 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

<table>
<thead>
<tr>
<th></th>
<th>Percent</th>
<th>Number of Methods Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>55.8</td>
<td>2</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>13.3</td>
<td>2</td>
</tr>
<tr>
<td>Water</td>
<td>7.4</td>
<td>2</td>
</tr>
<tr>
<td>Citrate (Possible)(^a)</td>
<td>8.2</td>
<td>1</td>
</tr>
<tr>
<td>Sodium ion (Possible)(^b)</td>
<td>6.4</td>
<td>1</td>
</tr>
<tr>
<td>Total(^c)</td>
<td>91.1</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) 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

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>24 hr.</th>
<th>72 hr.</th>
<th>0 hr.</th>
<th>Amino Acyl Residue (g/100g Glycoprotein)</th>
<th>Amino Acid (mol/10,000g Glycoprotein)</th>
<th>Nitrogen (% of Total N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>10.16</td>
<td>10.17</td>
<td>10.16</td>
<td>8.79</td>
<td>7.6</td>
<td>8.25</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.49</td>
<td>3.94</td>
<td>3.94</td>
<td>3.34</td>
<td>3.3</td>
<td>3.55</td>
</tr>
<tr>
<td>Serine</td>
<td>5.09</td>
<td>4.58</td>
<td>5.35</td>
<td>4.44</td>
<td>5.1</td>
<td>5.47</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>15.77</td>
<td>16.99</td>
<td>16.99</td>
<td>14.91</td>
<td>11.5</td>
<td>12.56</td>
</tr>
<tr>
<td>Proline</td>
<td>6.68</td>
<td>5.76</td>
<td>7.14</td>
<td>5.41</td>
<td>6.2</td>
<td>6.71</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.15</td>
<td>4.98</td>
<td>5.24</td>
<td>3.99</td>
<td>7.0</td>
<td>7.56</td>
</tr>
<tr>
<td>Alanine</td>
<td>4.06</td>
<td>3.64</td>
<td>4.26</td>
<td>3.40</td>
<td>4.8</td>
<td>5.17</td>
</tr>
<tr>
<td>Valine</td>
<td>4.44</td>
<td>5.32</td>
<td>5.32</td>
<td>4.51</td>
<td>4.5</td>
<td>4.93</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>1.00</td>
<td>--</td>
<td>1.00</td>
<td>0.86</td>
<td>0.8</td>
<td>0.92</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.73</td>
<td>--</td>
<td>1.73</td>
<td>1.53</td>
<td>1.2</td>
<td>1.23</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.08</td>
<td>3.96</td>
<td>3.96</td>
<td>3.42</td>
<td>3.0</td>
<td>3.24</td>
</tr>
<tr>
<td>Leucine</td>
<td>5.91</td>
<td>6.90</td>
<td>6.90</td>
<td>5.95</td>
<td>5.3</td>
<td>5.70</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.71</td>
<td>2.85</td>
<td>4.13</td>
<td>3.72</td>
<td>2.3</td>
<td>2.45</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.20</td>
<td>4.38</td>
<td>4.38</td>
<td>3.90</td>
<td>2.6</td>
<td>2.85</td>
</tr>
<tr>
<td>Ammonia</td>
<td>1.79d</td>
<td>1.48d</td>
<td>1.94d</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Lysine</td>
<td>3.64</td>
<td>4.71</td>
<td>4.71</td>
<td>4.13</td>
<td>3.2</td>
<td>6.90</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.04</td>
<td>2.08</td>
<td>2.08</td>
<td>1.83</td>
<td>1.3</td>
<td>4.32</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.25</td>
<td>5.15</td>
<td>5.35</td>
<td>4.80</td>
<td>3.1</td>
<td>13.26</td>
</tr>
<tr>
<td>Tryptophane</td>
<td>--</td>
<td>--</td>
<td>1.83</td>
<td>1.67</td>
<td>0.9</td>
<td>1.93</td>
</tr>
<tr>
<td>Totals</td>
<td>85.16</td>
<td>85.41</td>
<td>94.47</td>
<td>80.60</td>
<td>--</td>
<td>97.00</td>
</tr>
</tbody>
</table>

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 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 hydrolyzate. See Figure 6 for identification of peaks.
Table IV. Composition of the Carbohydrate Associated with Myrosinase.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Hydrolyzed Sugar $^a$ (g/100g Carbohydrate)</th>
<th>Average Sugar Content (g/100g Carbohydrate)</th>
<th>Alternate Qualitative Analysis $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hr.</td>
<td>48 hr.</td>
<td>72 hr.</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>15.2</td>
<td>17.6</td>
<td>16.2</td>
</tr>
<tr>
<td>L-fucose</td>
<td>2.6</td>
<td>2.4</td>
<td>2.2</td>
</tr>
<tr>
<td>D-xylose</td>
<td>5.9</td>
<td>4.8</td>
<td>(10.3)$^c$</td>
</tr>
<tr>
<td>D-mannose</td>
<td>(26.8)$^c$</td>
<td>31.9</td>
<td>33.0</td>
</tr>
<tr>
<td>D-galactose</td>
<td>(31.5)$^c$</td>
<td>28.2</td>
<td>25.9</td>
</tr>
<tr>
<td>D-glucose</td>
<td>10.9</td>
<td>13.9</td>
<td>11.7</td>
</tr>
<tr>
<td>D-glucosamine</td>
<td>2.7</td>
<td>1.1</td>
<td>0.9</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>4.9</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Totals</td>
<td>100.5</td>
<td>99.9</td>
<td>100.2</td>
</tr>
</tbody>
</table>

$^a$ 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°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 (pKₐ₃ = 10.5) and arginine (pKₐ₃ = 12.5) would possess positively charged groups (–NH₃⁺) 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 –NH₃⁺ 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 $2 \times 10^6$) 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 CaCl₂ 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-amyrosinase 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 μM.
Figure 11. Gel filtration of the pronase blank on Sephadex G-25.
--- Total carbohydrate; ---- O. D. 280 μM.

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).
Figure 12. Gel filtration of PM-25-1 on Sephadex G-75. — Total carbohydrate; --- O. D. 280 mμ.
Figure 13. Gel filtration of P-25-1 on Sephadex G-75. — — Total carbohydrate; --- O. D. 280 μm.

Figure 14. Gel filtration of P-25-2 on Sephadex G-75. — — Total carbohydrate; --- O. D. 280 μm.
Figure 15. Gel filtration of PM-25-2 on Sephadex G-75. — Total carbohydrate; --- O.D. $280\, \text{m}\mu$. 
Figure 16. Gel filtration of PM-75-2 (A) and PM-75-3 (B) on Sephadex G-15. ----- Total carbohydrate; --- O. D. 280 m."
Figure 17. Scheme for fractionation by gel filtration of the carbohydrate components in the pronase-myrosinase digest or in the pronase blank.
PM-75-1, PM-15-1 and PM-15-2 were analyzed for carbohydrate content by gas-liquid chromatography (Figures 18-20). These results are tabulated in Table V. Table VI gives the results of an amino acid analysis of the results of an amino acid analysis of PM-75-1 and PM-15-1 following a 24-hour hydrolysis. The Sephadex G-15 column was standardized by following the elution of mono-, di- and trisaccharides. This information suggested that carbohydrate in PM-15-2 consists of monosaccharides. Paper chromatography showed glucose to be the only CD-1-positive component.

<table>
<thead>
<tr>
<th>Table V. Carbohydrate Composition of the Pronase-Myrosinase Digest Fractions.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>L-Arabinose</td>
</tr>
<tr>
<td>L-Fucose</td>
</tr>
<tr>
<td>D-Xylose</td>
</tr>
<tr>
<td>D-Mannose</td>
</tr>
<tr>
<td>D-Galactose</td>
</tr>
<tr>
<td>D-Glucose</td>
</tr>
<tr>
<td>D-Glucosamine&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sialic acid&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>L-Ascorbic acid</td>
</tr>
<tr>
<td>Totals</td>
</tr>
</tbody>
</table>

<sup>a</sup> Obtained by gas-liquid chromatography as described in text.  
<sup>b</sup> Preparation contained 46.5% amino acid.  
<sup>c</sup> Preparation contained 15.2% amino acid.  
<sup>d</sup> Analyzed as the N-acetyl derivative.  
<sup>e</sup> N-acetyl-neuraminic acid.
Figure 18. Gas chromatogram of the trimethylsilyl derivatives from the 24-hour PM-75-1 hydrolysate. (1) Methyl α-L-arabinoside; (2) Methyl α-D-xyloside; (3) Methyl β-D-xyloside; (4) Methyl α-D-galactoside; (5) Methyl β-D-galactoside; (6) Methyl α-D-glucoside; (7) Methyl β-D-glucoside.
Figure 19. Gas chromatogram of the trimethylsilyl derivatives from the 24-hour PM-15-1 hydrolyzate. (1) Methyl L-fucoside; (2) Methyl α-D-xyloside; (3) Methyl β-D-xyloside; (4) Methyl α-D-mannoside; (5) Methyl 2-acetamido-2-deoxy-α-D-glucoside; (6) Methyl α-D-glucoside; (7) Methyl β-D-glucoside; (8) N-acetyl-neuraminic acid.
Figure 20. Gas chromatogram of the trimethylsilyl derivatives from the 24-hour PM-15-2 hydrolysate. (1) Possibly methyl 2-acetamido-2-deoxy-α-D-glucoside; (2) Methyl α-D-glucoside; (3) Methyl β-D-glucoside; (4) Possibly L-ascorbic acid; others not identified or suggested.
Table VI. Amino Acid Composition of the Pronase-Myrosinase Digest Fractions.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Amino Acyl Residue (mg/100 mg sample)</th>
<th>Amino Acids (μmoles/100 mg sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PM-75-1</td>
<td>PM-15-1</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>6.6</td>
<td>2.6</td>
</tr>
<tr>
<td>Threonine</td>
<td>2.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Serine</td>
<td>2.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>6.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Proline</td>
<td>1.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Valine</td>
<td>2.5</td>
<td>0.9</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Leucine</td>
<td>4.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.8</td>
<td>0.2</td>
</tr>
<tr>
<td>Ornithine</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.0</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td><strong>46.5</strong></td>
<td><strong>15.2</strong></td>
</tr>
<tr>
<td>Glucosamine</td>
<td></td>
<td>2.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Based on single samples hydrolyzed for 24 hours.

The discrepancy in the amounts of some sugars (particularly L-arabinose and D-mannose), which were present in myrosinase before and after pronase digestion, suggested that the pronase may have contained sugar-modifying enzymes, such as epimerases, oxidases, and decarboxylases. Pronase was checked for the presence of such enzymes by incubating it with individual solutions (6-10 mg/ml) of D-glucose, D-mannose, D-galactose, D-glucosamine, N-acetyl-neuraminic acid and D-galacturonic.
acid for 48 hours under the conditions used in previous pronase incubations. Analyses using paper chromatography showed no modification of any of these sugars by pronase.

Dissociation of Myrosinase

Myrosinase isolated during this investigation appeared to exist as a large molecular aggregate. This prompted an attempt to dissociate this aggregate into subunits. Such an experiment, if successful, might liberate adsorbed material (particularly carbohydrate in this case) by destroying the spatial arrangement necessary for adsorption or encapsulation of the carbohydrate. If the carbohydrate moieties present were covalently linked to the polypeptide chain, they would follow particular protein subunits during fractionation and, therefore, be partially characterized. Urea is one of several reagents capable of dissociating many proteins in solution and was used in the present investigation. The affect of urea on proteins is not understood, although most theories attempting an explanation postulate that urea competes successfully for sites necessary for hydrogen bonding within a protein aggregate. This idea appears to be applicable in many instances; however, it does not explain all phenomena observed in studies on association and dissociation of proteins (Reithel, 1963).

Ten milligrams of purified myrosinase were dissolved in 2 ml of 4 M urea solution and equilibrated 24 hours at 5°C. The solution was allowed to reach room temperature, applied to a Sephadex G-200 (58 x 1.6 cm) column equilibrated with 0.05 M sodium citrate buffer, pH 6.1, and eluted with the same buffer in 5-ml aliquots at a rate of 15 ml per hour. A second elution on
the same column was run in the same manner using a solution of purified myrosinase in 8 M urea (5 mg/ml). Similar elution curves were obtained from the two elutions. The latter elution curve is shown in Figure 21.

![Gel filtration of an 8 M urea solution of myrosinase on Sephadex G-200 (58 x 1.6 cm). The sample was eluted with 0.05 M citrate buffer, pH 6.1, at a flow rate of 15 ml/hr in 5-ml fractions.](image)

Figure 21. Gel filtration of an 8 M urea solution of myrosinase on Sephadex G-200 (58 x 1.6 cm). The sample was eluted with 0.05 M citrate buffer, pH 6.1, at a flow rate of 15 ml/hr in 5-ml fractions.

In some cases protein subunits are known to reunite when diluted or dialyzed free of urea. This could have happened during the elution with citrate buffer above. To minimize the association of subunits during elution, a new Sephadex G-200 column (46 x 1.6 cm) which used 8 M urea as the eluting agent was prepared. Five milligrams of purified myrosinase were dissolved in 1.5 ml of 8 M urea, equilibrated at 5°C for 24 hours and applied to the Sephadex G-200 column at room temperature. Two-milliliter
fractions were collected at a flow rate of 8 ml/hour. Each fraction was analyzed for protein and for carbohydrate. The resulting elution diagram is shown in Figure 22. Three groups of fractions were recovered from this elution and they were exhaustively dialyzed against sodium citrate buffer, pH 6.1. They were designated U-1 (fractions 13-16), U-2 (fractions 25 and 26), U-3 (fractions 29 and 30). Incubation of portions of these solutions alone, or in combination, with sinigrin solution at 37°C showed neither thiogluco- cosidase nor sulfatase activity. Gas-liquid chromatography of its carbohydrate showed U-1 to have a sugar composition similar to the carbohydrate of the original myrosinase preparation, and U-2 and U-3 gave evidence for minor amounts of glucose and galactose.
Figure 22. Gel filtration of an 8 M urea solution of myrosinase on Sephadex G-200 (47 x 1.6 cm). The sample was eluted with 8 M urea, at a flow rate of 8 ml/hr in 2-ml fractions. —— Carbohydrate by phenol-sulfuric acid method; —— Optical density at 280 mμ.
DISCUSSION

Extraction and Purification of Myrosinase

Results of this research appear to substantiate the two-enzyme character of the myrosinase system. Although actual separation of the two enzymes was not accomplished, the specific activity measurements during purification show a definite variance of thioglucosidase activity from sulfatase activity. It appeared that the thioglucosidase activity was more stable towards the conditions employed during purification. The specific activity ratio of the thioglucosidase to sulfatase increased from an initial 0.64 to 1.40 after the final purification step.

These results dispute the theory of a one-enzyme system previously reported (Nagashima and Uchiyama, 1959a; 1959b) which was partially based on the apparent 1:1 ratio between the specific activities of the two enzymes under various conditions. Nagashima and Uchiyama (1959b) isolated myrosinase from white mustard seed by water extraction followed by ethanol precipitation. Examination of Table I reveals a similarity in the specific activities of the two enzymes through the ethanol precipitation step; however, with added enzyme purification discrepancies in the activities resulted. Preliminary studies on the isolation and purification of myrosinase appeared to suggest a single-enzyme system. In later experiments the sinigrin concentration was increased for the assay incubations and yielded the results of Table I. Nagashima and Uchiyama (1959b) used an enzyme: substrate ratio larger than that used in the present work and, as a result may not have saturated the enzymes' active sites during the assay
period. In the present investigation most purification steps were performed at, or near, room temperature. This may have caused a preferential inactivation of the sulfatase. Inactivation may not have occurred under cold room conditions and the change in sulfatase activity may not have been noticed.

Failure to separate the thioglucosidase from the sulfatase allows room for speculation. The purification procedure of Gaines and Goering (1960) performed the ethanol precipitation step before the ammonium sulfate precipitation. In the present investigation these steps were reversed and, in some manner, may have prevented the ensuing separation on DEAE-cellulose. A private communication from another laboratory (Calderon, 1965) related failure to separate the enzymes by the method of Gaines and Goering. However, commercial preparations of DEAE-cellulose vary in their ion-exchange properties (Peterson and Sober, 1962) and could account for successful fractionation in one instance and no fractionation in another.

The high carbohydrate content in myrosinase as isolated in the present work may be the key to the problem of separation. Polysaccharides with their many polar groups would have great affinity for the polar protein molecules. Therefore, it is conceivable that, if the two enzymes were associated with a common carbohydrate moiety in the intact seed, this association may be difficult to disrupt. These enzymes must act simultaneously or at least sequentially when catalyzing the hydrolysis of sinigrin under in vivo conditions. Quite possibly these enzymes are held in a common vicinity by a carbohydrate matrix and the intact aggregate might be isolable. Dissociation studies on purified myrosinase in urea solution showed marked modification of the enzyme's elution from Sephadex G-200 (Figures 21 and
22). Nothing conclusive could be drawn from the data obtained; however, the apparent liberation of polysaccharide and the heterogeneity of the protein which was present suggested fragmentation of a protein-carbohydrate complex by urea. Pronase studies discussed later supported this idea.

The sedimentation pattern of purified myrosinase (Figure 2B) indicated a preparation of high purity, but showed a slight splitting of the migrating band. This ruled out the possibility of a homogeneous preparation. Disk electrophoresis appeared to explain the splitting in the sedimentation pattern. This technique utilizes the properties of gel filtration in addition to the usual electrophoresis parameters. The five enzymatically active bands resolved by electrophoresis appeared to constitute all of the protein applied to the gel. Therefore, the sedimentation pattern of myrosinase need not be interpreted as showing impurity in the enzyme preparation, but as a slight separation of various myrosinases present. (Emphasis should be made of the fact that the technique for isolating and assaying the protein bands from the polyacrylamide gel was crude and contamination between bands could have occurred.) The multi-enzyme preparation which is suggested by disk electrophoresis is in line with recent protein, particularly enzyme, research. Recent studies have indicated that one organism may produce several different enzymes with similar specificity. About 30 enzymes have been shown to exist as isozymes (Gregory, 1961). Additional research on specificity, specific activity, active site, inhibition, etc. would be necessary before accepting an isozymic nature for myrosinase. Carbohydrate was not followed during disk electrophoresis.
Chemical Composition of Myrosinase

The chemical nature of myrosinase when reported as a single enzyme system has never been discussed. Knowledge of the chemical nature of myrosinase could possibly elucidate some of the conflicting reports concerning its properties. Such information, when used in conjunction with molecular weight and proteolysis studies, might also yield further information as to the size and number of carbohydrate moieties known to be present.

Material balance was not achieved by the analyses run on purified myrosinase in this work. Approximately 91% of the preparation could be accounted for. Protein and carbohydrate were the major components. On the basis of the various types of compounds shown to be absent, it appeared legitimate to consider myrosinase in solution as a protein-carbohydrate complex containing 19.2% carbohydrate. Other materials expected to be present in the freeze-dried material were water of hydration and counterions. In the present case these counterions would of necessity include primarily citrate and sodium ions. The citrate ion is large enough to make a significant contribution, so its content was estimated as previously described. Sodium ion was estimated on the basis of the aspartic, glutamic, sialic and citric acid content. Sodium ion need not be the only cation present since other cations were present during various steps in the purification.

It was difficult to suggest other general types of compounds that could possibly be present. The fact that a 97% recovery of nitrogen was obtained in the amino acid analysis and that sialic acid and glucosamine were also present ruled out most other compounds commonly found associated with protein.
In common with other carbohydrases (Pazur et al., 1963), myrosinase was shown to contain large amounts of aspartic and glutamic acid and only small amounts of sulfur-containing amino acids. The significance of this fact is not known. In many glycoproteins the dicarboxylic amino acids appear to be the amino acids which link carbohydrate to the protein. Whether the carbohydrate is attached to protein through a covalent linkage or simply by adsorption, the free carboxyl groups of the dicarboxylic acids would certainly play an important role in stabilizing the protein-carbohydrate complex found in many carbohydrases. Conversely, the low content of sulfur-containing amino acids may point out that the cross-linking of peptide chains through sulfur atoms was not important for stabilizing the structure, and therefore, the activity of these enzymes. Such speculation is supported by the evidence that many enzymes are unstable when separated from their normal amounts of carbohydrate (Fischer and Stein, 1960).

Analysis of sugar residues by gas-liquid chromatography in this work appeared to be satisfactory for neutral sugars. Analyses for glucosamine and sialic acid were questionable. Extended hydrolysis in methanolic HCl appeared to destroy or modify both of these compounds. Glucosamine forms derivatives with two different retention times and one is apparently less stable than the other. The unstable peak is shown in Figure 6 while the more stable peak normally masked by neutral sugars began to appear in Figure 8. The sialic acid peak disappeared after a 48-hour hydrolysis. Sialic acid (N-acetyl-neuraminic acid) contains mannosamine. As the sialic acid content disappeared, the mannose content of myrosinase increased (Table IV). This suggests that one of the degradation products from
N-acetyl-neuraminic acid was mannose, or mannosamine was formed and had a retention time similar to mannose under the conditions used in the present investigation. Citrate ion gave three gas chromatogram peaks when treated by the procedure outlined previously. The most intense peak was that shown in Figures 6, 7 and 8. A much less intense citrate peak was the same as that of the α-D-xylose derivative. The interference of the citrate peak with the xylose peak is shown in Figure 8. Xylose content as calculated from the 72-hour hydrolysis was not included in calculating the average xylose content of myrosinase. A third citrate peak was usually less intense than the first two mentioned and occurs just prior to the peak for L-fucose.

Molecular Weight of Myrosinase

A molecular weight of 320,000 (± 15,000) was estimated for myrosinase by gel filtration techniques. This technique assumed a similar shape and degree of solvation for each protein included in the study. There is no reason why these assumptions should be true and, therefore, they probably were the major sources for error. A high degree of solvation would increase the apparent volume of a protein and reduce its ability to diffuse into the gel pores. Diffusion of a molecule is probably related to its shape. An attempt was made to get standards which might approximate the nature of myrosinase. Three of the five standards used for standardization of the Bio-Gel P-300 column were known to contain carbohydrate.

In the series of elutions which were run to determine the RBD values for myrosinase and the standard proteins, a single peak was obtained only in the elution pattern of myrosinase and phosphorylase. The multiple-peak elutions which result for the protein standards appear to be consistent with
present knowledge of dissociation and association of protein (Reithel, 1963).
In each case, some of the peaks obtained appear to represent particle sizes
which have been previously reported for that protein. Beta-amylase has
been shown to exist in four particle sizes (Enari, 1965). Catalase has been
reported to exist in many states of aggregation (Reithel, 1963); three par­
ticle sizes predominated in this work: 248,000, 175,000, 62,000. Hexokinase
was eluted predominately as one major peak which corresponded to its ac­
cepted molecular weight. Muscle phosphorylase contains inactive subunits
of 125,000 molecular weight. The phosphorylase preparation used in this
work was only slightly soluble when mixed with citrate buffer. This could
account for the single elution peak obtained. Elution of glucose oxidase
yielded a peak consistent with its reported molecular weight of 150,000
(Pazur et al., 1965). However, this component was not the major ingredient
in the enzyme preparation.

The possibility of contamination in the various protein standards was
real. Molecular weights of various peaks in each protein standard as esti­
mated from the present standard curve (Figure 9) correlated well with mole­
cular weights previously reported. Such results appeared to justify an es­
timation of the molecular weight of myrosinase in this manner.

Pronase and Dissociation Studies on Myrosinase

Digestion of myrosinase with pronase gave at least three distinct
carbohydrate-containing fractions after a series of fractionations by gel fil­
tration (Figure 17). Contaminating carbohydrate from pronase appeared
small. This was shown by gel filtration of a pronase blank in the same
manner as the pronase-myrosinase digest (Figures 12-15). The largest carbohydrate fragment (PM-75-1) was slightly retarded by Sephadex G-100 which would indicate a molecular weight of approximately 100,000. PM-75-1 was shown to contain 46% amino acid leaving 54% as carbohydrate. At least three possible structures are suggested: (1) the carbohydrate may be a series of oligosaccharide moieties linked to a polypeptide chain, (2) the carbohydrate is one moiety linked to an extensive polypeptide chain, or (3) the carbohydrate is a large polysaccharide associated with many short peptide groups. The non-specific proteolytic activity of pronase and the extended period of its incubation with myrosinase would seem to rule out (1) and (2). In addition, dissociation of the myrosinase in 8 M urea solution appeared to free this carbohydrate moiety of most protein. Arabinose, xylose, galactose and glucose were the monosaccharide units present in PM-75-1. Arabinose is not commonly present in glycoprotein (Spiro, 1963); however, it has been reported in β-glucosidase preparations (Veibek, 1950) and in a glycopeptide isolated from wheat flour (Kündig and Neukom, 1963). Xylose, galactose and glucose have been found in some established glycoproteins, but usually in conjunction with hexosamine, sialic acid and/or fucose (Spiro, 1963; Marler and Davidson, 1965). The percent arabinose in PM-75-1 was questionable and showed no relation to the arabinose content of intact myrosinase. Although the amino acid was not identified, gas chromatography of a standard amino acid mixture indicated that an amino acid had a retention similar to arabinose on a SE-30 column under conditions used in this work. Methanolic HCl may not have hydrolyzed this amino acid free
from myrosinase; however, after pronase digestion it was probably free to form a methyl ester and trimethylsilyl derivative.

PM-15-1 appeared to be the other carbohydrate moiety present to any extent in myrosinase. Sugar and amino acid content alone suggest a glycopeptide nature. Hexosamine, fucose, mannose, sialic acid and aspartic acid are common components of covalently-linked carbohydrate and peptide (Gottschalk, 1963; Spiro, 1963; Montreuil et al., 1962). In some cases glutamic acid (Li, 1963), serine and threonine (Blix, 1963) have been found to serve as the linking amino acids. The PM-15-1 fraction was present in both PM-25-1 and PM-25-2 indicating it was retarded by Sephadex G-25 (PM-75-2 in Figures 12 and 15). This suggested a molecular weight less than 4000. Elution from Sephadex G-75 placed PM-15-1 near the lower molecular weight limit (1000) for fractionation on this gel filtration medium.

PM-15-1 was shown to be heterogeneous by elution from a Sephadex G-15 column. This treatment, which allowed fractionation of materials with molecular weights less than 1500, suggested the presence of at least three carbohydrate fractions. Amino acid analysis showed PM-15-1 to contain 15% amino acid. This would imply that carbohydrate moieties which were present may have had a molecular weight range of 800-1200. Results included in Table V did not predict molecular weights of this size. Based on the assumption that one residue of fucose was present per carbohydrate grouping a molecular weight of 16,000 would be estimated. Using xylose or glucosamine as the basic sugar residue a molecular weight of at least 5000 would be expected. However, the evidence that PM-15-1 was heterogeneous probably explained this apparent discrepancy by allowing the possibility that
each carbohydrate moiety may have had a different sugar content. Gaines and Goering (1960) showed at pH 8.5 an electrophoretic mobility of $2.588 \times 10^{-5}$ cm$^2$ volt$^{-1}$ sec$^{-1}$ for the thioglycosidase and $-0.863 \times 10^{-5}$ cm$^2$ volt$^{-1}$ sec$^{-1}$ for the sulfatase. Huotari (1962) showed the thioglycosidase preparation to be free of sialic acid. This evidence when correlated with the results of the present work suggested a glycoprotein character for both enzymes present. Sialic acid at pH 8.5 would tend to impart a negative character to protein during electrophoresis. Carbohydrate moieties containing sialic acid were, therefore, probably recovered from the sulfatase molecule present. Assignment of the other sugars present to a particular enzyme was not possible from the results obtained. There were unexplained differences in the sugar content of intact myrosinase and sugars recovered after pronase digestion.

The third carbohydrate fraction from pronase digestion appeared to be monosaccharide. Relative intensities of the carbohydrate peaks in Figures 12 and 15 suggest that at least 50% of the carbohydrate detected by the phenol–sulfuric acid method was eluted as PM–75–3 (ultimately PM–15–2). It would be convenient to suggest possible carbohydrase activity in the pronase preparation which was used. However, the gas chromatogram from PM–15–2 (Figure 20) was not typical for the sugars known to be present in myrosinase. Paper chromatography showed only glucose to be present. This glucose was probably extracted from the CM–cellulose and Sephadex used during this work. Ascorbic acid was tentatively identified as one of the gas chromatography peaks. This is reasonable since ascorbic acid is known to be an activator for myrosinase (Nagashima and Uchiyama, 1959a). The fact
that the myrosinase preparation contained one carbohydrate moiety with an approximate molecular weight of 55,000 and other moieties of approximately 1000 molecular weight ruled out the possibility that a major amount of free monosaccharides was also in the preparation. If myrosinase has a molecular weight near 320,000 and is 19.2% carbohydrate, the carbohydrate moieties described would account for the total carbohydrate present. The number of the various carbohydrate components in PM-15-1 cannot be determined from the present data. The origin and nature of PM-15-2 was not resolved during this investigation.
SUMMARY

1. Myrosinase has been isolated from seeds of Brassica juncea by the consecutive steps of water extraction of the milled oil-free seeds, ammonium sulfate precipitation, ethanol precipitation, cellulose ion-exchange chromatography and gel filtration.

2. The enzyme preparation appeared to contain both a thioglucosidase and a sulfatase; however, the two enzymes were not separated. Purification techniques which were used yielded a 250-fold increase in the specific activity for the thioglucosidase, but only a 115-fold increase for the sulfatase.

3. Myrosinase as isolated in this investigation contained 19.2% carbohydrate. Proteolysis involving a Streptomyces griseus protease and dissociation in urea solution suggested that the carbohydrate was present as an associated high-molecular weight heteropolysaccharide and as several low-molecular weight moieties which were probably bound covalently to the protein.

4. The amino acid and sugar constituents of both the intact myrosinase and the carbohydrate fractions obtained by protease action were determined.

5. The molecular weight of myrosinase was estimated by a gel filtration method.

6. The significance of the results obtained were discussed in relation to those previously reported.
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